The dynamics of molecular components that regulate aphid-plant interactions

Claire Lynette Drurey

A thesis submitted for the degree of Doctor of Philosophy

John Innes Centre

University of East Anglia

December 2015

Abstract

Aphids are economically important insect pests, which feed on phloem sap using stylets. Aphids cause significant losses of crop yield, through draining plant resources and vectoring over 275 plant viruses. In plant-pathogen interactions, basal plant defense involving pathogen associated molecular pattern (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI) effectively fends off the majority of plant pathogens. I aimed to discover whether these mechanisms are also involved in the plant response to aphids.

I found that elicitors present within aphids can evoke PTI/ETI defense responses. In *Arabidopsis thaliana*, perception of aphid elicitors requires the Leucine-Rich Repeat Receptor-Like Kinase (LRR-RLK) BAK1, which is required for multiple PTI responses via interaction with other RLKs. I identified two RLKs which may detect aphid elicitors and provide specificity to aphid detection.

Successful aphid colonization of plants is thought to involve the suppression of PTI and ETI via effectors, leading to effector-triggered susceptibility (ETS). I investigated a *Myzus persicae* effector, Mp10, and found that it was required for success on Arabidopsis and could block immune signalling. A plant target for Mp10 was identified via a yeast two-hybrid screen. Further investigations suggest that the Mp10 target has previously unknown roles in immune receptor trafficking.

Mp10 induces ETI-like responses when expressed in plants, which I found were not dependent upon Mp10 effector action or salicylic acid. A yeast two-hybrid screen of candidate aphid effectors revealed interactions with plant resistance proteins, which may play a role in the aphid-plant interaction. Aphid effector proteins were also found to interact with each other, suggesting a role in the regulation of effector action and delivery into plants.

Taken together, the research described in this thesis has elucidated the roles of PTI, ETS and ETI in insect-plant interactions and identified specific plant and aphid proteins that are involved in these.

"Take wrong turns. Talk to strangers. Open unmarked doors. And if you see a group of people in a field, go find out what they're doing. Do things without always knowing how they'll turn out. You're curious and smart and bored, and all you see is the choice between working hard and slacking off. There are so many adventures that you miss because you're waiting to think of a plan. To find them, look for tiny interesting choices. And remember that you are always making up the future as you go."

Randall Munroe, http://xkcd.com/267

Acknowledgements

First and foremost, I would like to express my sincere thanks to my supervisor Saskia Hogenhout for her suggestions, guidance and constant encouragement and enthusiasm for the project during the four years I have spent in her laboratory. Together with my supervisory committee members, Cyril Zipfel and Mark Banfield, I feel I have been blessed with a trio of extremely knowledgeable, experienced and patient mentors to guide me towards becoming an independent scientist.

No woman is an island. One of the main reasons I have enjoyed my time at the John Innes Centre so much is because of the help, support and good humour of everyone around me. My particular thanks go to members of the Hogenhout Lab past and present, for help with planning and carrying out experiments as well as much-needed tea breaks. I would also like to thank the JIC insectary team for their constant care of the insects I have used throughout the last four years. Without their help I would have not been able to get half as much done as is documented within these pages.

Thank you to my fellow cohort of PhD students, whom I have shared the last four years with, and who have been a constant source of support, laughter and good cake. It hasn't always been easy, but it has definitely been an adventure.

I would like to acknowledge the 4th Norwich Brownies and leaders for their help in making me feel at home in Norwich, and keeping me sane during writing up. I have truly enjoyed all of our meetings, trips and camps.

I would also like to thank my partner Matthew Smith, who has kept me grounded throughout the whole experience, and has given me perspective (and tea) whenever I have needed it. I am also grateful to both of our sets of parents for the support they have given me throughout the years.

Finally, I would like to pay tribute to the roughly 30,000 green peach aphids I have collected, counted, frozen and ground over the past four years. The results I have produced show that it has not all been in vain.

Contents

List of Figures

List of Tables

List of Abbreviations

BAK1	BRI1-ASSOCIATED RECEPTOR-LIKE KINASE
Bp	base pair
CSP	Chemosensory Protein
Ct	cycle threshold
DAMP	Damage Associated Molecular Pattern
DMSO	dimethyl sulfoxide
DPI	days post infiltration
dsRNA	double stranded RNA
EDTA	ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FAC	fatty acid-amino acid conjugate
FLS2	FLAGELLIN SENSING2
GFP	green fluorescent protein
GLM	generalised linear model
GOX	glucose oxidase
GPA	green peach aphid
GUS	beta-glucuronidase
HAMP	Herbivore Associated Molecular Pattern
HR	hypersensitive response
MAPK	Mitogen-activated Protein Kinases
miRNA	microRNA
NBS-LRR	Nucleotide Binding Site-Leucine-Rich Repeat
Nt	nucleotide
NCBI	National Centre for Biotechnology Information
OD	optical density
ORF	open reading frame
OS	oral secretion
PAMP	Pathogen Associated Molecular Pattern
PCR	polymerase chain reaction
PRR	Pattern Recognition Receptor
PTI	PAMP-triggered immunity
qRT-PCR	(real time) quantitative reverse transcription PCR
RFP	red fluorescent protein
RNAi	RNA interference
ROS	reactive oxygen species
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
TAIR	The Arabidopsis Information Resource

Chapter 1

Introduction

1.1 Aphids: An Introduction

Aphids are piercing and sucking insects belonging to the order Hemiptera, suborder Sternorrhyncha which also includes whiteflies, psyllids and scale insects. There are over 4,000 species of aphid in ten families, and of these about 250 are serious pests in agriculture and forestry (1). Aphids are more speciose in the temperate zones of the world, where they colonise 25% of the existing plant species (2). Aphid species can be specialist, feeding on a restricted host range, or generalist plant feeders, feeding on multiple plant species from diverse families. The green peach aphid (GPA) *Myzus persicae*, is a generalist, and can feed on over 100 different plant species from over 40 different families, including the Brassicaceae and Solanaceae (3). This includes the model organisms used for investigating microbe-plant interactions; *Arabidopsis thaliana* and *Nicotiana benthamiana*. GPA is therefore widely used in experimental studies on the interaction between aphids and plants. Laboratories in the USA and UK (Hogenhout lab) have sequenced the genome of GPA (clone G006 from the Unites States and clone O from the United Kingdom, respectively). Though these are yet to be published, they are available to the research community via AphidBase (4). Another widely studied aphid species is *Acyrthosiphon pisum*, the pea aphid, for which there is a full genome assembly (5).

Aphids have life cycles with a high degree of phenotypic complexity (6). All-female parthenogenetic generations alternate with sexual generations (see Figure 1.1). Throughout the spring and summer, the aphid population consists only of females and reproduction occurs by parthenogenesis, with adults giving birth to large numbers of offspring (nymphs), which are presumed to be genetically identical to the mother. Aphid asexual reproduction is generationally telescopic, with the embryos of the next generation already beginning to develop within the bodies of the newly-born nymphs (7). The nymphs are also born alive (viviparous reproduction), which is unusual among insect species and facilitates more rapid development to reproductive maturity (8). There are several forms of aphids, with winged (alate) and unwinged morphs. When a food source fails, or is crowded by aphids, the stress response of aphids is to develop alates, enabling dispersal to nearby food sources. Though of limited flying ability, alates can disperse over a wide area and colonise other plant hosts (9). When alates land on a new host they seed nymphs, which can then pass through multiple generations of asexual reproduction. In favourable conditions, this strategy can result in explosive population growth. After repeated cycles of asexual reproduction, in many species short autumn day length induces the production of sexual females and males. After mating, the females lay eggs, which are able to withstand harsher winter temperatures. In

the spring, new asexual females emerge from the eggs and the cycle begins again. This cyclically parthenogenetic lifestyle enables adapted aphid populations to expand clonally when on a plentiful food source, but also maintain some genetic variability through sexual reproduction.

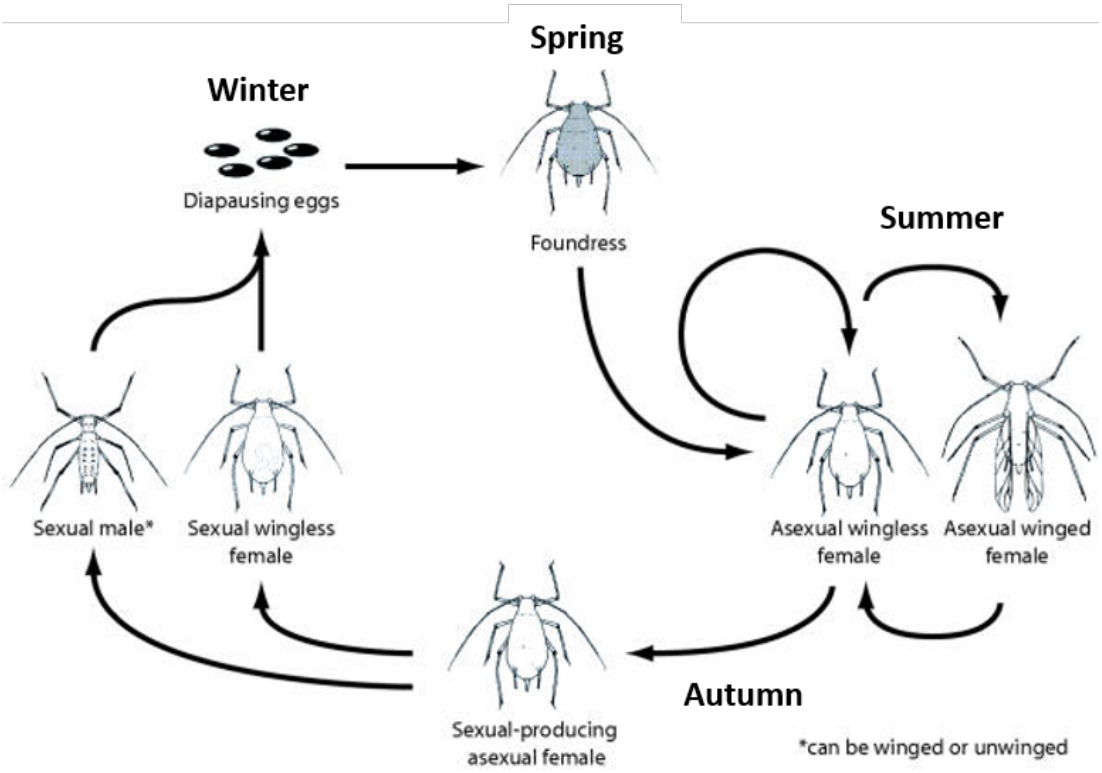


Figure 1.1: **Life cycle of the pea aphid, *A. pisum***. During the spring and summer reproduction is by parthenogenesis, then in the autumn there is a single generation of sexually-reproducing males and females which produce eggs. These eggs do not hatch until the following spring. Most aphids have a similar life cycle. Taken from Shingleton et al, 2003 (10).

Many aphids use a single plant species for oviposition (primary host), but feed upon plants from a wider range of species during the asexual part of their lifecycle (secondary hosts). This is often documented in the common name of the aphid, for instance GPA is also known as the peach-potato aphid, as it lays its eggs upon peach trees, but survives year round on other plants such as potatoes. Unlike other aphid species, though GPA lays eggs on its winter host, peach, they hatch before spring and the nymphs overwinter, sometimes growing to adulthood. Under environmental conditions with constant temperatures of over 18°C and long day lengths, the asexual cycle can continue indefinitely with no appearance of the sexual morph. It was believed that the clone of GPA found in the UK (O) has lost the ability to form sexual morphs as the peach trees that this aphid uses as a primary host are largely absent in the UK, leaving the aphids to cycle asexually indefinitely (11). However, we have found that switching to shorter day lengths and a temperature lower than 18°C in the lab induces the generation of sexuals in GPA clone O, suggesting that this is not true for

all UK clones. Perhaps GPA has adapted to use another species as a primary host in the UK.

1.1.1 Aphid feeding

Aphids feed from plant phloem using an adapted mouthpart that has morphed into a stylet bundle; a long tube through which phloem sap can be sucked (see Figure 1.2a). The stylet bundle has a complex anatomy, with two external mandibular stylets that protect and encompass two inner maxillary stylets. The mandibular stylets have sharp ridges that aid insertion of the stylet bundle into plant tissue, helping to anchor the stylets between probing thrusts. The inner maxillary stylets are pointed at the end, to aid the puncture of plant tissue and have a specific architecture, allowing them to fit tightly together inside the mandibular stylets (see Figure 1.2b)(12). The way the stylets are joined leaves two ducts through which phloem is taken up and saliva is secreted, named the food canal and the salivary canal. These canals are separated along most of the length of the stylets, but join shortly before the end in a common duct at the stylet bundle tip (13). An anatomical structure has been discovered inside the common duct, termed the "acrostyle" (14). The acrostyle contains specific cuticular proteins and has been implicated in the transmission of viruses by aphids, though it may also be involved in the control of fluid dynamics within the stylet, protein binding or the mechanical strength of the stylet itself (15; 14).

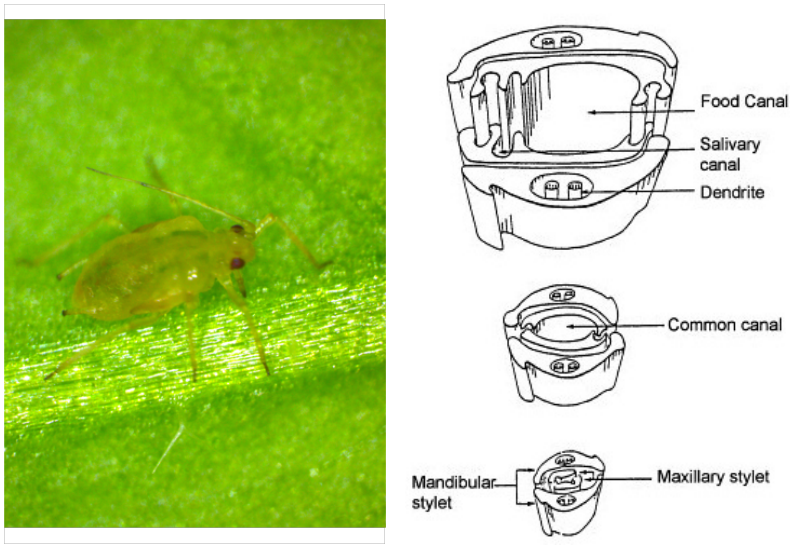


Figure 1.2: **Aphids feed from plant phloem using stylets**. 1.2a Photograph of *Myzus persicae* feeding from *Arabidopsis thaliana* with stylet visible. 1.2b Aphid stylet bundle structure, showing how the mandibular and maxillary stylets slot together to form the food and salivary canals. Taken from Taylor and Robertson, 1974 (12).

Aphids feed from sap present in the phloem sieve tubes, the contents of which include

carbohydrates, amino acids, lipids and minerals. Though phloem sap is nutrient rich, its essential amino acid content is very low (16). In order to provide the insect with the essential amino acids it needs, aphids undergo obligate symbiosis with *Buchnera aphidicola*. This bacteria is accommodated in specialised cells within the aphid haemocoel (body cavity), known as bacteriocytes (or mycetocytes), and is vertically-transmitted between aphid generations (17). Aphids can also contain one or more facultative symbiont. These have been documented to protect against entomopathogenic fungi and parasitoid wasps, affect aphid heat tolerance and even change the insects colour (18; 19). Some endosymbionts also have an effect on the plant colonization ability of aphids, for instance the facultative endosymbiont *Regiella insecticola* reduces *A. pisum* performance on *Vicia faba* and increases performance of certain *A. pisum* clones on *Trifolium pratense* (red clover) (20).

Aphids use their stylet to probe through plant tissues, from the plant surface to the sieve elements of the phloem using an intercellular pathway (21). During this process, the stylets puncture many cells, including those in the epidermis and mesophyll, before the phloem is reached (22) (see Figure 1.3). Each puncture includes a salivation phase and an ingestion (sometimes referred to as "tasting") phase. It is believed that the aphid uses the pH and sucrose concentration within these sampled cells as cues to navigate towards the sieve elements within plant tissues (23). Salivation after these initial punctures may also allow the introduction of salivary proteins that can modulate plant processes, including defense responses (24). This would allow inhibition of plant defense responses before phloem sap flow could be affected. Aphids make multiple probes on a plant surface before feeding from the phloem or retracting the stylets to feed elsewhere (25). This suggests that interactions between aphid and plant components at an early stage in probing, before the phloem has been reached, can determine the success of feeding.

Aphids seal puncture sites up after sampling using a type of saliva known as gelling or sheath saliva (22; 21). Sheath saliva gels almost immediately after leaving the stylet tip and forms a complete protective layer around the stylet, it also forms a flange around the site of stylet insertion on the plant surface (26). A continuous protective layer of sheath saliva may prevent the plant from recognising any molecules found upon the stylet, as well as sealing the puncture site (24). Sealing puncture sites maintains the turgor of the sieve element, which is important for the continued feeding of the aphid. Forming a tight seal around the site of penetration could also prevent the influx of calcium into the sieve element, which has been implicated in phloem occlusion (27). Aphids can feed continuously from a single sieve element for many hours, in some cases even days (25), showing that aphids are able

to prevent plant responses that would inhibit feeding, such as sieve element occlusion.

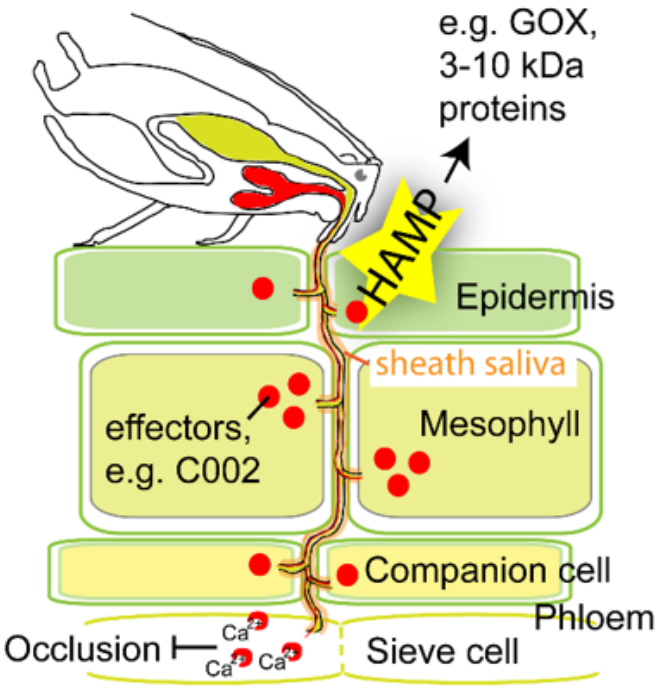


Figure 1.3: **Aphids use their stylets to probe through plant tissues in an intercellular pathway**. Multiple cells may be punctured en route to the phloem, with salivation and ingestion taking place in each punctured cell. Taken from Hogenhout and Bos, 2011 (24).

Aphids secrete a second type of saliva, termed called watery saliva, which does not gel but remains liquid. Watery saliva is injected into plant cells as soon as penetration occurs, before ingestion is begun (28). Watery saliva secretion is repeated in long feeding periods, so the aphid ingests phloem sap mixed with saliva. Together, this points to a role for watery saliva in allowing the aphid to feed from the phloem continuously. The saliva may maintain sap flow in the phloem as watery saliva is secreted when sieve element occlusion is induced by mechanical means (29). Watery saliva is also thought to keep the food channel in the stylet open by interacting with sieve element proteins in the sap (30).

As both gelling and watery saliva fulfil different roles in the plant-aphid interaction, they must also contain different components. Gelling saliva is composed of protein, phospholipids and conjugated carbohydrates (31). Structural proteins are found in the gelling saliva, including some rich in cysteines, which can be oxidized to form disulphide bonds, aiding solidification (30; 32). The structural sheath protein (SHP) in *A. pisum* gelling saliva is needed for formation of the salivary sheath and for aphid feeding (33). It is believed the SHP forms a vital role in the hardening of the sheath, which impacts the aphids ability to plug gaps formed when probing.

Watery saliva has a more complex protein composition, including calcium binding proteins which could prevent sieve element occlusion (29; 32). Several proteomics studies have been carried out on watery aphid saliva, including that from *A. pisum*, GPA and the Russian wheat aphid, *Diuraphis noxia*, and this has identified many protein components (32; 34; 35). Proteins found include a large number of enzymes, including hydrolases, such as pectinases, cellulases and glucosidases that together are thought to have roles in digestion (36; 34). Enzymes of the oxidoreductase class have also been identified, including peroxidases and phenol oxidases. These are able to detoxify defensive phytochemicals, suggesting a role in host plant colonisation (37; 38). Some proteins, such as glucose dehydrogenase and glucose oxidase (GOX) were found to be shared components of saliva between different aphid species that feed on plants from different families (34; 35; 39). This suggests that these salivary components may be essential to aphid feeding on plants in general. Differences were found between *D. noxia* saliva compared to that of GPA and *A. pisum* that may be linked to the phytotoxicity of *D. noxia* saliva on its monocot host. A difference in saliva composition was also seen between *D. noxia* biotypes, which are able to feed on wheat varieties containing different resistance genes, showing a link between saliva contents and aphid virulence (35; 40).

Some components of aphid saliva have been found to be essential in the feeding of aphids, such as the protein C002 from *A. pisum*. C002 is injected into leaves of the fava bean host when *A. pisum* feeds, and C002 knock down leads to difficulties in feeding and increased mortality of the aphid. This suggests that the aphid uses this protein to modulate plant processes in order to feed successfully (41; 42). The GPA C002, MpC002, is also required for success of GPA on plant hosts, as silencing it reduces aphid fecundity. Overexpressing MpC002 in *A. thaliana* and *N. benthamiana* increases aphid fecundity (43; 44; 45; 46). This suggests a conservation of C002 across different aphid species, and a requirement for colonization in many different plant species. Not all salivary proteins have shared functions between aphid species, as Mp1 and Mp2 from GPA both promote aphid colonization when expressed in Arabidopsis, but Ap1 and Ap2 from *A. pisum* do not (44). Large screens have found more predicted secreted salivary proteins like Mp1, Mp2 and C002 that could have roles in plant-aphid interactions, potentially acting as modulators of the plant immune response to aphids (46; 39). Conversely, studies have also found that some components of aphid saliva actually induce a defense response in the plant. De Vos et al. found that a 3-10 kD protein present in GPA saliva induced a defense response in *Arabidopsis thaliana*, leading to reduced aphid fecundity (47). As aphid saliva components determine both aphid resistance and susceptibility, investigation into aphid saliva can there-

fore shed light upon the interaction between aphid and plant.

1.1.2 Aphids as Agricultural Pests

Aphids are major agricultural pests, and are responsible for damage to crops in several ways; direct damage due to phloem drainage and plant reaction to aphid feeding, damage induced by other organisms growing on the honeydew that they secrete, and disease caused by the viruses that they vector.

Aphids directly influence the amount of resources plants have for biomass conversion by taking photoassimilates from the phloem sap. Hawkins et al. found that short term feeding of pea and cowpea aphids on several species of plants reduced the flux of photosynthetic product to the root, changed photoassimilate partitioning pattern in shoots and induced assimilate sources to become assimilate sinks (48). This shows that aphid feeding has an effect on resource flux within the plant, and so can affect plant productivity. Under heavy infestation conditions a large amount of photoassimilates can be taken up by aphid pests, leading to losses in crop yield (1).

Aphid feeding can induce leaf and fruit deformation and discoloration, this is thought to be due to enzymes found in aphid saliva such as pectinase, catalase and peroxidase (1). These enzymes can be phytotoxic and have been implicated in necrosis, stunting and vein-clearing symptoms (49). The feeding of some aphid species also causes proliferation of cells around stylet entry-sites, leading to gall formation. Galls can effect crop yield; in fruit trees they have been found to induce early leaf fall, which leads to a reduction in fruit size (1). The process behind gall induction by aphids is thought to involve the reprograming of host metabolism, including the manipulation of source-sink relationships in the plant, though the mechanisms behind this have yet to be elucidated (50; 51; 52). Gall forming aphids have not been found to produce or induce the plant hormones that gall forming pathogens such as *Agrobacterium tumefaciens* do (53; 54). The North American woolly apple aphid, *Eriosoma lanigerum*, can induce galls. It is found on elm trees in its native country, but has spread around the world and is now considered to be a major pest on apple crops in New Zealand. The aphid also disrupts xylem in the root, leading to problems with water conduction that reduce the growth of apple trees (55). One aphid species can therefore have many effects on its plant hosts.

The sap that aphids take up when they feed is high in sugars, not all of which can be

digested by the insect. High sugar concentrations also produce problems with osmoregulation in the insect digestive system. As a result of this, oligosaccharides are produced from ingested sucrose by aphids in order to reduce the osmotic pressure in the gut. These oligosaccharides and excess undigested sugars are excreted from the insect as "honeydew" (16). Under heavy infestation conditions, the honeydew secreted can coat the surface of the leaf, providing an ideal substrate for bacteria and fungi to grow on. Mould growth can block the leaf from obtaining light energy from the sun, reducing photosynthesis. Buildup of sooty mould fungus on pecan tree leaves as a result of honeydew deposition has been found to reduce light transmission by as much as 25%, which could have a serious effect on the yield of these trees (56). Honeydew on the surface of crops also reduces their marketability, making the crop undesirable to buyers.

As piercing and sucking insects, aphids also make ideal vectors for plant viruses. Two hundred and seventy five different plant viruses are known to be vectored by aphids. This is at least 50% of all insect-borne plant viruses, and includes many that cause diseases of major economic importance (57; 58). Viruses that are vectored by aphids include representatives from all plant virus families. Those from the family Potyviridae, genus Potyvirus are regarded as the most important due to the large number of virus species in this grouping, and the ease with which they are transmitted (59). As potyvirus transmission is non-persistant (virus stays in the stylet, and is not taken up into aphid tissues), both acquisition and transmission can occur over a short time of probing, before the phloem is even reached. This means that aphids that are not able to colonise a plant can still transmit potyvirus to it when probing. One hundred and ten potyvirus species are transmitted almost solely by aphids (60). Luteoviruses such as turnip yellows virus (TuYV) and potato leafroll virus (PLRV) are also economically important, and infect many crop plants, but these are transmitted in a circulative manner (virus passes through the aphid gut, and into the salivary glands), so are only transmitted when aphids feed from the phloem. GPA is one of the most agriculturally important aphid species due to its broad host range and ability to vector a wide range of plant viruses (over 100) (6).

The combined effects of aphids on plants, including direct damage and vectoring of viruses, can lead to large crop losses. In Britain, Tatchell (61) estimated that direct losses due to aphid damage were 8-16% in pea, 10-13% in wheat and 5% in potato. Yield losses of over 50% caused by the soybean aphid have been reported in the USA and China (62; 63). This makes aphids a serious threat to crop productivity, and so they need to be controlled in order to maintain yield.

1.1.3 Methods of Aphid Control

Due to the effects of aphids on their host plants, the control of aphids on crops is of importance in agriculture. Methods of aphid control include chemical control, such as spraying with insecticides, biotic methods of control, including use of predators or parasitoids that prey on aphids, and breeding cultivars that show resistance to aphids.

Chemical control of aphids currently consists of two different methods; spray-type insecticides, that are used to treat plants once they have grown, and systemic insecticides that travel from a treated area of the plant in the xylem and phloem (64). These systemic insecticides are often used as seed treatment, in which case the plant has the insecticide in it from the seedling stage and aphids are intoxicated after their first sap ingestion. Seed treatments allow plants to be protected when they are young and so more susceptible to negative effects upon aphid colonization, and crops do not have to be sprayed at the beginning of the growth season. As it affects the aphid after its first feed, this method of applying insecticides can also prevent the spread of some plant viruses (1). Seed application also only affects plant-feeding insects. Insects that land on plants but feed on aphids or are parasitoids of them are not directly affected, though they may eat intoxicated prey. Systemic pesticides may also be preferred as spray-type pesticides are often hard to apply and don't reach insects when they are hidden in flowers or underneath leaves. A drawback of using systemic insecticides is that this makes insecticide use general as seeds are always treated. In contrast spray-type insecticides may only be used when conditions suggest that aphid infestation is likely, and so their use can therefore be minimised in order to prevent development of resistance. This is also important as many insecticides can be harmful to the environment, leaching out of soils and into the water systems, where they can be toxic to aquatic life (65). Levels of insecticide can also build up in food chains, to the detriment of high trophic level feeders (65; 66). Insecticides such as neonicotinoids have also been linked to a reduction in the numbers of beneficial insects, such as pollinators (67; 68)

Another downside of chemical methods of control is that resistance can develop in aphid populations. By the mid-1980s, after use of insecticides such as pyrethroids became common in the 1960s and 70s, insecticide resistant clones had developed in around 20 aphid species across the world. Currently, at least 8 aphid species that are damaging to crop plants are resistant to one or more insecticide types in France (1). Insecticide resistance has been found to be frequent and diverse in GPA, with several different resistance

mechanisms identified so far. These include the overproduction of carboxylesterases that detoxify the insecticide before it can have an effect on the aphids nervous system (69), as well as changes to the target sites of insecticides (70; 71). Resistance mechanisms seem to have fitness costs though, and percentages of *M. persicae* populations with these adaptations fluctuate year-by-year. This maintains resistant aphids at manageable levels in most years, though the increasing genetic diversity of resistance is slowly reducing the amount of effective insecticides that can be used (72). In fact, *Myzus persicae* is the most widely insecticide resistant aphid species worldwide, with resistance reported to most classes of insecticide, including organophosphates, carbamates, pyrethroids, cyclodienes and neonicotinoids (73).There is therefore a clear need for methods of aphid control that do not require the use of insecticides.

Biotic methods are one such alternative method of aphid control. They do not cause harmful effects to the environment and take advantage of the large range of natural enemies that attack pests such as aphids. Classical biological control is used when pests have accidentally been introduced to an area. Natural predators from the pest insects homeland are introduced in order to control the pests population size. An example of this is in the control of woolly apple aphid in New Zealand orchards by the parasitoid wasp *Aphelinus mali*, from its native North America (74). This form of control can be effective over a long period of time, and can require little input if environmental conditions are right. However the introduction of another alien species into the environment may pose a problem as native species may also be predated on alongside invasive pests.

Augmentation is another strategy of biological control. It is used when the insect species is a native pest and involves the rearing and releasing of the aphids' natural enemies. It has had very limited success and currently the use of augmentation is limited to the control of a few insect species that attack flower and vegetable crops grown in glasshouses, such as use of the entomopathogenic fungus *Lecanicillium longisporum*, marketed as 'Vertalec' to control aphid numbers. Controlling insects in large areas such as fields with this method is much harder, and not economically viable due to the costs of rearing such a large amount of natural aphid enemies (75). There is also the issue of resistance, with the emergence of aphid lineages that have increased resistance to biological control methods (76). Interestingly, the mechanisms responsible for parasitoid resistance are distinct from those responsible for insecticide resistance; the resistance of *A. pisum* to the parasitoid wasp *Aphidius ervi* is linked to the presence of secondary symbionts in the aphid (77).

Further biotic methods include managing the habitat around crops so as to increase the levels of natural aphid enemies. This could involve sowing flowering plants whose nectar or pollen provides food for adult insects whose larvae are aphid predators, such as hoverflies. Managing the habitat around crops also limits aphid numbers by reducing interactions between aphids and the crop at a susceptible stage in its life cycle, for example the late sowing of winter cereals coincides with a reduced number of migrating aphids from nearby plants (1). Crops such as wheat and oilseed rape are often planted early in the year, at times when aphid populations are low. The increases in global temperatures that are predicted in the future may prevent this from being a successful means of aphid control, as milder winters will increase aphid survival and higher temperatures earlier in the year are more favourable to distribution of the aphids by flying from winter hosts (78).

Another strategy is to eradicate the presence of the aphid's alternative host near the crop fields, such as the removal of overwintering hosts. This has had some success in lettuce root aphid control in the USA, where Lombardy poplars have been removed from near lettuce fields. The aphids overwinter in galls on the poplars, so removing them prevents infection between years (79). The 'push-pull' strategy has been developed, in which intercropping cereal crops with an attractant plant, Napier grass, and a repellent plant, the legume intercrop *Desmodium spp.*, controls both the parasitic weed, striga, and stemborers. This has proven to be an effective method for pest control in maize farming in Africa (80).

Though biotic methods may be able to help control aphid populations, their action is not always fully successful. A method of control that is dependent upon a plant property, rather than one in the aphid or its enemy, can provide a much more reliable method of control. Breeding of cultivars resistant to aphids is one such method. Breeding aphid resistance has been occurring for a long time; woolly apple aphid resistant apple trees have been known since the middle of the 19th century and are now used all over the world (1). Mapping of aphid resistance genes is being carried out in labs throughout the world. Resistance loci have been found in tomato, soybean, melon, *Medicago truncatula* and many other plants (81; 82; 83; 84; 85). In some cases, these loci have been linked to genes, such as the *Vat* gene in *Cucumis melo* (melon), which has been found to give race specific resistance to the cotton-melon aphid, *Aphis gossypii* (83). *Vat* was found to encode a nucleotide binding-leucine-rich repeat (NBS-LRR) protein, a common structure found in plant defense proteins (86). The *Vat* resistance allele has been used extensively since the 1990s without the rise of resistant biotypes, despite the potential of the aphid to develop resistance (87). However the use of other resistance genes has not been so smooth. Breakdown of resistance

conferred by the *Nr* gene in lettuce to the lettuce aphid, *Pemphigus bursarius* occurred in Europe 10 years after the release of lines that utilized it (88). The raspberry resistance gene*Ag1* was also rendered useless after a resistance-breaking biotype of the larger raspberry aphid, *Amphorophora agathonica* appeared (89). Usually resistance breaking such as this occurs due to the introduction of a new aphid biotype to an area, rather than mutation in the existing population, which can explain why some resistance genes can be utilized for many years against one biotype in a country and then be overcome quickly. So far, the best studied insect-plant interaction system in terms of gene-for-gene interaction is that between the brown planthopper, (*Nilaparvata lugens*) and rice. Several NBS-LRR genes have been cloned, including *Bph14* and *Bph26* (90; 91).

Traditional methods of plant breeding have provided agriculture with successes in the past, but new methods are required to keep up with aphids, which can develop resistance to insecticides and resistance genes in plants. Other methods of aphid control, informed by chemical and molecular biology are therefore being pursued. Genetic modification provides a method for the quick introduction of resistance genes from sources that may not be able to be utilized by traditional breeding. Traditional breeding may also introduce undesirable traits, as has been found in breeding for aphid resistance on Brassicas. *Brassica fruticulosa* was found to have strong antibiosis against the mustard aphid, *Lipaphis erysimi* (92). However introgression of the resistance gene from this species into the crop plant *Brassica juncea* may lead to linkage drag and affect the crops agronomic traits (64). Genetic engineering can therefore be a preferred option as it is possible to transfer the resistance gene alone into the crop plant, without effecting other gene loci such as that controlling yield.

Genetic modification of some crop plants to give successful insect resistance has already occurred, with the introduction of *Bacillus thuringiensis* (Bt) toxin expression into crop plants such as maize, potato and cotton (93). Various types of these toxins have effects on Lepidoptera, Diptera, Coleoptera, Hymenoptera and nematodes specifically, so pest control can be targeted. Hemipterans are not susceptible to Bt-derived toxins, but work introducing an aphid gut-binding peptide to the protein sequence of the Bt cytolytic toxin Cyt2Aa enabled aphid targeting, suggesting that with some alteration Bt-toxins can also be successfully used against aphid pests (94). The use of virus coat proteins, which bind to components in the aphid gut to aid viral transmission, has enabled aphid-specific targeting of a spider-produced protein toxin (95). This shows that investigation of aphids on a molecular level can directly inform pest control strategies. Introduction of Bt-toxin producing crops has had various reported levels of success. An advantage is that synthetic

pesticides do not have to be applied to the crop, so Bt toxin expressing plants can be seen as more environmentally friendly. However, as with constant pesticide use, the presence of the constant selection pressure from the toxin can lead to the evolution of Bt-resistance (96). The use of non-Bt crop refuges can reduce the possibility of this occurring by providing plants that the insect can survive on, maintaining a susceptible population. Engineering plants that produce several types of toxin also reduces the chances of resistance developing (97).

Another example of genetic modification in crop plants against an insect pest is the introduction of proteinase inhibitors (PIs), which are components of the defense response against insects in some plant species. They display variable specificity against the digestive proteinases of insects and are induced as an immune response to wounding. Constitutive expression of these PIs has been considered as a resistance mechanism. A PI was used in the first attempt to develop an insect resistant crop; cowpea trypsin inhibitor was engineered into tobacco (*Nicotiana tabacum*), conferring resistance against the chewing herbivore *Manduca sexta* (98). Cowpea trypsin inhibitor in tobacco has also been found to be effective against a range of other lepidopteran pests (93). PIs that are effective against aphid pests have also been identified; potato proteinase inhibitors I and II have been shown to act as effective proteins for the control of three species of cereal aphids (99). Genes such as this are prime candidates for transferal into crop species by genetic modification to increase aphid resistance.

Genetic modification also enables the application of research on aphid chemical ecology. Aphids produce an alarm pheromone upon attack by aphid predators, which signals to other members of the same species to move away (100). Aphid predators have evolved or learned to use this alarm pheromone as a cue for prey location (101; 102). The main component of aphid alarm pheromone is the sesquiterpene (E)-β-farnesine (EBF) (102). Some plants naturally produce EBF in order to protect themselves against aphids; for example the wild potato, *Solatium berthaultii* releases EBF from glandular hairs, which repels aphids at short distances (103). Plant breeders have been able to obtain hybrid potatoes with these glandular trichomes, which gives farmed potatoes effective protection against aphids (104). However, not all crop species have wild relatives with this property. The metabolic pathway for EBF production via sesquiterpene synthase has been identified (105; 106). Expressing sesquiterpene synthase in Arabidopsis causes emission of pure EBF, which has an effect on the behavior of aphids and a parasitoid (107), though aphids do become habituated to EBF over several generations. EBF-habituated aphids show higher predation levels by

ladybird *Hippodamia convergens*, suggesting that constitutive EBF emission from plants may control aphid populations by increasing predation rather than repelling aphids (108). Transgenic wheat lines that produce EBF have been developed that show aphid repellence and increased foraging of a natural enemy in the lab. However field trials using these lines showed no reduction in aphid numbers, though this may be due to low insect numbers in the field trial year (109). Refinements are needed before this is used as a method of crop protection, but it demonstrates how deeper understanding of the aphid-plant interaction is able to inform agricultural methods.

1.2 Plant Immunity

Much is still to be uncovered about the interaction between insect and plant host on a molecular level. Figure 1.4 shows a scheme for this interaction, outlining what was known before the research described in this thesis started. In the remainder of the introduction I will discuss each section in turn.

1.2.1 Perception of pests and pathogens

Plants have constitutive defense responses, such as maintaining levels of anti-herbivory compounds in tissues and producing spines, hairs and trichromes to deter herbivores (112). However, there are several costs associated with mounting a defense response against a pest or pathogen (113). As resources are limited, investment of plant resources into immunity leads to a trade-off between growth and development. Relying on constitutive defense responses alone would therefore be costly to the plant, leading to a need for defenses that are induced only in the presence of the pest or pathogen. The first layer of plant immunity therefore relies on the specific detection of conserved pathogen- or herbivore-associated molecular patterns (PAMPs or HAMPs). Plant-derived molecules released when pests or pathogens attack plants can also act as elicitors of defense responses. These are known as damage associated molecular patterns, or DAMPs. I shall refer to PAMPs, HAMPs and DAMPs jointly as elicitors, which includes any compound that triggers plant immune responses (114; 115).

Elicitors from insects which cause immune responses in plants have been identified; these have mainly been found in oral secretions (OS) of chewing insects. Fatty acid-amino acid conjugates (FACs) are the best studied, and have been isolated from several lepidopteran species (115). FACs are formed of two groups; one fatty acid and one amino acid (either

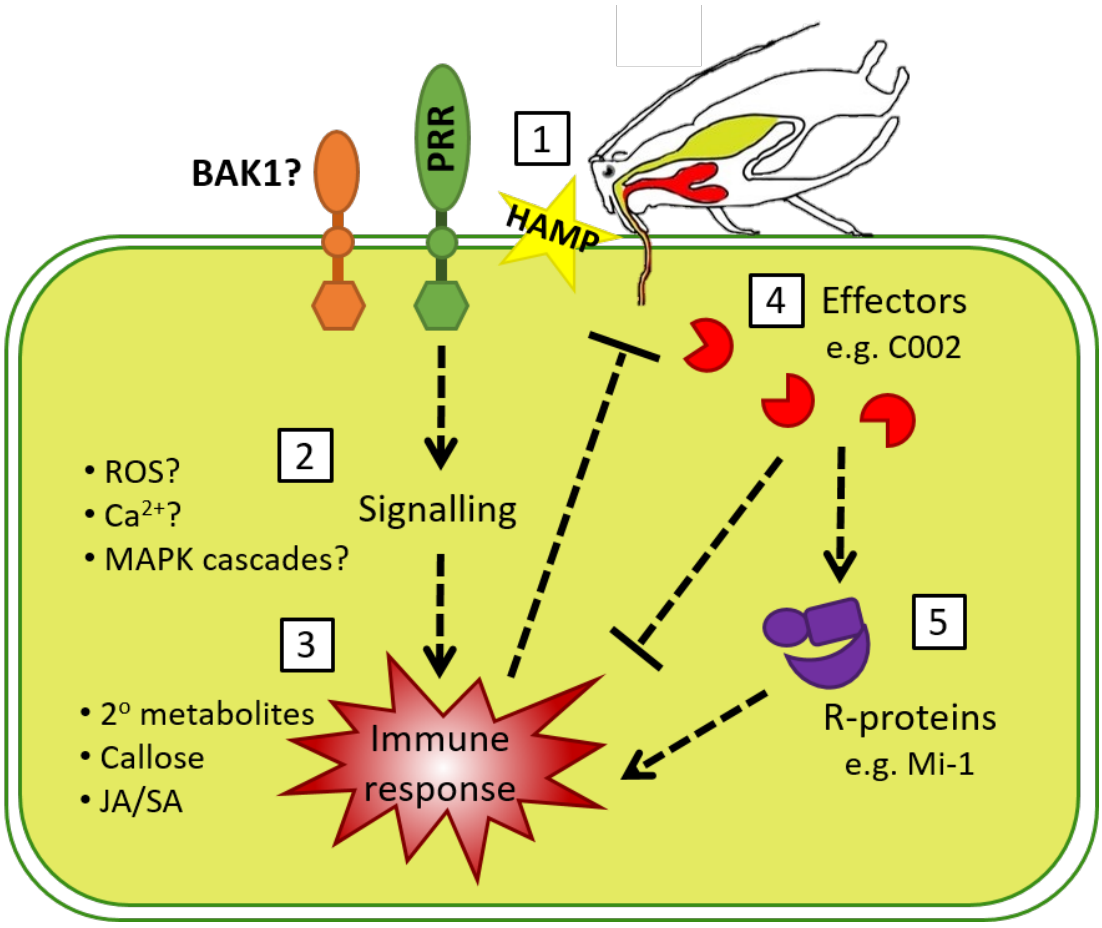


Figure 1.4: **Model of plant-aphid interactions**

1. Whole aphids and aphid saliva contain components that act as Herbivore Associated Molecular Patterns (HAMPs). In plants, elicitors such as this are detected through pattern recognition receptors (PRRs). BAK1 is required for defense responses against *Manduca sexta* in *Nicotiana attenuata* (110), so may have a role in insect perception, including of aphids.
2. Downstream of perception, intracellular signalling is required for initiation of a defense response (PAMP Triggered Immunity, PTI). Reactive oxygen species, calcium bursts and MAP kinase cascades are implicated in plant responses to insects.
3. Components of the plant immune response induced by insects or required for effective resistance include the production of secondary metabolites (camalexin, glucosinolates) and herbivore-induced plant volatiles, callose deposition and the phytohormones salicylic and jasmonic acids.
4. Aphids introduce proteins along with their saliva when feeding. These proteins may act as effectors and interfere with the plant immune response to enable colonisation. C002 is one such aphid effector (41; 42).
5. Resistance (R)-proteins may detect the presence of insect effectors, and reinstate the immune response, leading to effector-triggered immunity (ETI). Mi-1 is an R-gene that confers resistance to root-knot nematodes, potato aphids and sweet potato whitefly (111).

glutamine or glutamic acid). Interestingly, the fatty acid is plant derived and the amino acid is from the insect. The two are combined to make FACs in the insect midgut, and are then present in oral secretions (116). The plant therefore perceives a molecule that is in part plant-derived. FACs in *Manduca sexta* OS in combination with leaf wounding trigger increased salicylic acid-induced protein kinase (SIPK) activity, jasmonic acid levels and trypsin proteinase inhibitor activity (TPI) in *Nicotiana attenuata* (117). SIPK and jasmonic acid are components of plant immune signaling and TPI are proteins involved in anti-herbivore defense (118; 115; 119). FACs are found in crickets (*Teleogryllus taiwanemma*) and fruitflies (*Drosophila melanogaster*), so could be present more widely in insect species, and be used by plants to detect a wider range of insects than lepidopterans alone (120). Ideal elicitors should be shared amongst pathogens or pests of the same type in this way, and should also be needed by the pest so as to be evolutionarily conserved and unlikely to change in response to selection pressures induced by plant recognition. As FACs play an essential role in insect nitrogen metabolism, they provide an ideal elicitor for plant perception as FACs may be difficult for insects to change or stop producing (121).

Other elicitors identified in OS include caeliferins, glucose oxidases (GOX) and inceptins (122; 123; 124; 125). Interestingly, inceptins are also plant-derived elicitors, composed of a fragment of chloroplastic ATP synthase subunit (123; 126). Though elicitors such as these from chewing insects have been identified, less is known about potential elicitors from piercing and sucking insects such as aphids. Experiments using whole Russian wheat aphid (*D. noxia*) have shown that the proteins from the aphids alone when injected into plants can cause defense responses (127). Salivary components of GPA between 3 and 10 kD induce defense responses in Arabidopsis, quantified as a decrease in aphid fecundity on saliva-infiltrated leaves (47). GOX has been found in aphid saliva (34), so may be acting as an elicitor from both aphids and lepidopterans (124; 125).

Plant-derived molecules released when pests or pathogens attack plants can also act as elicitors of defense responses. These are known as damage associated molecular patterns (DAMPs). An example of this is systemin, a peptide found only in Solanaceae species including potato, tomato and pepper (128). Systemin is cleaved from a precursor protein, prosystemin, upon wounding by chewing plant pests and induces the synthesis of both jasmonic acid and proteinase inhibitors, as well as increasing the release of plant volatiles that attract parasitoid wasps (129; 130; 128). Aphids may also cause DAMP production in plants; glycoproteins that are produced in wheat infested with *D. noxia* can induce defense responses in wheat plants that have not been exposed to the aphids, suggesting the

production of a compound in wheat under aphid infestations that activates immunity like a DAMP (131). The damage-associated molecular pattern peptide 1 (AtPep1), is a well-studied DAMP in Arabidopsis that is derived from the precursor protein AtproPep1. The transcription of its gene is strongly induced in response to cell wall degradation, wounding, jasmonate, ethylene or general elicitor recognition (132). Pathogen attack leads to production of Pep1 via cleavage of AtproPep1. Pep1 is then perceived by immune receptors, leading to a heightened immune response, including induction of the defense gene defensin (132). Receptor-mediated defense-signaling peptides such as AtPep1 and systemin are used by plants to amplify signaling initiated by wounding and molecular patterns from pathogens and pests, ensuring an effective response to biotic stresses.

Plants detect elicitors using pattern recognition receptors (PRRs). These are generally plasma membrane associated via a transmembrane helix domain that anchors the receptor to the membrane. Many PRRs found so far are receptor-like kinases (RLKs) or receptor-like proteins (RLPs); the latter differs from RLKs by lacking an intracellular kinase domain. RLKs and RLPs are sorted into families based on their extracellular domain, which is responsible for elicitor binding. Extracellular domains include those with leucine-rich repeats (LRRs), carbohydrate-binding domains, lectin-binding domains and lysin motifs (LysM) (133). A LRR-RLK, SR160 from wild tomato (*Lycopersicon peruvianum*) has been identified as a PRR for the DAMP systemin (134), but some studies have thrown this into question, suggesting that systemin is principally perceived by another, as yet unknown, receptor (135). So far SR160 is the only PRR implicated in the perception of insect pests, though given the identification of several different insect elicitors, it is likely that plants possess a variety of PRRs able to detect them. Intriguingly, a locus in rice that confers resistance to the brown planthopper (*Nilaparvata lugens*) has been found to be a cluster of three genes encoding plasma-membrane localised lectin receptor kinases (called OsLecRK1-RK3) (136). These LecRKs may therefore act as PRRs for the planthopper, perceiving HAMPs or DAMPs to activate plant immunity. Further analyses of the biochemical function of these receptor proteins is needed to verify true receptor function.

In *Arabidopsis thaliana*, the best studied PRRs are the LRR-RLKs FLAGELLIN SENSING2 (FLS2) and EF-TU RECEPTOR (EFR), which detect the bacterial PAMPs flg22 and elf18/elf26 respectively (137; 138). Flg22 is a 22 amino-acid fragment of the flagellar protein of bacteria, and the elf peptides are derived from bacterial elongation factor EF-Tu (139; 140; 141). Flg22 has been adopted for use in investigating PTI due to the consistent responses that it causes. The DAMP Pep1 is also perceived by LRR-LRKs. In

this case, there are two: PEP RECEPTOR1 (PEPR1) and PEPR2 (142). FLS2, EFR and PEPR1/2 all require interaction with the LRR-RLK BAK1/SERK3 (BRI1-ASSOCIATED RECEPTOR-LIKE KINASE/SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3) for immune signaling to occur. BAK1 is also needed for the immune responses triggered by other elicitors including peptidoglycans, lipopolysaccharides and the oomycete PAMP INF1 (143; 144). BAK1 can therefore be seen as a central regulator of the plant response to elicitors, interacting with individual receptors and then activating defense pathways.

BAK1 was originally identified as the co-receptor of BRI1 (brassinosteroid-insensitive 1), a cell membrane located receptor for the steroid phytohormones brassinosteroids (BR). Upon BR binding, BRI1 and BAK1 physically associate, leading to full activation of BR signaling (145). It was later found that BAK1 also has a role in plant immune signaling, physically associating with FLS2 and EFR after the PRR has bound its respective PAMP (146). *A. thaliana* and *N. benthamiana* plants defective in BAK1 are less sensitive to flg22 treatment, on top of the defects caused in BR signaling (143). This leads to the need for the study of BAK1 functions independently of each other. A BAK1 mutant, *bak1-5*, is deficient only in immune signaling, and not BR signaling or cell death responses (147). This mutant is therefore used in investigations into plant immunity. Not all defense responses are dependent on BAK1. The response to fungal chitin in *A. thaliana* is dependent on the LysM-receptor kinase CERK1, which does not interact with BAK1 (148).

The method of flg22 perception and induction of immune signaling by FLS2 and BAK1 has been well studied, and many of the steps involved are known. The current model for perception of flg22 (see Figure 1.5) begins with the binding of the PAMP to FLS2 at its external LRR portion independently of BAK1 (138; 149). Binding to flg22 induces association of FLS2 with BAK1, and the two proteins intracellular kinase domains interact, leading to trans-phosphorylation events between FLS2 and BAK1 (146; 149; 150). BAK1 then phosphorylates the receptor-like cytoplasmic kinase BOTRYTIS-INDUCED KINASE-1 (BIK1), which in turn transphosphorylates the FLS2-BAK1 complex to fully activate it. Phosphorylated BIK1 is released from the FLS2-BAK1 complex to activate downstream immune signaling (151). Interestingly, BIK1 has also been implicated in Arabidopsis resistance to GPA, though it was seen to have a negative effect on plant defense against the aphid, suggesting BIK1 may not necessarily act in the same pathways in insect immunity as those seen for pathogens (152).

After elicitor activation, downregulation of PRR signaling is needed to prevent excessive

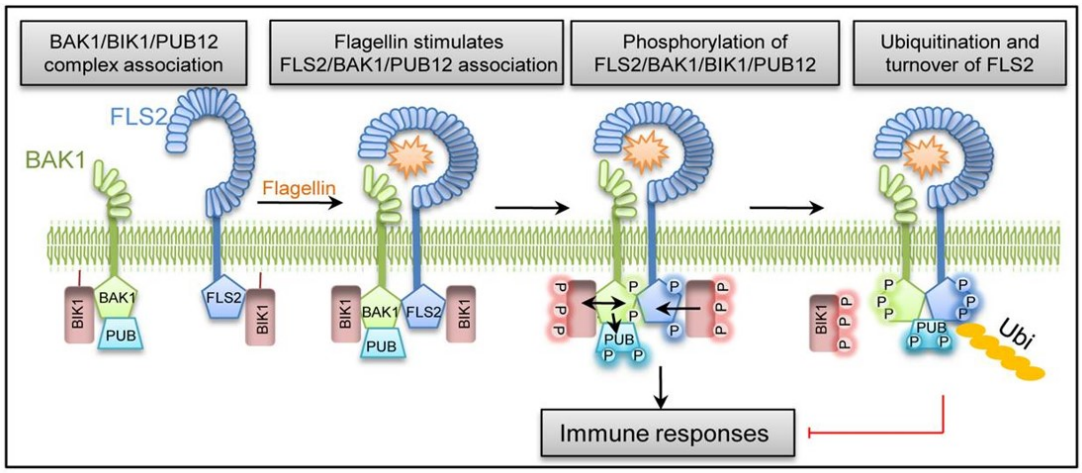


Figure 1.5: **The model for perception of flg22 by FLS2 and BAK1**. Adapted from Lu et al. 2010 (151).

or prolonged activation of immune responses which would be detrimental to the plant host. This is believed to occur via the addition of ubiquitin groups, which marks a protein for degradation or relocalisation (153). Ubiquitination is mediated by a three step enzymatic cascade that consists of the activating (E1), conjugating (E2) and ligating (E3) enzymes (154). It is the E3 ubiquitin ligases that specify substrate specificity. The plant U-box (PUB) E3 ubiquitin ligases PUB12 and PUB13 interact constitutively with BAK1 via its intracellular domain. After BAK1 activation in the presence of flg22, BAK1 phosphorylates PUB12 and PUB13 causing the E3 ligases to associate with and ubiquitinate FLS2 (155). This is linked to a decrease in FLS2 levels upon flg22 stimulation. The ubiquitination is believed to lead to FLS2 degradation via endocytosis of the receptor and intracellular trafficking to the vacuole (156; 157).

Interestingly, despite a lack of identified insect PRRs, BAK1 has been linked to the plant response to insect herbivores. BAK1 is involved in the response of *N. attenuata* to its specialist herbivore *Manduca sexta*, as NaBAK1 silenced plants accumulate less jasmonates (110). However, MAPK activity was not affected in these plants, in contrast with flg22-induced defense responses, where BAK1 is required for activation of MAP kinase signaling (143). This again suggests that although pathogen immune components such as BAK1 are involved in the response to herbivory, they do not necessarily function in the same capacity to both biotic threats. As aphids are piercing and sucking insects, compared to the chewing herbivore *M. sexta*, their method of feeding may induce less wounding responses and more pathogen-like responses, with BAK1 being involved in a similar way upstream of MAPK cascades. This is something which requires further investigation.

In summary, though elicitors from insects have been identified that induce immune

responses in plants, their exact method of perception is unknown. In plant-pathogen interactions, elicitors are detected by cell membrane localised PRRs, some of which interact with the co-receptor BAK1. BAK1 was found to be required for the hormonal defense response to the chewing insect *M. sexta*. I found in my own investigations that protein elicitors from GPA were capable of inducing immune responses in Arabidopsis, some of which were dependent on BAK1 (Chapter 3). This suggested that there are PRR/s for the aphid. I investigated PRR candidates that were induced by aphid exposure and found two mutants that lose the defense response to GPA, suggesting that they are involved in aphid perception (Chapter 4). This data contributes to the evidence that insects are perceived by their plant hosts using PRRs that detect elicitors.

1.2.2 Early signaling components of elicitor triggered immunity

After the plant has perceived elicitors using cell surface receptors and their signaling partners, multiple downstream events are set into action. These have been widely studied in the context of PAMP perception, and immune responses downstream of PAMP recognition are referred to as PAMP-triggered immunity, or PTI. Components that form a part of PTI are also found to be shared in the responses to insect elicitors.

The earliest documented plant responses to herbivore contact are ion fluxes at the cell membrane in the damaged site. Herbivore feeding leads to calcium influx around the wounded zone which is different to that induced by wounding alone (158; 159; 160). This suggests that there is a degree of specificity to the response which may, in part, be defined by the insect elicitor. In support of this, FAC elicitor N-acyl-glutamines from *Spodoptera littoralis* were found to trigger calcium influx when supplied alone to soybean cells (161). GPA on Arabidopsis and *Diuraphis noxia* on wheat have both been found to induce increased expression of calcium binding proteins that are involved in plant defense signaling, suggesting that calcium fluxes also have a role in the defense response to aphids (162).

In PTI, the first changes to occur are also large ion fluxes across the plasma membrane. The largest of these is calcium influx from the apoplast causing a rapid increase in cytosolic calcium concentrations, termed the calcium burst (163; 164). These calcium fluctuations are an integral part of the plant defense response (165). Cyclic nucleotide gated channels (CNGCs) facilitate calcium influx into the cytosol (166). Glutamate-like receptors (GLRs) have also been proposed to function in the calcium flux of plant defense signaling (167; 168; 169). The link between elicitor perception by PRRs and calcium eleva-

tion is not fully understood. Work on Pep perception by PEPR1 and PEPR2 suggests that these receptors may activate CNGCs by cyclic nucleotide signaling, as they have a guanylyl cyclase catalytic domain that is capable of producing cGMP (170). There are differences between PRRs though, as PEPR perception requires an extracellular calcium source and a functional CNGC2, whereas flg22-induced FLS2 signaling does not need these, suggesting intracellular calcium stores are used (170). The receptor-like cytoplasmic kinase BIK1 has recently been implicated in control of flg22-induced calcium influx, though the components BIK1 interacts with in order to do this are unknown (171). Little is known about the channels involved in the calcium response to insects, though several GLRs known to mediate calcium entry into plant cells have been identified as required for wound-induced signaling (172; 173).

Another early defense response is the rapid and transient production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide, singlet oxygen and hydroxyl radicals. These act in several parts of the defense response both as secondary messengers during defense signal transduction and as direct antimicrobial agents (174). ROS release is induced by herbivore feeding and forms part of the early signaling response to both chewing and phloem feeding insects (175; 176; 177). In Arabidopsis, the ROS response to PAMPs is due to the action of respiratory burst oxidase homologues AtRBOHD and AtRBOHF (178). Both *rbohD* and *rbohF* mutant plants show increased susceptibility to GPA (179; 177), suggesting that the ROS produced by these NADPH oxidases is also required for an effective defense response against aphids.

The steps between PRR activation and ROS production in flg22/EF-Tu perception have been identified (see Figure 1.6). It is BIK1 that provides the link between the PRR/elicitor complex and RBOHD activation. After phosphorylation by the PRR complex, BIK1 directly interacts with and phosphorylates RBOHD, leading to its activation and ROS production (180; 171). Interestingly, calcium fluxes also contribute to RBOHD activation. Calcium affects RBOHD function via the activation of calcium-dependent protein kinases (CPKs), which also phosphorylate and activate RBOHD (181; 182). Phosphorylation by CPKs occurs at separate locations to phosphorylation by BIK1 (180). Calcium can also directly bind to RBOHD via EF-hand motifs in the cytosolic N-terminal region of the protein and activate ROS production. However RBOHD phosphorylation has to occur prior to calcium-binding in order for RBOHD activation to take place (182; 183). It is suggested that BIK1 phosphorylation therefore "primes" RBOHD for regulation via calcium (180). Many cellular events use calcium as a signal, so this may act as a failsafe to ensure that the immune

related ROS burst is only triggered upon BIK1 activation after elicitor perception.

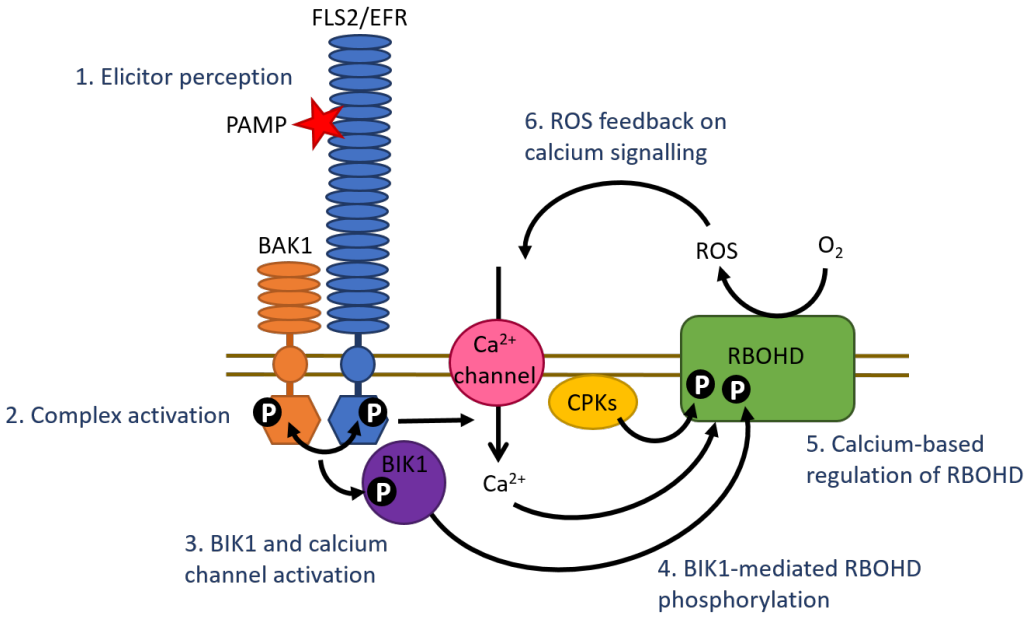


Figure 1.6: **Interaction between calcium and ROS signaling and RBOHD activation in PTI**. P = phosphorylation. Adapted from Kadota et al. 2014 (180).

ROS may feed back on calcium signaling. In some systems, ROS have been found to activate calcium channels (167). ROS is also able to stimulate CPK5 phosphorylation of RBOHD (181). This interaction of calcium and ROS signaling has been identified as a potential means for cell-to-cell signal propagation in plants, with ROS acting as the potential extracellular signal (167; 184). Interestingly, electrical signals have also been identified as having a role in cell to cell defense signaling. Several GLRs, GLR3.2, 3.3 and 3.6, have been identified that have a crucial role in long-distance signaling in response to wounding (172). These channels are capable of controlling cytosolic calcium influxes (173), and are responsible for wound activated surface potential charges (WASP) that are linked to defense responses in tissues away from the wound site (172). Immune signaling is therefore not just important for activating plant defense responses locally, but also for inducing systemic responses.

Segonzac et al. found that after the calcium influx, two distinct branches of signaling occur in *N. benthamiana*, with one leading to ROS production and the other to the activation of mitogen-activated protein kinases (MAPKs) and downstream transcriptional changes leading to defense against a bacterial pathogen (185). MAPK cascades form an integral part of the plant defense response. These involve the sequential phosphorylation of various MPKs (MAP kinases) within the cytoplasm. MAPK cascades act as key components in the regulation of transcriptional changes in cells that have detected a pathogen. In Ara-

bidopsis, MPK6 and MPK3 are both phosphorylated upon flg22 treatment, leading to the activation of the defense-related WRKY transcription factors (186). Their orthologs in tobacco and *N. benthamiana*, SIPK and WIPK (salicylic and wound activated protein kinases) are also activated in response to pathogen detection (187; 188). Activation of SIPK and WIPK in *Nicotiana attenuata* occurs in response to the elicitors found in *Manduca sexta* oral secretions, leading to an increase in jasmonic acid biosynthesis (189). Grasshopper oral secretions increase both calcium levels and MPK3 and MPK6 activity in Arabidopsis (190), showing conservation of these signaling components in the response to biotic stresses.

MAPK cascades are not only induced by ROS but can also regulate ROS production (191). In *N. benthamiana*, ROS production by the NADPH oxidase RBOHB in response to the oomycete elicitor INF1 requires two MAPK cascades (192). Recently, another signaling pathway has been identified upstream of MAPK activation involving Arabidopsis heterotrimeric G-protein subunits, which are involved in the recognition of a bacterial protease elicitor (193). G-protein subunits are also needed for the ROS burst response to other pathogens (194; 195), suggesting that G-proteins may be one of the main players in signaling downstream of elicitor perception. The involvement of G-proteins in plant responses to insects has not yet been studied.

To sum, calcium and ROS bursts and MAP kinase cascades are all induced by insect feeding. These signaling components also play a vital role in the defense response to pathogens, where their interactions have been better characterised. I found that a ROS burst is induced to aphid elicitors independent of insect feeding and that the NADPH oxidase RBOHD was required for the ROS response to aphids (Chapter 3). I also found an involvement of the G-protein β subunit in the ROS response to GPA, indicating G-protein regulation of aphid elicitor responses (Chapter 4). This work has identified key signaling pathways that are involved in the recognition of aphids on a molecular level and suggests a conservation of components between signaling in response to aphids and pathogens.

1.2.3 Plant defense responses

After initial calcium and ROS bursts, and MAPK cascades leading to the activation of transcription factors, defense responses downstream include hormonal signaling, the deposition of callose and production of antimicrobial compounds such as glucosinolates and camalexin. Callose is a polysaccharide composed of glucose residues joined by β-1,3-linkages that is deposited between the cell wall and membrane after pathogen attack. It forms a matrix that

antimicrobial compounds can be deposited in, allowing focused delivery of these chemical defenses to specific sites of attack. The buildup of callose also reinforces the cell wall, helping to prevent invasion of the cell. Callose is synthesised by a group of enzymes known as callose synthases. In *Arabidopsis thaliana* twelve genes encoding putative callose synthases have been found called glucan synthase-like 1-12 (AtGSL1-12) (196; 197). GSL5 is responsible for the synthesis of wound- and pathogen-induced callose in leaf tissue, and loss of function mutants in this gene are unable to synthesise callose at sites of fungal infection (198). Unexpectedly, *gls5* mutants are more resistant to some pathogens, rather than more susceptible (199). This was found to be because callose suppresses the salicylic acid signaling pathway, showing that there is regulation between different defense responses. Not only do growth and pathogen response need to be balanced in the plant, but different forms of defense response too.

Callose deposition in plants has been found in response to the PAMPs flg22, EF-Tu and chitin, amongst others, as well as in response to DAMPs, such as oligogalacturonides. Callose is also deposited in response to aphid feeding, though it is not known whether this is a wounding response or a reaction to elicitor perception (175). The callose response to aphids in barley is dependent on the species of aphid, with the Russian wheat aphid inducing stronger deposition than bird cherry-oat aphid (200). This suggests that some aphid species can prevent or attenuate the plant callose response. Callose deposition on the sieve plates of phloem in rice is an important mechanism of resistance against the brown planthopper. This callose-mediated resistance is linked to the action of two resistance genes, Bph14 and Bph15 (201; 90; 202).

ROS produced by RBOHD have been found to be upstream of callose deposition, tying it into the PTI response as a whole (203). Callose deposition is also influenced by the phytohormone abscisic acid (ABA), which is implicated in the response to pathogen attack. Defense against the necrotrophic fungus *Plectosphaerella cucumerina* in *Arabidopsis* is dependent upon both callose synthesis and ABA signalling (204). ABA does not directly regulate callose deposition, but modulates the speed and intensity at which it is laid down. ABA therefore has a priming role in callose deposition. In contrast, other groups have reported that ABA suppresses callose deposition in plants after elicitor challenge (205). Luna et al. found that the effect of ABA on callose deposition changed between repressive and stimulatory depending on the growth conditions of the plant (206). The differences seen in ABA-callose interaction are therefore due to environmental conditions, allowing adaptation to abiotic stresses. The pathways controlling callose deposition also differ depending on the

PAMP the plant is challenged with; flg22-induced deposition is dependent on H_2O_2 production, whereas the callose response to chitosan is not (206). These results demonstrate that callose deposition is a complex response, not just dependent upon the perception of a pathogen, but the type of pathogen and the abiotic conditions that the plant is subjected to.

Phytohormones in addition to ABA, such as salicylic acid (SA) and jasmonic acid (JA) also have a role in the plant immune response to pathogens. They enable systemic signals to be propagated throughout the plant (207). Jasmonic acid is seen to play a central role in regulating plant defense responses to herbivores (208). Tissue-chewing insects induce JA accumulation and mutants affected in JA synthesis or signaling show compromised resistance to a wide range of insects including caterpillars, beetles, thrips and leafhoppers (209; 208). In response to pathogens, it is thought that SA signaling triggers resistance against biotrophic or hemibiotrophic pathogens, and a combination of JA and ethylene signaling activates defense pathways against necrotrophic pathogens (210). JA and SA pathways are antagonistic to each other; elevated biotroph resistance therefore leads to enhanced necrotroph susceptibility and vice versa (207). Diezel et al. found that the balance between JA and SA responses to lepidopteran herbivores differs on whether the herbivore is a specialist or a generalist, with specialists inducing JA responses and generalists SA responses (211). The balance of SA and JA during plant-pest/pathogen interactions can therefore be seen to be important in the outcome of infection.

The gaseous phytohormone ethylene also plays a role in insect defense. Ethylene is required for cysteine-protease-mediated resistance to the corn leaf aphid (*Rhopalosiphum maidis*) in maize independently of JA (212). In *N. attenuata*, ethylene is induced upon attack by the specialist herbivore *Manduca sexta* (211). This ethylene burst suppresses SA production, allowing JA-mediated defense activation. It is suggested that this plant response allows JA defense induction in situations where SA is induced by the herbivore. For example, *Arabidopsis* resistance to GPA is mainly jasmonate dependent, as the aphids do better on jasmonate-insensitive mutants (213; 214). However GPA also induces a SA response (213). This is suggested to be a strategy of the aphid to prevent the plant from mounting an effective defense by using the antagonism between the SA and JA pathways (215). In this way, aphids can manipulate the plant defense response to allow continuous feeding. Manipulation of hormones in order to take advantage of crosstalk between defense pathways is also carried out by plant pathogens (216). For example the biotrophic bacterial pathogen *Pseudomonas syringae* produces the phytotoxin coronatine, which acts as a JA mimic. This suppresses SA-dependent defenses which are required for full resistance to the

bacteria (217; 218).

Major players in the plant defense response to insects are secondary metabolites. Evolution of the huge diversity of plant secondary metabolites is thought to be due to coevolution with herbivores (219). In plants of the order Brassicales, to which *Arabidopsis thaliana* belongs, glucosinolates are the main defense compounds. Around 120 different glucosinolates have been identified, with *A. thaliana* having at least 37 (220; 221). Glucosinolates are derived from glucose and an amino acid, generally methionine, tryptophan or phenylalanine. Upon plant damage, glucosinolates that are stored within plant cells come into contact with the enzyme myrosinase, which converts glucosinolates to their active form as isothiocyanates (222), which are deterrents to generalist herbivores (223). Although glucosinolate production is constitutively active, herbivore (including aphid) feeding increases glucosinolate levels indicating that plants also regulate production of these toxic compounds upon insect perception (224; 225; 226). Glucosinolate hydrolysis has a role in innate immunity against pathogens. The atypical myrosinase PENETRATION2 (PEN2) is induced by PAMP treatment and is required for antifungal defense (227; 228). PEN2 products are believed to act as signaling molecules or activators of callose deposition, as well as having a direct antimicrobial effect (205).

GPA avoids plants with higher glucosinolate contents than normal due to overexpression of the IQD1 transcription factor, which is normally induced on aphid infestation (229). This suggests glucosinolates play a role in aphid host plant selection. Though aphids do not cause much tissue damage when feeding, breakdown products of indolic glucosinolates are present in aphids and their honeydew, suggesting that glucosinolates are broken down in aphids in a myrosinase independent manner, or may be altered in the phloem prior to ingestion (230). The breakdown produces have antifeedant effects against GPA. GPA is not only repelled by glucosinolates, but also experiences reduced growth and fecundity with increased glucosinolate content in *Arabidopsis* defense-related mutants (231). Interestingly, the cabbage aphid *B. brassicae* sequesters plant glucosinolates and uses them in combination with aphid-synthesized myrosinases for protection against predators such as hoverflies and ladybirds (232; 233; 234). In this way, a part of the plant defense process has been co-opted to perform as a part of the aphids own defense. Nevertheless, even *B. brassicae* reproduction is negatively correlated with the glucosinolate content of plants (231).

Although glucosinolates are a well-known set of secondary metabolites in Arabidopsis, there are also other compounds present that may have an effect on aphids. Indole glu-

cosinolates and camalexin, an Arabidopsis phytoalexin, are both derived from tryptophan. There is evidence that camalexin may have a role in defense against some aphid species. PAD3, a cytochrome P450 monooxygenase which catalyses the final steps in the biosynthesis of camalexin (235; 236; 237), is induced by aphid feeding, leading to higher camalexin levels in the plant (175; 47). Camalexin has an effect on aphid fitness, as aphid fecundity increases on *pad3* mutant lines of Arabidopsis (175; 238; 235). Camalexin is also induced by microbial elicitors, and is required for resistance to microbial pathogens in Arabidopsis (239; 240; 241).

Direct chemical defenses can be effective at discouraging insects from feeding on plants, or actively affecting the insect in a negative way as they feed upon the plant. Plants also use indirect chemical defenses against insects, such as herbivore induced plant volatiles (HIPV), which are released into the air after attack from herbivores (242). The volatile blend can be made up of many different compounds, with common compounds including terpenoids, fatty acid derivatives, phenyl propanoids and benzenoids (243). Emission of HIPVs can lead to the attraction of carnivorous arthropods or parasitoids which prey on the herbivore that is eating the plant. Soybean plants infested with soybean aphids (*Aphis glycines*) release the compound methyl salicylate, which attracts the predatory seven-spotted ladybird, *Coccinella septempunctata* (244). Hoverflies also detect plant volatiles; (Z)-3-hexenol, an aldehyde HIPV released by *Vicia faba*, was found to increase the chances of hoverfly oviposition (245). The aphid alarm pheromone E-β-farnesene (EBF) is detected by hoverflies and used as a cue for aphid presence on *Vicia fabia* plants (245). Interestingly, some plants are able to synthesise EBF, which acts as an aphid deterrent (103; 246). Volatiles are involved in pathogen resistance. Green leaf volatiles are induced by *Botrytis cinerea* infection in Arabidopsis, and contribute to resistance against the fungus (247). The mode of action is unknown, but could be direct antifungal properties of the volatiles, or increased activation of defense responses such as accumulation of phytoalexins or cell wall reinforcement (248; 249). As with direct chemical defenses, some aphids can overcome indirect defenses. The pea aphid *Acyrthosiphon pisum* is able to feed on its host plant, *Vicia faba*, without inducing detectable changes in plant volatile organic compound emission. In co-infestations with beet armyworm caterpillars, *A. pisum* is able to reduce caterpillar-induced HIPVs, showing an active inhibition of the plant HIPV response (250).

Multiple defense responses are induced downstream of the signaling events that take place after elicitor perception, including callose deposition, phytoalexin synthesis and volatile release. I found that exposure to aphid elicitors caused PEN2-dependent callose deposition

and induction of defense genes such as PAD3, which is involved in camalexin synthesis (Chapter 3). Camalexin is an important component of Arabidopsis defense against GPA (Chapter 3). I also found that PAD3 was required for the GPA-induced ROS burst (Chapter 4), suggesting feedback on immune signaling. Results in this thesis show that defense responses shared with the response to pathogens can occur in response to aphid elicitors alone, and not just wounding caused by feeding. Not only are signaling pathways shared in the plant response to insects and pathogens, but downstream defenses are as well.

1.2.4 Effectors

Despite plant perception of elicitors leading to immune signaling and defense responses, herbivores and pathogens are still able to colonise their plant host. This suggests that pests and pathogens must be able to block the plant immune response to elicitors, either by suppressing plant defense signaling or avoiding detection. In plant-pathogen interactions many molecules, mainly proteins, have been found that fulfil this function, and they are termed effectors.

Gram-negative bacteria inject effector proteins through their type III secretion systems to aid pathogenesis in both animals and plants (251). Biotrophic fungi and oomycetes have also been found to deliver effector proteins inside plant cells. They do this with the use of haustoria; specialised hyphal structures that form within plant cells but remain surrounded by a modified cell membrane, known as the extrahaustorial membrane (EHM) (252; 253). How effectors move across the EHM after secretion by a filamentous pathogen is still not understood, though this may differ for different effectors and involve both host and pathogen factors (253). Plant parasitic nematodes can also deliver proteins to the inside of plant cells by using their feeding organs, stylets (254). Herbivores are able to manipulate plant defense responses; for instance the spider mite *Tetranychus evansi* is able to suppress SA and JA signaling pathways in tomato, as well as reducing the release of plant volatiles (255). This suggests that herbivores are also capable of introducing effectors into plants to allow successful colonisation. It is thought that insects introduce effectors into plants when they feed, in saliva. It is known that aphids secrete saliva into the plant as they probe and before taking up any phloem sap (28). The oral secretions of chewing herbivores contain elicitors which are perceived by the plant, so effectors may also be introduced in this way.

One such effector found in oral secretions of *Manduca sexta* is glucose oxidase (GOX). This enzyme acts as an elicitor in tomato (125), but in *Nicotiana tabacum* suppresses the

production of nicotine, preventing resistance against the insect (256; 257). GOX is also present in the saliva of other insect species, including caterpillars and aphids, so may also be used for defense suppression by other insects (258; 34). Aphid saliva suppresses plant defenses, such as the clogging of sieve elements (29). Occlusion of sieve elements in response to insect feeding is thought be due to an influx of calcium caused by wounding (27). Aphid saliva contains calcium-binding proteins that may prevent this calcium signaling in the phloem and enable continued feeding (29).

Screening of proteins in aphid saliva has found several candidate effectors, including some calcium-binding proteins (39; 46). An aphid protein found in *A. pisum* saliva, C002, is essential for the feeding of the aphid on fava bean (41). C002 does not match any proteins found in insects outside of the Aphididae family, suggesting a specific role in aphid feeding. Bos et al. (46) also identified C002 as a potential effector, and showed that overexpression of the GPA C002 in *N. benthamiana* enhanced aphid performance, supporting the idea that C002 is important in aphid virulence. GPA also shows increased fecundity on *A. thaliana* stably expressing MpC002 (44). Other candidate effectors, Mp1 and Mp2, also increase aphid fecundity when expressed in *A. thaliana*, though the *A. pisum* homologs Ap1 and Ap2 do not (44). Mp10 was identified as a potential GPA effector by Bos et al. Mp10 is able to suppress the ROS burst elicited by flg22, indicating a suppression of the plant immune system (46). Recently, another protein found within aphid saliva, macrophage migration inhibitory factor (MIF), was also found to be required for aphid success. Expression of MIF in *N. benthamiana* prevents elicitor-induced plant immune responses, such as callose deposition and defense gene induction (259).

Though insect effectors such as GOX, MIF and C002 have been identified, their plant targets are still unknown. Effectors from plant pathogens have been found to target a range of plant immune processes via interaction with different plant targets (260). The receptors involved in elicitor recognition are a target for several different effectors. One of the best studied bacterial effectors, AvrPtoB from *Pseudomonas syringae*, targets and binds to BAK1 (261). This interferes with flg22-induced BAK1-FLS2 association, leading to a suppression of defense responses and allowing bacterial colonisation of the plant (262). As BAK1 is also a signaling partner in the immune responses to other elicitors, multiple other defense pathways can be effected. *P. syringae* has another effector that targets BAK1, HopF2, showing that BAK1 is an important target (263). AvrPtoB is also able to affect immunity by ubiquitinating the immune receptors CERK1 and FLS2, which targets them for degradation (264; 265). Effectors can therefore have more than one target in the plant.

The signaling components involved in plant innate immunity are targeted by pathogen effectors. *P. syringae* effector AvrPphB is a cysteine protease that targets and cleaves receptor-like cytoplasmic kinases (RLCKs), including BIK1 (266). BIK1 has a central role in RBOHD activation downstream of elicitor perception, which leads to ROS production (180; 171). AvrPphB action therefore interferes with elicitor-induced immune signaling. Corn smut *Ustilago maydis* inhibits the ROS burst upon host plant invasion via the action of its secreted effector Pep1, which directly targets maize peroxidase (POX12) (267). The MAPK cascade is also targeted by pathogen effectors at different steps in the signaling cascade. HopAI1 from *P. syringae* encodes a phosphothreonine lyase that alters the threonine residues of MAPKs, including MPK3 and MPK6 (268). This irreversibly blocks phosphorylation and so prevents defense signaling.

Another area that pathogen effectors are known to target is vesicle trafficking, which is how antimicrobial compounds are directed towards the site of pathogen infection (269). Effectors can target proteins involved incellular transport of these compounds, for instance, the *P. syringae* effector HopM1 targets the Arabidopsis protein MIN7, an ARF-GEF (adenosine diphosphate ribosylation factor, guanine nucleotide exchange factor) involved in vesicle trafficking, including that involved in callose deposition. HopM1 binds MIN7 and acts as an adapter to recruit plant ubiquitination machinery, which leads to MIN7 degradation by the 26S proteasome (270). The *Blumeria graminis* effector candidate BEC4 interacts with an ARF-GAP (ADP ribosylation factor-GTPase-activating protein) and an ubiquitin conjugating enzyme from barley (271). ARF-GAPs act in opposition to ARF-GEFs in the control of vesicle trafficking (272), and the one identified in barley is required for full resistance to powdery mildew infection (271). This suggests that BEC4 also targets vesicle trafficking to interfere with defense and allow fungal invasion of the plant.

Investigations into pathogen effectors have found plant targets throughout the plant immune pathway, from elicitor perception to secretion of antimicrobial compounds. Though several potential insect effectors have been identified, their targets have not. I investigated the GPA effector Mp10, and found that it was required for aphid success on Arabidopsis and could block immune signaling to both the bacterial elicitor flg22 and GPA elicitors (Chapter 5). I also investigated Mp10 structure and found two residues important for its effector function (Chapter 5). A plant target for Mp10 was identified via yeast two hybrid, and initial investigations suggest that it may be involved in immune receptor trafficking (Chapter 6). My research has thus shed further light on the molecular interaction between

plant and aphid.

1.2.5 Effector-triggered immunity

Effectors interfere with plant innate immunity, leading to effector-triggered susceptibility (ETS). Plants have responded to pathogen use of effectors with the evolution of cytoplasmic resistance (R)-proteins, which can recognise the presence of effectors in the plant cell either directly or indirectly. This reinstates the immune response, leading to effector-triggered immunity (ETI) which is generally associated with programmed cell death, also known as a hypersensitive response (HR). (273). As a result of the selection pressure that ETI creates, pathogen isolates can evolve that either lose or alter the effector that causes an immune response. New plant receptors for these can evolve in turn. Coevolution such as this continues, with continuous selection in the pathogen for new effector proteins that will overcome ETI, and selection for new R-proteins that will reinstate ETI in the plant. Plant immunity can therefore be seen as a zigzag model, where resistance and pathogenicity evolve in a gene-for-gene (or protein-for-protein) manner (see Figure 1.7) (273; 274).

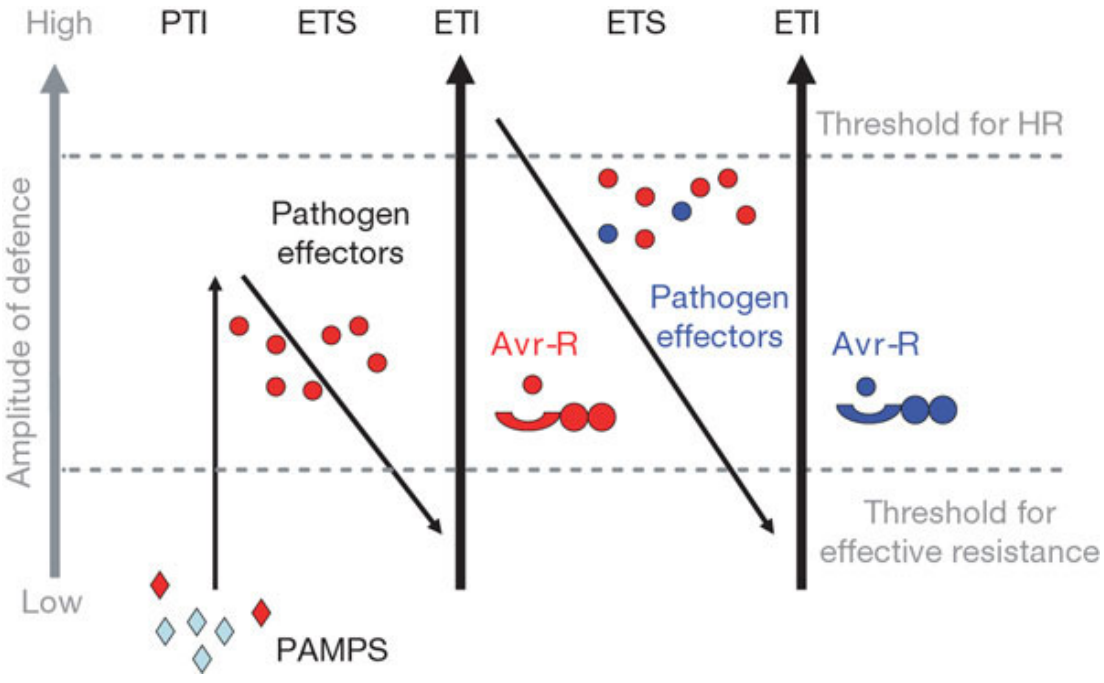


Figure 1.7: **The zigzag model of plant immunity**. In phase 1, plants detect PAMPs via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, leading to effector-triggered susceptibility (ETS). In phase 3, an effector is recognized by an NBS-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the recognised effector, and perhaps gained new effectors which can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. Taken from Jones and Dangl, 2006. (273).

Not much is known about the signaling involved in ETI, though SA and JA seem to

have a role, as well as transcription factors from the WRKY and TGA families (273). These components are also involved in PTI, suggesting that PTI and ETI are overlapping responses (275). The main assumed difference between PTI and ETI is that the immune response in ETI occurs earlier than in PTI, as well as acting for longer and more aggressively, for instance leading to the cell death response (275). However, PAMPs such as flg22 also cause HR responses (276). 'Weak' ETI has also been described, in which resistance is not as strong as other ETI responses, for instance the resistance in *Arabidopsis* against *P. syringae* mediated by RPS4 (277; 278). This, taken together with the fact that many parts of the signaling pathways in both PTI and ETI overlap, suggests that perhaps PTI and ETI are not distinct defense responses, but a continuum of defense (278).

R-proteins are generally intracellular receptor proteins of the nucleotide binding-leucine rich repeat (NBS-LRR) type, with a coiled-coil (CC) or toll interleukin 1 (TIR) domain at their N-termini (279). Several genes encoding NB-LRR proteins have been found that confer resistance to insect herbivores; Mi-1, Vat and Bph14, 15 and 26. Mi-1 confers resistance to herbivores both above and below ground, (280; 81; 281; 282), Vat confers resistance to the melon-cotton aphid (*Aphis gossypii*) (83; 86) and Bph14/15/26 give resistance to the rice brown planthopper (*Nilaparvata lugens*) (90). These resistances are limited to specific clones of the insect species, so some insect biotypes are still be able to evade or suppress the defenses in plants with the R-genes, depending on the presence of possible effectors (24). This agrees with the model of gene-for-gene resistance in plants (273).

Mi-1 was the first cloned insect R-gene, giving resistance against root-knot nematodes, whiteflies, a psyllid and the potato aphid, *Macrosiphum euphorbiae* (81; 280; 281; 282). Mi-1 is a cytoplasmically-located coiled-coil type NB-LRR, with 2 coiled coil domains at its N-terminus. Mutational studies have found that this N-terminus has a role in preventing the cell death response when no pathogen is present, as without it there is constitutive cell death (283). The cell death response mediated by Mi-1 is important in defense against root-knot nematodes, but does not seem to be involved in defense against potato aphids, which can access the phloem of Mi-1 plants but show limited feeding and seem to die from starvation (284). Various proteins and signaling pathways are required for Mi-1 dependent resistance, including SA and MAPK pathways, WRKY-type transcription factors and SlSERK1, a tomato member of the SERK family of which BAK1 is also a member (285; 286). This shows that the Mi-1 R-gene response is tightly linked to plant defense responses, as these proteins and pathways are shared.

Though plant responses involved in Mi-1 mediated resistance are well characterised, the insect effector/s that the NB-LRR detects from insects are unknown. Effector protein/R-gene pairs have been found in plant-pathogen interactions. This work has discovered that detection by R-genes can be through direct interaction of the R-gene and effector or by indirect R-gene detection of plant cell perturbation that is caused by the effector. An example of direct detection is that between the RRS1-R gene in Arabidopsis, which confers resistance to several strains of the bacteria *Ralstonia solanacearum* (287). RRS1-R physically interacts with the *R. solanacearum* effector PopP2 in the plant cell nucleus, leading to induction of ETI (288).

Indirect detection of effectors by R-proteins is described as the "guard hypothesis", as R-proteins have been found to monitor specific plant proteins (guardees) (289; 290). An example of such a guardee is RIN4. RIN4 is implicated in both PTI and ETI immune responses, and specifically functions with the plasma membrane H^+ATPase to regulate stomatal aperture during bacterial attack, preventing pathogen entry (291; 292; 293; 294). In Arabidopsis, RIN4 is guarded by two NBS-LRR proteins; RPM1 and RPS2 (291; 295). RIN4 is targeted by the *P. syringae* cysteine protease effector AvrRpt2, which cleaves RIN4 at two sites (296). In the absence of RPS2, this suppresses PTI and promotes bacterial growth (297). However, in cells where RPS2 is present, cleavage of RIN4 activates RPS2 leading to ETI (298). The bacterial effectors AvrRpm1 and AvrB also target RIN4, in this case inducing its phosphorylation and leading to ETS (293). It is RPM1 that guards RIN4 in this case. The R-protein detects phosphorylation of RIN4, inducing ETI (299; 300). This system shows that multiple effectors can evolve to target the same host target, and in response to this multiple plant R-genes can develop which detect the different modifications that effectors cause. RIN4 also represents an example of a molecular hub in plant immunity, as its role in both PTI and ETI makes it an ideal target for effectors (301).

R-proteins also use decoys in order to detect effectors. These decoy proteins are non-functional in immune pathways but contain similar domains to effector targets, which effectors bind to (302). Kinases play crucial roles in PTI, both as PRRs and PRR-associated co-receptors, and so represent important targets for effectors (303). Plants have therefore evolved R-proteins to guard against perturbations to kinases involved in PTI. An example of this is the Pto-Prf complex found in tomato. Pto is a serine/threonine protein kinase decoy, which interacts with the NBS-LRR Prf (304). The Pto protein interacts directly with the bacterial effectors AvrPto and AvrPtoB, which both target immune receptors. Effector interaction by Pto triggers Pto/Prf complex activation, leading to ETI and the hypersensi-

tive response (305). Disguising pathogen receptors as effector virulence targets is therefore an effective means of maintaining resistance.

R-genes which mediate effector-triggered immunity act in various ways to restore plant resistance against biotic threats. R-proteins confer resistance to insect pests, suggesting that insect effectors are also detected by plants. These effectors have yet to be identified. Mp10 induces ETI-like responses when expressed *in planta* (46). I found that these defense responses were not dependent upon Mp10 effector action or SA (Chapter 5). A yeast two-hybrid screen of candidate aphid effectors revealed several interactions with plant R-proteins, which may be relevant for plant-insect interactions (Chapter 7). Results from this thesis strengthen the argument that effectors produced by aphids are capable of being recognised by the plant.

1.2.6 Focus and aims of research described in this thesis

Aphids induce defense responses in the plants that they feed upon, yet are also able to successfully colonise many species of plant. Saliva has been identified as an important component in the colonisation of plants by insects, providing a source of potential elicitors as well as containing proteins that may act as effectors to suppress plant defense responses. The identification of several classical NBS-LRR proteins that confer resistance to some aphid species suggests that effectors produced by aphids are detected by the plant in order to trigger ETI. These aspects of plant-aphid interactions mirror the PTI, ETS and ETI identified in plant-pathogen systems, though the molecular basis for plant-aphid interaction is unclear. Given the known involvement of plant immunity in plant-aphid interactions, we set out to investigate the interaction between aphids and the plant immune system at a molecular level. The primary aim of this research was to identify how aphids may both trigger and suppress plant immunity.

1.2.7 Overview of thesis contents

I began the investigation of the interaction between aphids and the plant immune system by examining whether GPA contains elicitors that induce defense responses in the model plant species *Arabidopsis thaliana* (Chapter 3). Having found that defense responses are induced by elicitors from GPA, I then attempted to characterise the elicitors based on chemical properties and the nature of the plant responses. I also investigated plant components that are involved in the perception of GPA elicitors. The LRR-RLK BAK1 was tested as a

potential coreceptor, and other receptor candidates were identified by screening RLPs and RLKs that were induced in Arabidopsis by insect exposure (Chapter 3 and Chapter 4). This screen identified three RLKs that may play a role in the defense response against aphids (Chapter 4). Together, this data provides evidence that aphids elicitors are perceived by plants using PRRs.

I also explored the immune signaling involved in the response to aphids, investigating whether ROS and components of ROS signaling identified in plant-pathogen interactions were involved downstream of aphid perception. I found that ROS were induced in response to GPA elicitors (Chapter 3). I also identified components in Arabidopsis that are required for the ROS response to aphids; the NADPH oxidase RBOHD and a plant G-protein subunit (Chapter 3 and Chapter 4). This work identified key signaling pathways that are involved in the recognition of aphids on a molecular level.

To discover the nature of the plant response to aphid perception, I looked at downstream defense responses induced by aphid elicitors, including the deposition of callose and induction of defense genes involved in the synthesis of camalexin (Chapter 3). Camalexin was found to be an important component of Arabidopsis defense against GPA (Chapter 3). I also investigated the link between these downstream responses and immune signaling, finding evidence of feedback responses (Chapter 4). Results in this thesis show that defense responses can occur to aphid elicitors alone, and components of the defense response to them are shared between insect herbivores and pathogens.

Having found that GPA elicitors were able to induce plant defense, I investigated the immune suppression activities of an aphid effector, Mp10. Mp10 has homology to chemosensory proteins (CSPs) found across insect species. I found that Mp10 was required for aphid success on Arabidopsis and could block the immune signaling response to elicitors (Chapter 5). I also investigated Mp10 structure by conducting mutant analysis and found two residues important for its effector function (Chapter 5). I used existing crystal structures of insect CSPs to model the structure of Mp10, and localised the two residues to areas within the CSP binding pocket, that may also be exposed to the protein exterior. A plant target for Mp10 was identified via yeast two hybrid, and initial investigations suggest that it may be a novel protein involved in immune receptor trafficking (Chapter 6). My research has thus shed further light on the interaction between plant and aphid at a molecular level, and uncovered a potential new component of immune receptor regulation.

Mp10 induces ETI-like responses when expressed in *Nicotiana benthamiana*, and I found that these defense responses were not dependent upon Mp10 effector action or salicylic acid (Chapter 5). A yeast two-hybrid screen of candidate aphid effectors revealed several interactions with plant R-proteins, which may be relevant for plant-insect interactions (Chapter 7). These findings strengthen the argument that effectors produced by aphids are capable of being recognised by the plant.

This study has shed light onto the complex molecular interactions that occur between plants and the aphids that feed on them. There is a key role for insect elicitor perception by PRRs, which induce defense signaling that utilizes components shared with the response to plant pathogens. Aphid ability to suppress these defenses, and also activate ETI was studied by focusing on one effector. In conclusion, this thesis provides a base of evidence supporting the roles of PTI, ETS and ETI in the plant-aphid interaction.

1.2.8 Contributions to thesis

All experiments in this thesis were conducted by me, unless acknowledged in the legend accompanying the figure. Contributions of those who shared data, expertise or knowledge are listed at the start of each results chapter (Chapters 3 to 7). Contributions of both plasmids and plant lines are acknowledged in Chapter 2.

Chapter 2

Materials and Methods

2.1 Insect maintenance conditions

Myzus persicae

A stock colony of the green peach aphid (GPA) *Myzus persicae* (RRes genotype O) (46) was continuously reared in 52 cm x 52 cm x 50 cm cages containing up to six Chinese cabbage plants (*Brassica rapa*, subspecies *chinensis*) with a 14 h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$ at 18°C) and a 10 h night (15°C) photoperiod.

Before use in aphid performance experiments, aged *Myzus persicae* were produced by placing 10-20 adults from the *B. rapa* colony onto a 5-week old *Arabidopsis thaliana* Col-0 plant. These were caged onto the plant inside clear plastic tubing (10 cm diameter, 15 cm tall) (Jetran Tubing, Bell Packaging, UK), which was pushed inside the soil of the pot and capped at the top with white gauze-covered plastic lid. After 24 hours, the adults were removed, leaving nymphs which were all of the same age. These were then allowed to mature to the stage at which they were needed in the experiment. This was generally 10 days, at which the nymphs would be fully grown aphids and begin producing nymphs.

Acyrthosiphon pisum

A stock colony of the pea aphid, *Acyrthosiphon pisum*, was continuously reared in 52 cm x 52 cm x 50cm cages containing up to four broad bean (*Vicia faba*) plants with a 14 h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$ at 18°C) and a 10 h night (15°C) photoperiod.

Brevicoryne brassicae

A stock colony of the cabbage aphid *Brevicoryne brassicae* was continuously reared in 24 cm x 54 cm x 47 cm cages containing *B. rapa* plants with a 14 h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$ at 18°C) and a 10 h night (15 °C) photoperiod.

Sitobion avenae

A stock colony of the grain aphid *Sitobion avenae* was continuously reared in 52 cm x 52 cm x 50 cm cages containing up to 6 oat (*Avena sativa*) plants with a 14h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$ at 18 °C) and a 10h night (15 °C) photoperiod.

2.2 Plant line creation and growth conditions

Plant growth conditions

Arabidopsis thaliana

All *Arabidopsis thaliana* plants used for experiments were germinated and maintained on Scotts Levington F2 compost (Scotts, Ipswich, UK). Arabidopsis seeds were vernalized for one week at 4-6°C and grown in a controlled environment room (CER) with a 10 h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$) and a 14 h night photoperiod and a constant temperature of 22°C. Most plants were used at 5 weeks of age.

Nicotiana tabacum and *Nicotiana benthamiana*

Nicotiana tabacum (variety Petit Gerard) and *Nicotiana benthamiana* plants used for experiments were germinated on Scotts Levington F1 compost (Scotts, Ipswitch, UK) and transferred after 12 days to square black plastic pots (base measurement 5 cm x 5 cm, top measurement 7.5 cm x 7.5 cm, height 8 cm) containing Scotts Levington F2 compost (Scotts, Ipswitch, UK). All plants were grown in a CER with a 16 h ($120\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$) day and 8 h night at a constant temperature of 22°C.

Vicia faba

Vicia faba plants were grown in Scotts Levington F2 Compost (Scotts, Ipswitch, UK) in a greenhouse. Three seeds were planted at a depth of 1.5 cm in square plastic pots (5 x 7.5 x 8 cm) and covered in foil until germination had taken place. Plants were attached to stakes as they grew.

Plant lines

Arabidopsis mutants

The following Arabidopsis mutants are all in the Col-0 background unless specified otherwise and were obtained from the laboratories indicated. The *bak1-5* mutant (147) was originally provided by Ben Schwessinger (Dr. Cyril Zipfels group, The Sainsbury Laboratory (TSL), Norwich, UK) to Dr. David Prince (John Innes Centre (JIC), UK). The mutant was then maintained within the Hogenhout Lab. The *cyp79b2/cyp79b3* (306) double mutant, *pad3* (307) and *dcl1-9* (308) mutant lines were originally provided by Prof. Jean-Pierre Metraux (Department of Biology, Plant Biology, University of Fribourg, Switzerland), Dr.

Alexandre Robert-Seilaniantz (Prof. Jonathan Joness group, TSL, UK) and Dr. Fuquan Liu (Cell and Developmental Biology, JIC, UK) respectively to Dr. Graeme Kettles (JIC, UK) and then maintained within the Hogenhout lab. The *pen2-1* mutant in the *glabrous1* background (228) and the *AtrbohD* mutant (178) were obtained from Prof. Jonathan Jones (TSL, UK). The *agb1-2* (309), *sobir1-12* (310), *rlp23* and *etp1-1,2,3* mutants were provided by Prof. Cyril Zipfel (TSL, UK). The *crk6*, *crk7-1,2*, *crk13* and *crk30* mutants were obtained from Prof. Silke Robatzek. T-DNA insert mutants in AT1G35710, AT1G51830, AT3G20190, AT5G35390, AT5G39000 and AT5G49770 were provided by NASC (the European Arabidopsis stock centre).

T-DNA insert mutants were tested for homozygosity using DNA extraction followed by genotyping PCR (Section 2.3). The resultant products as seen by agarose gel electrophoresis and EtBr-staining were used to identify plants that were homozygous for the T-DNA insert.

Arabidopsis silencing lines

Arabidopsis thaliana plants expressing dsRNA for various genes were developed in the Hogenhout lab, JIC, UK. dsRack1 and dsGFP lines in the Col-0 background were created by Dr. Marco Pitino and Dr Alexander Coleman (43). I performed the crosses to place these into the *bak1-5* background. The dsMp10 lines in both Col-0 and *bak1-5* backgrounds were dipped by Dr. David Prince (2.2) and confirmed as mutants by me. I created the dsMpOS-D1 lines in Col-0 and *bak1-5* backgrounds.

***Nicotiana benthamiana* lines**

The SLJR15 *N. benthamiana* line stably expressing the calcium reporter protein aequorin was obtained from Dr. Cecile Segonzac (Zipfel group, TSL, UK) (185). The NahG line stably expressing the NahG transgene for salicylate hydroxylase was provided by Prof. Silke Robatzek (TSL, UK).

Plant line creation

Creation of stable transgenic *Arabidopsis thaliana* lines

To create dsMp10 and dsMpOS-D1 Arabidopsis lines, Col-0 and *bak1-5* plants were grown in square plastic pots (base 6 x 6 cm, top 8.5 x 8.5 cm, height 9.5 cm) containing Scotts Levington F2 compost (Scotts) plus grit, with five plants per pot. The plants were grown in a CER with 16 h day ($120\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$) and 8 h night at 20°C to allow flowering. Flowering plants were cut to encourage increased bolt number. Once the plants were flowering again they were transformed by floral dip (311). T1 seeds were collected and sown on plastic trays (38 x 24 x 5.6 cm) of Scotts Levington F2 compost (Scotts), grown in a greenhouse and sprayed with phosphinothricin (BASTA) to select for transformants. T2 seeds were collected from the surviving plants, sterilised and germinated on 0.8% Murashige and Skoog (MS) agar containing 20 mg/ml phosphinothricin and grown for 10 days at 18 hour day ($120\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$) and 6 hour night photoperiod and a constant temperature of 22°C. Plants were screened for evidence of a single insertion (alive:dead ration of 1:3) and then taken forward to T3 where they were again screened on MS agar plates with phosphinothricin. Independent lines with 100% survival at this stage, representing lines homozygous for the insert, were selected. These were then assayed for their ability to silence aphid genes.

Crossing *Arabidopsis thaliana*

Arabidopsis lines were crossed when unopened buds were present. Small and sharp tweezers were used to remove sepals, petals and stamen from around the central carpel. Mature stamen from the genotype to be crossed with were then plucked from the flower and used to pollinate the isolated carpel. Crosses were carried out in both directions. The bare carpel was wrapped in clingfilm for two days for protection, after which it was unwrapped and allowed to mature into a silique. Seeds from this were grown up and allowed to self-fertilise. The resulting progeny were checked for the presence of both parental mutations/genes.

In the *dcl1/pad3* cross, plants were checked for the presence of T-DNA insertions in both the DCL1 and PAD3 genes. DNA was extracted from the plant and used in genotyping PCR reaction (Section 2.3). The length of the resultant products as seen by agarose gel electrophoresis and EtBr staining were used to identify plants that were homozygous for both *dcl1* and *pad3*.

In *bak1-5* crosses to create RNAi lines, the presence of both the homozygous *bak1-5* mutation and the pJawohl8-RNAi vector were tested for. This was first via BASTA selection for the presence of the pJawohl8 Gateway vector (as for floral dipping). bak1-5 genotyping was carried out as described by Schwessinger et al. (147). DNA was extracted from the plant and used in genotyping PCR reaction (Section 2.3) to amplify the region around the BAK1 gene. The PCR product was cut using the restriction enzyme Rsa1 (New England Biolabs, Massachusetts, USA) and the subsequent restriction pattern seen via agarose gel electrophoresis used to identify *bak1-5* homozygotes.

2.3 DNA methods

DNA extraction

Rough genomic DNA extraction was used to isolate DNA from Arabidopsis to be used in PCRs diagnostic for genotype. 2-3 leaves were taken from each plant and frozen in liquid nitrogen. Leaf tissues were ground, then 400 µl of DNA extraction buffer (200 mM TrisHCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.4% SDS (w/v)) was added and mixed. The solution was centrifuged at 13,000 rpm for 5 minutes, and 300 µl of supernatant was transferred to a fresh tube. 300 µl of isopropanol was added to precipitate DNA and centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded and the pellet washed with 500 µl of 70% ethanol and dried. The pellet was then re-suspended in 100 µl sterilised water.

DNA Sequencing

Sequencing reactions were carried out in one of two ways:

1. Sequencing reactions were carried out in a final volume of 10 µl, using BigDye Terminator v3.1 (Life Technologies, Carlsbad, CA, USA) and vector primers, according to the manufacturers instructions. These ready reactions were submitted to Genome Enterprise Limited (The Genome Analysis Centre, Norwich, UK) for sequencing on Life Technologies 3730XL capillary sequencers. Sequences were analyzed with CodonCode Aligner software (CodonCode Corporation, Dedham, Massachusetts).

2. Plasmid samples of 50-100 ng/μl in 15 µl and 2 µl of 5mM vector specific primer were

sent directly to Eurofins Genomics for value read sequencing (Eurofins, Ebersberg, Germany). Sequences were analysed with CLC Bio Genomics Workbench software (CLC Bio, Qiagen).

PCR Methods

All PCR reactions were carried out in an Eppendorf Mastercycler® pro thermocycler (Eppendorf, Stevenage, UK).

Genotyping PCR

PCR was carried out using GoTaq polymerase (Promega) in 20 µl volumes. Each reaction contained 0.2 µl GoTaq, 2 µl 10 x GoTaq buffer, 0.5 µl of each 10 mM primer, 0.5 µl of 10 mM dNTPs, 15.3 µl dH_2O and 1 µl of Arabidopsis DNA. PCR program used was: 60 sec at 95°C, followed by 35 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec, with a final 10 mins at 72°C. Annealing temperature was altered according to primer melting point.

Cloning PCR

This was used to amplify genes from Arabidopsis or aphid cDNA, or non-Gateway plasmids present in the lab. Phusion polymerase (Finnzymes, Vantaa, Finland) was used in a 50 µl volume. Each reaction contained 0.5 µl Phusion, 10 µl 5 x HF Phusion buffer, 1.5 µl DMSO, 1 µl of each 10 mM primer, 1 µl 10 mM dNTPs, 35 µl dH_2O and 1 µl of cDNA. PCR programme used was: 60 sec at 98°C, followed by 35 cycles of 98°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec, with a final 10 mins at 72°C. Annealing temperature and elongation time were altered according to primer melting point and length of the target gene respectively.

Colony PCR

Colony PCR was used to confirm the presence of insert-containing plasmid in bacterial colonies. One primer specific for the vector was used in combination with one specific for the insert. PCR was carried out using GoTaq polymerase (Promega) in 20 µl volumes. Each reaction contained 0.2 µl GoTaq, 2 µl 10 x GoTaq buffer, 0.5 µl of each 10 mM primer, 0.5 µl of 10 mM dNTPs and 15.3 µl dH_2O. A small amount of bacterial colony was transferred to the PCR tube and mixed to provide the DNA template. PCR program used was: 3 minutes at 98°C, followed by 30 cycles of 95°C for 60 sec, 55°C for 30 sec and 72°C

Table 2.1: Primers used for genotyping PCR

Gene/Mutant line		Primer	Designed by/Source
WT DCL1	F	AATGGGCATCAGCCGTTTAC	Graeme Kettles (312)
	R	CTCTTTGCATGAGCCGGTC	
dcl1-9	F	CTCCGTTCAATTTACTGATTGTAC	Graeme Kettles (312)
	R	TTGAATGGTGCCCGTAACTTTCG	
pad3-1	F	CGAGGAATCGCCGTAATCTCGCC	Claire Drurey
	R	ACCGCTTCAGCCTTTAGCACAA	
BAK1 dCAPs	F	AAGAGGGCTTGCGTATTTACATGATCAGT	Schwessinger et al. (147)
	R	GAGGCGAGCAAGATCAAAAG	
PRK4/GK 065H10	F	GTGCGTTGAAATCATTGTACTTGTC	Carlos Caceres
	R	GCTTCTGTTGGCTAACAACG	
AT5G39000/SALK 007108	F	CTGGAGCCAAGTACACGATC	Carlos Caceres
	R	CCATCATCTACCGTGCTTTAGGTTC	
AAT1G51830/SALK 009061	F	GCTGTTGACATTACGCTTTGTGG	Carlos Caceres
	R	CAATCCCAAAGTAAGATAGG	
PRK1/SALK 054149	F	GGCTATCCTCAAGTTCAAGG	Carlos Caceres
	R	CGGTTAAGCAAGAAGATCACTCC	
AAT1G35710/SALK 067463C	F	AGAACCACGGGGACAAAATAGAGAG	Carlos Caceres
	R	CACGTTACAGAGTACAAGAG	
PRK1/SALK 074439	F	CGTTAGGAAAACCAAGTTTGG	Carlos Caceres
	R	GTCTCTTTCTCATGCAAAGC	
AT5G49770/ GK 585B07	F	CTAATGGGTCCCTTAGAGAC	Carlos Caceres
	R	GATTCGTTTGAGTACACTGGGG	
ASALK LBb1.3		ATTTTGCCGATTTCGGAAC	SALK Genotyping Project
GK O8474		ATAATAACGCTGCGGACATCTACATTTT	GABI-Kat Project (313)

for 90 sec, with a final 10 mins at 72°C. Annealing temperature was altered according to primer melting point.

Site directed mutagenesis PCR

Site directed mutagenesis was carried out by PCR to change specific codons of Mp10 and MpOS-D1, as well as to clone AMSH2.3 from AMSH2.1. Phusion polymerase (Finnzymes) was used in these PCRs, in 50 µl volumes. Each reaction contained 0.5 µl Phusion, 10 µl 5x HF Phusion buffer, 1 µl of each 10 mM primer, 1 µl of 10 mM dNTPs and 5, 10 or 25ng pDONR207 plasmid with gene insert to amplify from. The rest of the volume was made up with dH_2O. PCR program used was: 60 secs at 95°C, followed by 18 cycles of 95°C for 30 sec, 55°C for 40 sec and 72°C for 3 minutes, with a final 10 mins at 72°C. Annealing temperature and elongation time were altered according to primer melting point and length of the pDONR207 containing the desired insert. The restriction enzyme Dpn1 (New England Biolabs) was used to treat the products of the reaction and remove the template DNA.

Table 2.2: Primers used for site directed mutagenesis PCR

Mutation		Primer	Designed by
Mp10 Y40A	F	CTCGCCTACACCACAAAAGCTGACCATATTGACATCGAC	Claire Drurey
	R	GTCGATGTCAATATGGTCAGCTTTTGTGGTGTAGGCGAG	
Mp10 Y40F	F	CGCCTACACCACAAAATTTGACCATATTGACATCGAC	Claire Drurey
	R	GTCGATGTCAATATGGTCAAATTTTGTGGTGTAGGCG	
Mp10 W120A	F	GGAAACAGCTTCTTGACAAAGCTGACCCTAAGCGTGAATATTTC	Claire Drurey
	R	GAAATATTCACGCTTAGGGTCAGCTTTGTCAAGAAGCTGTTTCC	
Mp10 W120Y	F	GAAACAGCTTCTTGACAAATATGACCCTAAGCGTGAATATTTC	Claire Drurey
	R	GAAATATTCACGCTTAGGGTCATATTTGTCAAGAAGCTGTTTC	
MpOS-D1 F28Y	F	GAAAAGTACACAACTAAATATGATAACTTTGACGTGGAC	Claire Drurey
	R	GTCCACGTCAAAGTTATCATATTTAGTTGTGTACTTTTC	
MpOS-D1 Y108W	F	GATCGTTTGACCGCCAAATGGGACCCATCGGGCGAATAC	Claire Drurey
	R	GTATTCGCCCGATGGGTCCCATTTGGCGGTCAAACGATC	
AMSH2.1 to AMSH2.3	F	GATAGCTCTAATTACGGGATATTTAAGCTAACGGACCCTGGAGG	Christine Wilson
	R	CCTCCAGGGT CCGTTAGCTT AAATATCCCGTAATTAGAGC TATC	

2.4 Cloning

Gateway cloning

The desired DNA sequences were amplified via cloning PCR (Section 2.3) using the appropriate template and primers containing attb adapter sequences (Table 2.3). A small amount of the PCR product was analysed for size on an EtBr-stained 1% agarose gel. If the fragment was identified as having the correct size, the fragment was then amplified using attB adapter

primers. The PCR product was again analysed for size, then purified by ethanol precipitation and cloned into pDONR207 or pDONR221 (Invitrogen, Carlsbad, USA) using BP Clonase II (Invitrogen) following the manufacturers instructions. Reactions were transformed into *Escherichia coli* (DH5α) cells. Positive clones were identified via colony PCR (Section 2.3). Clones with confirmed inserts were grown overnight and plasmids DNA purified using the QIAprep Spin Miniprep Kit (Qiagen). The sequence was verified by DNA sequence analysis.

LR Clonase II (Invitrogen) was used according to the manufacturers instructions to clone desired DNA sequences into Gateway destination vectors. Positive clones were again identified using colony PCR (Section 2.3). Clones with inserts of the correct size were grown overnight and plasmid DNA purified using QIAprep Spin Miniprep Kit (Qiagen).

Table 2.3: Primers used for Gateway cloning

Mp10	F	AA AAA GCA GGC TCC ATGGCGCCGCAAAAAGATGCTGTG	David Prince
	R	A GAA AGC TGG GTC AAATTTGACA ACACCTTTTT TC	
Mp11	F	AA AAA GCA GGC TCC ATGATC GGC GGA TGT CCG GAA TTC	Claire Drurey
	R	A GAA AGC TGG GTC GTTCAAGCTC CTCCTCCTTC	
Mp15	F	AA AAA GCA GGC TCC ATGGATGAATTTACTATAGAAACTA	Claire Drurey
	R	A GAA AGC TGG GTC ACCGCAGAAA AACATTTCG	
Mp17	F	AA AAA GCA GGC TCC ATGCAA TAC TCC GCT CCA GCT TAC	Claire Drurey
	R	A GAA AGC TGG GTC GTATGCTGGC TTGTATGCTG G	
Mp19	F	AA AAA GCA GGC TCC ATGGCG GAA ACG CAA CAA CAG GGG	Claire Drurey
	R	A GAA AGC TGG GTC TTTCGGATGT TTCACCGCG	
Mp20	F	AA AAA GCA GGC TCC ATGGCC AAC TTG ACC GCC GCC GTC	Claire Drurey
	R	A GAA AGC TGG GTC CAAAGTATGG TAGGCACCAC C	
Mp21	F	AA AAA GCA GGC TCC ATGGGC GAC GTC ATT ATT CAA AAA AGG	Claire Drurey
	R	A GAA AGC TGG GTC ACATTTGTCA CTGGTGGCAA G	
Mp28	F	AA AAA GCA GGC TCC ATGGCT CAC TGT CAT CAC GAA GGT G	Claire Drurey
	R	A GAA AGC TGG GTC ATAACTATTA TTTAGTGTG	
Mp30	F	AA AAA GCA GGC TCC ATGCAA CAA TAT CAA CCC ACA ACT CCT	Claire Drurey
	R	A GAA AGC TGG GTC CTTAATTTTG TTGTAAGCTG G	
Mp31	F	AA AAA GCA GGC TCC ATGGGT AAA AAG GTG AGC GAT AAG	Claire Drurey

Gene		Primer	Designed by
	R	A GAA AGC TGG GTC TTGATTTTTC ATTGCTTCAT C	
Mp32	F	AA AAA GCA GGC TCC ATGGAG TCA GAC AAC GAA GTC	Claire Drurey
	R	A GAA AGC TGG GTC ATTTGGAAGT ATATTTTGC	
Mp33	F	AA AAA GCA GGC TCC ATGGGC ACA AAA CTT CTT CCT G	Claire Drurey
	R	A GAA AGC TGG GTC GTTTTTCTTG ATTTCTTTC	
Mp35	F	AA AAA GCA GGC TCC ATGAGA AAC GTG CAT CAA ACG ACG	Claire Drurey
	R	A GAA AGC TGG GTC ATCATCTGGA AACTTAAGAA C	
Mp39	F	AA AAA GCA GGC TCC ATGATT AGT ATC AAT CCG TTT AAA G	Claire Drurey
	R	A GAA AGC TGG GTC ACGAGTGTTG CCACTCTTGC AG	
Mp40	F	AA AAA GCA GGC TCC ATGTCG GAT AAG TTC TTT CAA ACC	Claire Drurey
	R	A GAA AGC TGG GTC AATGATTATG TCGTTGTCGG G	
Mp41	F	AA AAA GCA GGC TCC ATGCAA AAA CAA GAA CCA TCA GG	Claire Drurey
	R	A GAA AGC TGG GTC AACTTGACGT TGGATTAAG	
Mp42	F	AA AAA GCA GGC TCC ATGAATACAGTTAAAAAAGGTGAAGT	Claire Drurey
	R	A GAA AGC TGG GTC AACTCCACCA GATTCTGATG C	
Mp43	F	AA AAA GCA GGC TCC ATGCAA TAT GCA CCA GCA CCG CCG	Claire Drurey
	R	A GAA AGC TGG GTC TGCCGATTGT TTCTGAGTAC	
Mp44	F	AA AAA GCA GGC TCC ATGGAA GAA GCC CCA AAA GCC GAA G	Claire Drurey
	R	A GAA AGC TGG GTC AAAACTACGT TTGTTGCCGG	
Mp45	F	AA AAA GCA GGC TCC ATG CAA GTT ATG TGC AGT CAA GAC	Claire Drurey
	R	A GAA AGC TGG GTC GGCCATAACC ACCGGTCGAG	
Mp46	F	AA AAA GCA GGC TCC ATGCAC AAA TTA ATA AAA GTC G	Claire Drurey
	R	A GAA AGC TGG GTC ATTGACTCCA AATTGTTGG	
Mp47	F	AA AAA GCA GGC TCC ATGGCTCCTGCTGAAACAATAATTGG	Claire Drurey
	R	A GAA AGC TGG GTC ATCCAAAGCA TCTGAGATAG	
Mp49	F	AA AAA GCA GGC TCC ATGGCA ATA CCC ATT AAT TGT CC	Claire Drurey
	R	A GAA AGC TGG GTC ATCGCATCTT TTTGCTCCTC C	
Mp50	F	AA AAA GCA GGC TCC ATGAAG TCT GAC AGT GAA ATT GAT TTG	Claire Drurey
	R	A GAA AGC TGG GTC ATATCCATAA ATAATTGTTC	
Mp51	F	AA AAA GCA GGC TCC ATGAAT GAA ATT AAC GTC AAA C	Claire Drurey

Gene		Primer	Designed by
	R	A GAA AGC TGG GTC ATTTCTTGTC GTTCTCTTTC G	
Mp53	F	AA AAA GCA GGC TCC ATGGAT GTG AGT CAA CAA CAA CAA G	Claire Drurey
	R	A GAA AGC TGG GTC ATTGAGCTTT TCGGATTTCA TG	
Mp54	F	AA AAA GCA GGC TCC ATGGGA AAA GTG CCA TCT TCA G	Claire Drurey
	R	A GAA AGC TGG GTC AATGTATTCA ATTCTCATAT TG	
Ap1	F	AA AAA GCA GGC TCC ATGT CTTTGTACCAACCTCCTCC	Marco Pitino
	R	A GAA AGC TGG GTC CAAAAGCTCT CTATCGATAGG	
Ap2	F	AA AAA GCA GGC TCC ATGG AAGAAGTTACTGAGTTGG	Marco Pitino
	R	A GAA AGC TGG GTC AGCCTTAGAA GATCCGTTC	
ApC002	F	AA AAA GCA GGC TCC ATGGAAGTTAG ATGCGATTGG	Marco Pitino
	R	A GAA AGC TGG GTC AAAACGTCGA AGGAAACTTC C	
AMSH1	F	AA AAA GCA GGC TGC ATGGGGTCGT CTTTTGAGAC	Christine Wilson
	R	A GAA AGC TGG GTA CTATCTGAGATCAATGACATC	
AMSH2.1/2.3	F	AA AAA GCA GGC TGC ATGGTAACGC TCTCGTCTCC ATC	Christine Wilson
	R	A GAA AGC TGG GTA TTAACGTAGATCAAAAATCTCG	
AMSH2.2	F	AA AAA GCA GGC TGC ATGTTCATAT CTCAGAAAGG	Christine Wilson
	R	A GAA AGC TGG GTA TTAACGTAGATCAAAAATCTCG	
AMSH3	F	AA AAA GCA GGC TGC ATGAAGATTG ATCTGAACAA GG	Christine Wilson
	R	A GAA AGC TGG GTA TTAGCGGAGATCGAGGACTTC	
attB (adapter)	F	G GGG ACA AGT TTG TAC AAA AAA GCA GGC T	Invitrogen
	R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GT	
attB (colony)	F	ACAAGTTTGTACAAAAAAGCAGGC	Invitrogen
	R	ACCACTTTGTACAAGAAAGCTGGG	

Cloning into the Potato Virus X (PVX) Vector, pGR106

The ORFs minus signal peptide of Mp10 and Mp10 mutants were amplified from pDONR207 via cloning PCR (2.3) using primers that introduced a ClaI (forward primer) or NotI (reverse primer) restriction site (primers listed in Table 2.4). A small amount of PCR product was analysed for size using 1% agarose gel electrophoresis and EtBR staining. The remaining PCR product was cleaned using the QIAquick PCR purification kit (Qiagen) according to the

manufacturers instructions. Cleaned PCR products and the pGR106 vector were digested using ClaI and NotI (New England Biolabs), then cleaned using agarose gel electrophoresis and the QIAquick gel extraction kit (Qiagen) according to the manufacturers instructions. Digested Mp10 was ligated into ClaI/NotI digested pGR106 using T4 DNA ligase (Invitrogen). Ligations were transformed into *E. coli* (DH5α). Colonies were tested for presence of the insert using colony PCR (2.3) and digestion. Products were analysed via agarose gel electrophoresis to ensure insert presence of the correct size. Clones with verified inserts were grown overnight and plasmid DNA purified using QIAprep Spin Miniprep Kit (Qiagen).

Table 2.4: Primers used for cloning Mp10 into pGR106

Gene		Primer	Designed by
Mp10	F	GGA ATCGAT G GCGCCGCAAAAAGATGCTGTG	Jorunn Bos (46)
	R	GGA GCGGCCGC TTAAAATTTGACAACACCTTTTTTC	

Transformation of bacteria by electroporation

Prior to electroporation, electrocompetent cells were thawed on ice for 5-10 minutes. 100ng of plasmid was added to 20 µl of electrocompetent cells. Cells-plasmid mixes were transferred to pre-chilled electroporation cuvettes (Cell Projects Ltd., Kent, UK) and pulsed in a Bio-rad MicroPulser™ electroporator (Biorad) (125V, capacitance 25 μF, resistance 200 Ω). Cells were recovered from the cuvette using 200 µl of liquid Super Optimal Broth with Catabolite repression (SOC) media and transferring the suspension to a sterile Eppendorf tube. The bacteria were incubated for 1-2 hours at 37°C (*E. coli*) or 28°C (*Agrobacterium tumefaciens*). The bacterial solution was then plated on LB-agar plates containing the appropriate antibacterial selection. Bacteria was left to grow overnight (*E. coli*) or for 48 hours (*A. tumefaciens*). Colonies were tested for presence of the plasmid and insert using colony PCR (2.3) and agarose gel electrophoresis of resulting products. Permanent freezer stocks were made of positive colonies and stored at -80°C.

Plasmids used in this study

All plasmids were generated as described above with the exception of the following:

- AvrPtoB containing vector (pCB302-3) (264) was originally obtained from Dr. John Rathjen (TSL, Norwich, UK).
- The Mp10 containing pCB302-3 and empty vector control used alongside AvrPtoB

in ROS burst assays was provided by Dr. Jorunn I. B. Bos (JIC, Norwich, UK). Dr. Bos also provided the pGR106 plasmid.

- pDONR207 and pJawohl8-RNAi containing full length Mp10 and MpOS-D1 were generated and provided by David Prince (JIC, Norwich, UK).
- pDONR207 with Mp1, Mp2, Ap1, Ap2, MpC002 and ApC002 was provided by Marco Pitino (JIC, Norwich, UK).
- The 35s:GFP expressing pB7WG2 vector used as a control in many experiments was generated and provided by Dr Akiko Sugio (JIC, Norwich, UK).
- NbAMSH2 in pB7WGF2 and pK7WIWG2 (II) was generated and provided by Friederike Bernsdorff (JIC, Norwich, UK).
- The pK7WIWG2 (II) GUS control was provided by Sam Mugford (JIC, Norwich, UK).
- The pUBQ10 vector, with RFP-GUS and RFP-ARA6 was provided by Prof Silke Robatzek (TSL, Norwich, UK). I used pUBQ10 to generate RFP-Mp10 via LR reaction, as described above.
- The AtFLS2 and SlFLS2 in pCAMBIA2300 (with FLS2 promoter) were kindly provided by Jenna Loiseau (TSL, Norwich, UK) from the lab of Prof. Silke Robatzek.
- pICH86988-RRS1 (His and FLAG tag), pBIN19-RPS4 (HA tag), pK7FWG2-AvrRps4 and pK7FWG2-AvrRps4 E187A (GFP tag) were provided by Dr. Panagiotis Sarris, from the lab of Prof. Jonathan Jones (TSL, Norwich, UK).
- Cloning of candidate aphid effectors into pGAD and pLexA plasmids for use in yeast two-hybrid assays, and generation of Mp10 Y40F W120Y, VPS2.1, VPS24.1 in pDEST22/32 was carried out by Carlos Caceres.

A full list of plasmids generated and used in this thesis can be found in Appendix C.

2.5 RNA methods

RNA extraction

Aphid and leaf samples were ground in chilled 1.5 ml Eppendorf tubes using disposable pellet pestles (Sigma-Aldrich, St Louis, MO, USA). Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) and included a DNaseI treatment (RQ1 DNase set; Promega, Madison, WI, USA). Resulting RNA was analysed for purity via agarose gel electrophoresis

and EtBR staining, and NanoDrop 2000 spectrophotometer (Thermo Scientific, Loughborough, Leicestershire, UK). RNA samples with A260/A280 ratios between 1.9 and 2.1 were used.

cDNA synthesis

cDNA was synthesised from $1\ \mu g$ RNA using the M-MLV-RT Kit (Invitrogen, Carlsbad, CA, USA) and oligo-dT primer, following the manufacturer's instructions.

qRT-PCR

cDNA from aphid and leaf samples was diluted 1:10 with distilled H_2O before using for qRT-PCR. Each reaction consisted of $20\ \mu l$ containing 25 ng of cDNA and $0.5\ \mu g$ of each primer (listed in Table 2.5) added to SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in a single well of a 96-well plate white ABgene PCR plate (Thermo Scientific). Reactions for the target and reference genes and corresponding controls were combined in one 96-well plate, which was placed in a CFX96 Real-Time System with a C1000 Thermal Cycler (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). PCRs were carried out using the following thermocycle: 3 min at 95°C, with 40 cycles of 30 s at 95°C, 30 s at 60 °C, 30 s at 72°C, followed by melt curve analysis: 30 s at 50°C (65-95°C at 0.5°C increments, 5 s for each).

To identify genes that remained stable across a range of mock, flg22 and GPA extract-exposed Arabidopsis leaf disc RNA samples I used geNORM analysis (314) within qbase^{PLUS} software (Biogazelle, www.biogazelle.com) on a selection of candidates previously identified as superior Arabidopsis reference genes (315). From this analysis, I found that the Arabidopsis genes GAPDH (At1g13440) and TIP41 (At4g34270) remained the most stable, so these were used as reference genes for my Arabidopsis investigations (Table 2.5). geNORM analysis of aphid reference genes for Mp10, MpOS-D1 and MpRack1 expression analysis had already been carried out for investigations by Alex Coleman and David Prince (316; 317) and found that L-27, β-tubulin and actin were the most stable genes across aphids fed on dsRNA plants. All primers were tested for efficiency and specificity under the qRT-PCR conditions used before being used.

To calculate the relative expression levels of target genes, mean C_t values for each sample-primer pair combination were calculated from three replicate reaction wells. Mean C_t values were then converted to relative expression values using (efficiency of primer pair)$^{-\delta Ct}$ (318). The geometric mean of the relative expression values of the reference genes was calculated to produce a normalization factor unique to each sample (314). This

normalisation factor was then used to calculate the relative expression values for each gene of interest in each sample. Data was incorporated into a generalised linear model (GLM) and pairwise differences between the lines was investigated within the model. For display of data, mean expression values were rescaled such that aphids fed on dsGFP plants or leaf discs exposed to water represented a value of 1. Statistical analyses were conducted using Genstat v.17 (VSN International, Hemel Hempstead, UK).

Table 2.5: qRT-PCR primers

Organism	Gene	Use		Primer	Designed by/source
A. thaliana	FRK1	Defense marker gene	F	TGCAGCGCAAGGACTAGAG	Cecile Segonzac (319)
			R	ATCTTCGCTTGGAGCTTCTC	
A. thaliana	CYP81F2	Defense marker gene	F	AATGGAGAGAGCAACACAATG	Graeme Kettles (238; 312)
			R	ATACTGAGCATGAGCCCTTTG	
A. thaliana	PAD3	Defense marker gene	F	TGCTCCCAAGACAGACAATG	Chassot et al., 2008 (320)
			R	GTTTTGGATCACGACCCATC	
A. thaliana	GAPDH	Reference gene	F	AGGTCAAGCATTTTCGATGC	Graeme Kettles (312)
			R	AACGATAAGGTCAACGACACG	
A. thaliana	TIP41	Reference gene	F	TCCATCAGTCAGAGGCTTCC	Graeme Kettles (312)
			R	AAGAAAGCTCATCGGTACGC	
M. persicae	Mp10	Target for knock down	F	GGTCGGAGCGCCGCAAAAAG	David Prince (316)
			R	TTGGAACCCAAAACTTGGTCGATGT	
M. persicae	MpOS-D1	Target for knock down	F	ACCAACGAAGGCCGAGAATTGAGG	David Prince (316)
			R	GGCGGTCAAACGATCAAAGTCAGT	
M. persicae	MpRack1	Target for knock down	F	GGACGTACCACTCGTCGTTT	Alex Coleman (317; 45)
			R	CATGATACCCAATCGCTGTG	
M. persicae	β-tubulin	Reference gene	F	CCATCTAGTGTCGCTGACCA	Alex Coleman (317; 43)
			R	GTTCTTGGCGTCGAACATTT	
M. persicae	Actin	Reference gene	F	GGTGTCTCACACACAGTGCC	Marco Pitino (43)
			R	CGGCGGTGGTGGTGAAGCTG	
M. persicae	L-27	Reference gene	F	CCGAAAAGCTGTCATAATGAAGAC	Alex Coleman (317; 43)
			R	GGTGAAACCTTGTCTACTGTTACATCTTG	

2.6 Preparation of aphid extract and saliva collection

Whole GPA extracts

Whole GPA extract was prepared by collecting apterous late instar and adult aphids from the Chinese cabbage stock cage, freezing in liquid nitrogen, and then grinding to a fine powder using a pre-chilled mortar and pestle. The powder was then transferred to a 50 ml Corning tube (Corning, New York, USA) on ice using a pre-chilled metal spoon. Sterile distilled water was added to the ground powder in the quantity of 1 ml water per 0.02 g (wet weight) of aphid and mixed to suspend. Assays carried out with "whole" aphid extract used this crude mixture suspended in water. For callose assays and candidate receptor ROS assays, whole extract spun at 13000 rpm for 10 minutes was used.

GPA extract for treatment with proteinase K, boiling and protease inhibitors was prepared using a modified version of the protocol, similar to that of Lapitan et al. (127). Aphids were collected, frozen, ground, and transferred to a Corning tube (Corning) as above. At this point, sterile 0.025 M potassium phosphate buffer (KH_2PO_4, pH 6.8) was added to the powder in the quantity of 1 ml buffer per 0.02 g (wet weight) of aphid, and mixed to suspend. The extract was then placed in Eppendorf tubes and centrifuged at 13000 rpm for 15 mins at 4°C. The supernatant was then removed and placed in fresh tubes and processed further.

Treated extract

GPA supernatant was boiled for 10 mins in order to denature the proteins. In order to degrade proteins, GPA supernatant was also treated with proteinase K at $1\ \mu\mathrm{l}$ of 100 mg/ml proteinase K (Sigma-Aldrich, St. Louis, USA) per $500\ \mu\mathrm{l}$ supernatant and incubated at 37°C for 30 mins. To inhibit proteases present within the aphid extract, Protease Inhibitor Cocktail, leupeptin (provided by the lab of Mark Banfield) or phosphoramidon (all Sigma-Aldritch) was added to GPA supernatant at concentrations of 1:100, $50\ \mu\mathrm{mol}$, and $10\ \mu\mathrm{mol}$ respectively. To investigate the role of calcium chelation on the ROS burst, EDTA was added to GPA extract to a concentration of $5\ \mathrm{mmol}$.

Extracts of other aphid species

Species other than GPA were collected from their respective stock cages and processed as described above in order to obtain the crude whole aphid extract.

GPA saliva collection

GPA saliva was collected using a parafilm sachet as described previously (47; 34). Two 500 ml plastic tumblers (Sainsburys Supermarkets Ltd., London, UK) had several holes pierced in them using a needle (Terumo, Surrey, UK). 0.2 g of adult *M. persicae* (approximately 1000 individuals) from the stock cage were added to one tumbler, which was then sealed using a thin layer of parafilm (Brand, GMBH, Wertheim, Germany). 1 ml of sterile distilled water was pipetted onto the parafilm and kept in place by the addition of another parafilm layer over the top of it. A second tumbler was set up without the aphids as a no-aphid control. Both tumblers were placed under a sheet of yellow plastic (Lincoln Poly-

thene Ltd., Lincoln, UK) to enhance feeding activity and kept in a CER with a 14 h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$ at 18°C) and 10 h night (15°C) photoperiod. After a period of 24 hours the saliva/water was collected from between the parafilm layers and used as described.

2.7 Aphid assays

GPA induced resistance assays

Induced resistance assays were carried out using a modified protocol as described by De Vos and Jander (47). Experiments were carried out on five-week old Arabidopsis plants in black plastic pots (base 3.5 x 3.5 cm, top 5.5 x 5.5 cm, height 5.5 cm) in a CER with an 8 h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$ at 18°C) and 16 h night (16°C) photoperiod. The first fully expanded leaf of each plant was infiltrated with aphid extract diluted 1:10 with distilled water or a water control using a needless 1 ml syringe (Terumo, Surrey, UK). The infiltrated leaves were marked and the plants used for aphid reproduction assays 24 hours later. One adult GPA of 10 days old was placed in a clipcage which was then placed over the infiltrated leaf. Plants were then returned to the experimental CER and left for 10 days, after which time the number of aphids in each clipcage was counted. The experiment was repeated to give four independent replicates. Aphids produced per clipcage was analysed using a GLM with Poisson distribution, taking repeat and tray into account as variables. To find any significant differences, pairwise differences between the lines was investigated within the model. Data shown are predicted means and their associated standard errors from within the model. Statistical analyses were conducted using Genstat v.17 (VSN International, Hemel Hempstead, UK).

GPA survival and fecundity assays

GPA survival and fecundity assays were carried out as described by Pitino et al. (43). Experiments were conducted on four-week old plants in round one litre pots containing Scotts Levington F2 compost (Scotts) and kept in a CER with an 8 h day ($90\ \mu\mathrm{mol}\ m^{-2}sec^{-1}$ at 18°C) and 16 h night (16°C) photoperiod. Each plant had five adult GPA added to it from the stock colony and contained using clear plastic tubing (13 cm diameter, 10 cm tall) (Jetran Tubing) capped at the top with white gauze-covered plastic lids. After 48 hours, all adult aphids were removed from test plants (Day 0). After another 72 hours, excess nymphs were removed from the plants, leaving 5 nymphs per plant. The number of offspring produced on the 11th and 14th days of the experiment were counted and removed,

and the number of surviving adults was counted on day 14. In the case of experiments on Arabidopsis RNAi lines, surviving adult aphids were snap frozen in liquid nitrogen in batches of 5 insects. These were then used in qRT-PCR experiments to confirm silencing. To calculate the number of nymphs produced per aphid on the plant, the nymphs counted at days 11 and 14 were totalled and then divided by the number of surviving adults on that plant. Each experiment included 5 or 6 plants per genotype that were randomly assigned to trays that could hold a maximum of 10 plants. Experiments were repeated to create data from four independent biological replicates. The data on nymphs produced per adult and percentage adult survival was analysed using a generalised linear model (GLM) with Poisson distribution, taking both repeat and tray into account as variables. To find any significant differences, pairwise differences between the lines was investigated within the model. Data shown are predicted means and their associated standard errors from within the model. Statistical analyses were conducted using Genstat v.17 (VSN International, Hemel Hempstead, UK).

Acyrthosiphon pisum survival assays on Arabidopsis

Experiments were conducted as described by Prince et al. (321). Aphids were kept in a CER with an 8 h day ($90\ \mu\mathrm{mol}m^{-2}sec^{-1}$ at 18°C) and 16 h night (16°C) photoperiod. Two mature *Vicea faba* plants between three and four weeks old were placed in a 24 x 54 x 47 cm cage and 50 adult *A. pisum* from the stock cage added to each. After 24 hours all adults were removed from the plants, leaving the nymphs which formed an aged population of *A. pisum*. These were left to mature for 10 days, after which time 5 adult aphids were placed in a clipcage which was then attached to the youngest fully expanded leaf of an Arabidopsis plant. The Arabidopsis plants were placed into the experimental CER and the number of aphids alive in each clipcage was recorded on days 2 to 7. The time point at which 50% of *A. pisum* were still alive on wild-type Col-0 Arabidopsis was calculated. This time point (4 days) was then used to find the survival on the other genotypes. Each experiment included 5 plants of each genotype, and was repeated to give 4 independent replicates. The results were analysed using a GLM with Poisson distribution, taking repeat into account as a variable. Statistical analyses were conducted using Genstat v.17 (VSN International).

GPA assays for silencing on dsMp10 and dsMpOS-D1 Arabidopsis lines

To identify Arabidopsis dsMp10 and dsMpOS-D1 lines that reduced expression of *Mp10* and *MpOS-D1* in aphids, I carried out assays using several different plant lines to check

expression levels in aphids colonising the plants. Experiments were conducted on four-week old plants in one litre round pots containing Scotts Levington F2 compost (Scotts) and kept in a CER with an 8 h day ($90\ \mu\mathrm{mol}m^{-2}sec^{-1}$ at 18°C) and 16 h night (16°C) photoperiod. Each plant had 20 adult GPA added to it from the stock colony and contained using clear plastic tubing (13 cm diameter, 10 cm tall) (Jetran Tubing) capped at the top with white gauze-covered plastic lids. After 48 hours, all adult aphids were removed from test plants, leaving nymphs. These were left to develop for 8 days, then collected in batches of 5 aphids and snap frozen in liquid nitrogen, to be used for RNA extractions and qRT-PCR experiments to investigate levels of silencing.

2.8 Arabidopsis defense assays

Defense assays were carried out using the peptide flg22 (QRLSTGSRINSAKDDAAGLQIA) (EZBiolab, Carmel, Indiana, USA) (139) (provided by the lab of Prof. Cyril Zipfel, TSL, Norwich) and aphid extracts which were produced as described above.

Callose assays

Callose deposition in leaves was assayed using a method that involved infiltrating the leaf with the substance of interest, and then harvesting leaf discs a set period of time later for inspection under a microscope after staining. The first two fully expanded leaves of 5-week-old Arabidopsis plants were infiltrated using a needleless 1ml syringe with buffer (control), 100 nM flg22 (in buffer) and 20 mg/ml GPA extract (in buffer). After 24 hours, one leaf disc was taken from each infiltrated leaf using a circular cork borer with a diameter of 5 mm. To remove chlorophyll from the leaf discs, the discs were placed in 70% ethanol for 1 hour, 95% ethanol with chloroform overnight (18 hours) and 100% ethanol for 2 hours. The discs were then rehydrated for 30 minutes in 70% ethanol, 30 min in 50% ethanol and 30 min in 67mM K_2HPO_4 at pH 9.5. Staining with 0.1% aniline blue in 67mM K_2HPO_4 at pH 9.5 was carried out for one hour. Leaf discs were mounted in glycerol and viewed under a Nikon Eclipse 800 microscope (Nikon, Japan) using a UV filter (band pass (BP) 340-380nm, long pass (LP) 425nm). An image was taken of the entire field of view of the centre of each leaf disc under 10x magnification. The images were analysed using ImageJ (National Institutes of Health, USA) to count callose deposits. Statistical analyses were conducted within a GLM using Genstat v.17 (VSN International).

Defense gene induction

Two Arabidopsis leaf discs were taken from each of the two youngest fully expanded leaves of five-week old Col-0 plant using a circular cork borer with a diameter of 6 mm. The leaf discs were floated on water overnight in 96-well plates (Grenier Bio-One, Stonehouse, Glouchestershire, UK). Before the experiment began the water was removed and leaf discs were exposed to $100\ \mu l$ of water (control), 100 nM flg22 (in water) and 20 mg/ml GPA extract (in water) for 1 hour. After this time, eight leaf discs under the same treatment were pooled together, generating one sample, and snap frozen in liquid nitrogen for RNA extraction and cDNA synthesis. Measurement of the induction of defense genes in these samples was then measured via quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assays, described above.

Arabidopsis ROS Burst Assays

Flg22 was used at a concentration of 50nM throughout investigations, and GPA extract, and variations of it, were used at 5mg/ml.

One leaf disc was taken from each of the two youngest fully expanded leaves of five-week old Arabidopsis plants using a circular cork borer with a diameter of 4 mm. 8 leaf discs were taken per Arabidopsis line/elicitor. The leaf discs were floated on $200\ \mu l$ water overnight in 96-well plates (Grenier Bio-One). Before conducting the assay, the water was removed from the wells and replaced with $100\ \mu l$ of the assay solution. This solution contained $100\ \mu g$/ml horseradish peroxidase (hrp) (Sigma-Aldritch) and 21 nM of the luminol derivative 8-amino-5-chloro-7- phenylpyrido [3,4-d] pyridazine-1,4(2H,3H) dione (L-012) (Wako, Osaka, Japan)(322) alongside flg22, aphid extract or water/buffer controls. ROS burst assays to proteinase K were conducted with $100\ \mu g$ of proteinase K (Sigma-Aldrich). Luminescence was captured using a Photek camera system (Photek, St Leonards on Sea, East Sussex, UK) and analysed using company software and Microsoft® Office Excel (Microsoft, London, UK). Experiments were repeated at least three times on different days to generate independent biological replicates. Data was exported into Genstat v.17 (VSN International) for statistical analysis within a GLM.

2.9 Effector investigations in *Nicotiana* species

Agrobacterium-mediated transient expression in *N. benthamiana* and *N. tabacum*

A. tumefaciens strain GV3101 was used for all transient expression assays in *N. benthamiana*. Cultures from freezer stocks were grown overnight at 28°C with gentle shaking in LB media using the appropriate antibiotics. Cultures were then pelleted and resuspended in infiltration buffer (10 mM $MgCl_2$ (Sigma-Aldrich), 10 mM 2-(N-Morpholino) ethanesulfonic acid (MES) (Sigma-Aldrich), pH 5.6) to give an OD_{600} of 0.3, unless otherwise stated. Acetosyringone (Sigma-Aldrich) was added to each culture to a concentration of $100\ \mu\mathrm{mol}$ to enhance transformation. Cultures were left for at least one hour before infiltration into the two youngest, fully expanded leaves of three and a half to four and a half-week old *N. benthamiana* or *N. tabacum* 'Petit Gerard' plants using a needless 1 ml syringe (Terumo). Up to six (generally 4) different constructs were infiltrated into one leaf, and infiltrated areas were marked with permanent marker. Leaves were left for 48 hours before samples were harvested, unless otherwise stated.

N. benthamiana ROS burst assays

A. tumefaciens was used to transiently express the protein of interest alongside controls in *N. benthamiana* leaves as described above. Two leaf discs were taken from each infiltration site using a circular cork borer with a diameter of 4mm. 8 leaf discs were taken per construct. The leaf discs were floated on $200\ \mu\mathrm{l}$ water overnight in 96-well plates (Grenier Bio-One). Before conducting the assay, the water was removed and replaced with $100\ \mu\mathrm{l}$ of assay solution. This solution contained $100\ \mu\mathrm{g}$/ml horseradish peroxidase (hrp) (Sigma-Aldritch) and 21 nM of the luminol derivative L-012 (Wako)(322) alongside 50 nM flg22 or 5 mg/ml whole GPA extract. Luminescence was captured using a Photek camera system (Photek), and data was retrieved and analysed as described for the Arabidopsis ROS burst assays.

N. benthamiana ROS burst assays after RNAi

A. tumefaciens was used to transiently express a silencing construct (pK7WIWG2 (II)) against NbAMSH2 alongside a RNAi GUS control in *N. benthamiana* leaves. Leaves were left for 4 days before leaf discs were taken, and used in ROS burst assays as described above.

N. benthamiana calcium burst assays

A. tumefaciens was used to transiently express the protein of interest alongside controls in the leaves of SLJR15 *N. benthamiana*, which stably express the calcium reporter protein aequorin (185). Two leaf discs were taken from each infiltration site using a circular cork borer with a diameter of 4mm. 8 leaf discs were taken per construct. The leaf discs were incubated in the dark overnight in 96 well plates (Grenier Bio-One) containing $200\ \mu l$ of $12.5\ \mu mol$ coelenterazine (Biosynth AG, Staad, Switzerland) in each well. Before the assay, the coelenterazine was removed and $100\ \mu l$ of 50 nM flg22 was added to each well. Luminescence was captured using a Photek camera system (Photek).

N. benthamiana chlorosis assays

A. tumefaciens was used to transiently express Mp10, MpOS-D1 and variants of the two proteins in *N. benthamiana* leaves, as described above. A SPAD 502 plus chlorophyll meter (SPADmeter)(Spectrum Technologies, Aurora, Illinois, USA) was used to measure the chlorophyll content of leaves at 3, 5 and 7 days post infiltration. Readings from at least 3 independent biological replicates were grouped, and analysed within a GLM, taking repeat into account, in the Genstat v.17 package (VSN International).

A. tumefaciens was also used to introduce the PVX-based expression vector pGR106 containing Mp10 and MpOS-D1 sequences into two and a half to three and a half-week old *N. benthamiana* plants. Infiltrations into the leaves were carried out as described above. Systemic PVX symptoms were scored 14 days post inoculation.

HR assays in *N. tabacum*

To investigate activation of a HR response, *A. tumefaciens* was used to transiently express tagged forms of the R-proteins RSP4 and RRS1 alongside Mp19, Mp21 and Mp44.1/3 in *N. tabacum* leaves, as described above. AvrRps4 and AvrRps4 E187A were included as positive and negative controls respectively. Each culture was mixed so its individual OD_{600} in the end mixture was 0.5. Leaves were checked for the presence of a HR response (cell death) from 48 hours after infiltration, with HR seen in the positive AvrRps4 control by 3 days after infiltration.

To investigate HR inhibition, *A. tumefaciens* was used to transiently express tagged forms of RSP4, RRS1 and AvrRps4 alongside Mp19, Mp21 and Mp44.1/3 in *N. tabacum*

leaves. GFP and AvrRps4 E187A were included as controls. Each culture was mixed so its individual OD_{600} in the end mixture was 0.4. Leaves were inspected for HR as described above.

2.10 Protein methods

Sample preparation

For analysis of proteins in *N. benthamiana*, two leaf discs (10 mm diameter) were taken from the infiltrated area and frozen in liquid nitrogen at the same time samples were taken to be used in assays. For analysis of proteins in aphids, 10 whole aphids, 20 aphid heads or 40 µl of aphid saliva was collected and frozen in liquid nitrogen. Samples were ground, extracted in 40 µl 4x NuPAGE LDS sample buffer (Invitrogen) and boiled for 10 minutes. 10 µl was loaded into the SDS-PAGE gel.

SDS-polyacrylamide gel electrophoresis and electroblotting

SDS-polyacrylamide gel preparation methods were followed according to Laemmli, 1970 (323; 324). 12% or 15 % gels were used, depending on the size of protein investigated. Gels were run in Mini PROTEAN III gel tanks (Bio-Rad) filled with Tris-glycine SDS Running buffer (Thermo Scientific). All gels included a molecular size marker; NEB protein marker, broad range or NEB blue protein standard, broad range (both New England Biolabs). Electrophoresis was continued until the loading dye band migrated to the bottom of the gel. Proteins were transferred to 0.45 µm Protran BA85 nitrocellulose membrane (Whatman) using the Mini PROTEAN III system (Bio-Rad) according the manufacturers instructions. Transfer took place for 90 minutes at 250 mA.

Immunodetection

The nitrocellulose membrane containing immobilised, denatured proteins was blocked for 40 minutes at room temperature with blocking buffer (5% (wt/v) milk powder in 1x phosphate buffered saline and 0.1% (v/v) Tween-20 (Sigma Aldrich)) with gentle agitation on a platform shaker. The membrane was then incubated in blocking buffer with the primary antibody at for 1 hour at room temperature (RT) or 4°C overnight. The membrane was washed 4 times with blocking buffer before being incubated with peroxidase-conjugated

secondary antibody, for 1 hour at RT or 4°C overnight. The membrane was then washed 4 times with washing buffer (1x phosphate buffered saline and 0.1% (v/v) Tween-20). Bound antibodies were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore, Watford, UK). The membrane was then either exposed onto Super RX film (Fujifilm, Dusseldorf, Germany) and developed, or visualised using the ImageQuant LAS-500 chemiluminescence imager (GE Healthcare Life Sciences, Little Chalfront, UK). Protein loading was visualised using Ponceau S solution (0.1% (wt/v) in 5% acetic acid (Sigma Aldritch)).

Co-immunoprecipitation

Co-immunoprecipitation assays were performed using protein transiently expressed in *N. benthamiana* leaves. Agroinfiltration was performed as described above, with GFP-tagged AMSH1/2/3 and FLAG-tagged Mp10/MpOS-D1 mixed, ensuring a final OD_{600} of 0.3 for each culture. Two entire leaves were infiltrated for each construct pair to provide sufficient material. Leaf discs were removed from each leaf prior to freezing in liquid nitrogen, and Western blots were performed with these samples to ensure adequate protein expression prior to co-immunoprecipitation. Following this verification, the remaining sample was ground in liquid nitrogen using a pestle and mortar. Cold extraction buffer (150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol (v/v), 10 mM EDTA, 20 mM sodium fluoride, 10 mM DTT, 0.5% (wt/v) polyvinylpolypyrrolidone, 0.1 % Triton-X, protease cocktail inhibitor (Sigma Aldritch)) was then added to ground samples at 2.5 ml/g and incubated on ice for 30 minutes. Samples were then centrifuged at 3,200x g at 4°C for 15 minutes, and the resulting supernatant was filtered through $0.45\ \mu\mathrm{m}$ filter (Sartorius Stedim UK Limited) using a needleless syringe (Terumo). 2 ml filtered extract was added to $20\ \mu\mathrm{l}$ equilibrated GFP-binding affinity resin (GFP-Trap M; Chromotek GMBH, New York, USA), and a sample was taken as 'input'. Protein extract and GFP-trap beads were incubated at 4°C overnight on a rotating wheel. Samples were pelleted by centrifugation at 2,700x g for 2 minutes and pellets were washed with 1 mL TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% Tween-20). Washes were repeated 3 times using a magnetic stand to pellet GFP-binding resin. All steps were performed at 4°C using ice-cold buffer. Following the final wash, all buffer was carefully removed using a syringe fitted with a 27G needle (Terumo), and the resin was resuspended in to $30\ \mu\mathrm{l}$ 4x NuPage LDS sample buffer (Invitrogen) and prepared and used in gel electrophoresis as described above.

Antibodies

Polyclonal antibodies were generated against Mp10 and MpOS-D1 by Genscript (New Jersey, USA). The predicted mature Mp10 and OSD1 proteins (minus signal peptide) were

expressed in E. coli with N-terminal 6Xhis tags by Genscript BacPower^{TM} and FoldArt^{TM} technologies to express and purify the target protein. Protein quality and purity was verified by SDS-PAGE and western-blotting using anti-his-tag antibody. Anti-sera was raised in chicken (Mp10) or rabbit (OSD1). Specific antisera were affinity purified using immobilized recombinant protein. Sensitivity and specificity of the antibodies were tested by Western blotting using a dilution series of the pure recombinant proteins on blots probed with a series of different dilutions of the antisera (carried out by Sam Mugford, detailed in Chapter 5). Antibodies were used at 1:1000 (Western blotting) or 1:100 (immunogold labeling).

Other primary antibodies used were anti-GFP (polyconal rabbit, Santa Cruz Biotech, Texas, USA) and anti-FLAG (monoclonal mouse, Sigma-Aldrich), which were used at 6:10,000 and 1:10,000 dilutions respectively. Anti-mouse, anti-rabbit and anti-chicken HRP-conjugated secondary antibodies (all Sigma Aldrich) were all used at 1:10,000.

2.11 RNAseq investigations

Two leaves of four week old Col-0 and *bak1-5 A. thaliana* plants were placed in a clip cage containing either 10 GPA, 10 *Acyrthosiphon pisum* or 4 *Bemisia tabaci* (2 male and 2 female). Five plants were treated with each insect species, with 2 clipcages per plant. Control plants were clipped with empty cages. Leaves were harvested, brushed free of insects and snap frozen in liquid nitrogen after 48 hours. Samples were ground and RNA was extracted using RNeasy Mini Kit (Qiagen), before being checked for purity and 3 samples of each sumitted to The Genome Analysis Centre (TGAC, Norwich, UK).

Illumina truseq libraries, with an average insert size of 500 bp, were constructed according to the manufactures protocol. Libraries were sequenced on an Illumina HiSeq2000, with 50 bp single end reads. Four samples were pooled on each lane, generating 40 to 70 million reads per sample. Quality control was done using FastQC (fastqc-0.11.2, http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/), and sequences were screened for contamination using an in-house pipeline called Kontamination. Alignment of RNA-seq reads to transcriptome reference (Arabidopsis thaliana TAIR 10 assembly) was done using TopHat (tophat -2.0.11, http://tophat.cbcb.umd.edu/) with min-anchor-length 12. Differential expression analysis was run using Cuffdiff (cuffdiff-2.1.1, http://cufflinks.cbcb.umd.edu/manual.html) treating the 18 samples as 6 different conditions with 3 replicates each.

2.12 Yeast two-hybrid

Yeast two-hybrid Arabidopsis protein library construction

RNA was extracted using the RNeasy Mini Kit (Qiagen) from *A. thaliana* leaves exposed to GPA, *Acyrthosiphon pisum*, *Bemisia tabaci*, Aster and maize leafhoppers and phytoplasma (Aster Yellows-Witches Broom) then pooled. A sample of phloem sieve element RNA was also added. The RNA was submitted to Dualsystems Biotech (Switzerland) (now Hybrigenics (France)). A normalised cDNA library was constructed from the Arabidopsis RNA in the pGADHA vector by Dualsystems. The library contained an estimated 500,000 clones, with an average insert size of 1.6 kb.

Yeast two-hybrid protocol

Yeast strains used were:
NMY51 for the insect-exposed Arabidopsis library screen using the DUALhybrid yeast two-hybrid system (Dualsystems Biotech, Schlieren, Switzerland) with pGAD-HA (prey, AD) and pLex-AN (bait, BD).
Ma V203 for confirmation assays using the Gateway compatible ProQuest^{TM} two-hybrid system (Invitrogen) with pDEST22 (prey, AD) and pDEST32 (bait, BD).

Transformation of yeast

Yeast cells were transformed as described by Fromont et al. (325). Yeast cells were cultured to an OD_{600} of 0.5 in 100 ml of YPAD (yeast extract-peptone-dextrose) media at 28°C with gentle shaking. After this, the cells were washed twice with sterile water, using centrifugation at 2500 g for 5 minutes at room temperature to pellet cells before removal of supernatant. Cells were finally re-suspended in 1 ml dH_2O. 100 µl of yeast cells were added to 360 µl transformation mixture containing 240 µl polyethylene glycol (PEG), 36 µl lithium acetate (LiOAc), 50 µl boiled salmon sperm DNA (Invitrogen), dH_2O and 1 µg plasmid. The yeast/transformation mix mixture was incubated at 30°C for 1 hour, before being centrifuged at 10,000 g for 30 seconds. The supernatant was removed and the yeast cell pellet re-suspended in 500 µl dH_2O. 250 µl was plated onto synthetic defined (SD) media lacking the appropriate amino acid to select for the plasmid, and incubated at 28°C for 3-5 days.

Successful transformants were picked and used to inoculate SD media lacking the appropriate amino acid. Cells were grown to an OD_{600} of 0.6-0.7, then cultures were centrifuged

and washed as above. Pellets were re-suspended in $600\ \mu l$ LiAc/TE master mix (0.1M LiOAc, 1x TE pH7.5). Transformation reactions were then set up using $600\ \mu l$ yeast cells, $100\ \mu l$ boiled salmon sperm DNA, $7\ \mu g$ plasmid and $2500\ \mu l$ PEG/LiOAc master mix (0.1M LiOAc, 1x TE pH7.5, 50% PEG). Transformation mixes were incubated at 30°C for 45 minutes, then $160\ \mu l$ DMSO was added and mixed, followed by an incubation at 42°C for 20 minutes. Cells were pelleted at 700g for 5 minutes, resuspended in 3 ml YPAD, and incubated at 28°C for 90 minutes. The culture was then centrifuged at 700g for 5 minutes, re-suspended in 5 ml 0.9% NaCl and $300\ \mu l$ plated onto SD plates lacking the appropriate amino acids to select for both plasmids. Plates were incubated at 28°C for 3-5 days to allow colonies to grow.

AMSH-Mp10 confirmation assays were carried out using a high throughput method carried out in 96-well plates, using the same reagents and solutions.

Checking protein-protein interaction via yeast two-hybrid

Single colonies were taken from plates with yeast transformed for both constructs, and suspended in $200\ \mu l$ of 0.9% NaCl. $5\ \mu l$ of each colony suspension was pipetted out onto SD plates lacking the appropriate amino acids to select for both plasmids, and for protein interaction. Plates were left at 28°C for 2-5 days to allow colonies to grow, at which point images were taken.

2.13 Imaging

Confocal imaging

Leaf discs were taken from *N. benthamiana* expressing fluorescently tagged genes of interest using a circular cork borer (9 mm diameter). Leaf discs were mounted in water and imaged using the Leica SP5 II confocal microscrope (Leica Microsystems, Milton Keynes, UK). GFP was excited using the 488-nm argon laser, and fluorescence emissions were captured between 500 and 550 nm. RFP was excited at 561 nm, and emission taken between 580 and 620 nm. The sequential scan mode was used for simultaneously imaging GFP and RFP. For GFP only images, chloroplast autofluorescence was captured between 700 and 800 nm. Images were taken using a 63X water immersion objective and processed using the Leica LAS-AF software package (Leica Microsystems).

Immunogold labelling

Leaves of 4 week old *A. thaliana* were exposed to 1-week old GPA for 24 hours. After this time, aphids were removed from the leaves, and the leaves were submitted to Elaine Barklay (Bioimaging, JIC, Norwich) for fixing and immunogold labelling.

Samples were fixed in 4% formaldehyde/0.5% glutaraldehyde in PBS and left at 4°C overnight. The samples were then embedded using the progressive lowering of temperature (PLT) method using the Leica EM AFS2 (Automatic Freeze Substitution) equipment (Leica Biosystems, Milton Keynes, UK). Sectioning was carried out on a Leica UC6 ultramicrotome to produce ultrathin sections of approximately 90 nm which were picked up onto 200 mesh gold grids which had been pyroxilin and carbon coated. The sections were labelled according to an Aurion protocol (www.Aurion.nl). TEM - Grids were viewed in a FEI Tecnai 20 transmission electron microscope (FEI UK Ltd, Cambridge, UK) at 200 kV and imaged using an AMT XR60 digital camera (Deben, Bury St Edmunds, UK) to record TIF files.

Chapter 3

The plant response to aphids shares components of PAMP-triggered immunity

Contributors: Claire L. Drurey, David C. Prince, Simon Lloyd, Cyril Zipfel and Saskia A. Hogenhout

Parts of this chapter were published in Prince et al. (2014) and Kettles et al. (2013) (Appendix A and B)

3.1 Introduction

Typically, plant defenses against insects are discussed in terms of static constitutive defenses, such as physical barriers formed by spines and trichomes, and active induced defenses, like the production of volatiles upon insect feeding (326). For inducible defense to occur, the plant must perceive the insect to trigger regulatory signaling and an appropriate response. Though complex pathogen-recognition mechanisms that trigger plant innate immunity have been identified in many plant species, less is known about how plants perceive and respond to insect-derived signals (115).

Investigations into *Manduca sexta* feeding on *Nicotiana attenuata* have revealed that BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1) is required for phytohormone responses and secondary metabolite production after herbivory (110). BAK1 is a key regulator of several leucine-rich repeat-containing surface-localized pattern recognition receptors (PRRs) and plays a central role in plant innate immunity and brassinosteroid signaling (148). Oral secretions from *Manduca sexta* activate both salicylate- and wound-induced protein kinases (SIPK and WIPK), types of mitogen activated protein kinase (MAPK), which are needed for the phytohormone response to feeding and transcriptional upregulation of defense related genes (118). The WRKY transcription factors WRKY3 and WRKY6 are upregulated by *Manduca sexta* oral secretions and are needed for jasmonic acid-mediated herbivore defense (327). WRKY transcription factors, BAK1 and MAP kinases have signaling roles in immune responses to plant pathogens as well (328). This suggests that components of the plant defense pathways to microbial plant pathogens and herbivores are shared.

In the perception of pathogens by plants, the first step is the recognition of conserved elicitors known as pathogen associated molecular patterns (PAMPs). These PAMPs are recognized by pattern recognition receptors (PRR) in the cell membrane, which then activate signaling pathways, leading to induction of defense responses. The immune response triggered by PAMPs is termed PAMP-triggered immunity, or PTI. PAMPs perceived by plants are varied (329). They may be peptides, such as the bacterial PAMP flg22 derived from flagellin, or polysaccharides such as chitin from fungi and oligogalacturonides, which are released from the plant cell wall upon tissue damage (139; 140; 330; 331; 332).

Insect herbivores are likely to release elicitors analogous to PAMPs, often named herbivore-associated molecular patterns or HAMPs. Examples of elicitors from insects that can ac-

tivate plant defenses include glucose oxidases (GOX) and fatty acid-amino acid conjugates (FACs) found in oral secretions of lepidopterans, which are capable of inducing hormonal, ROS and MAP kinase signaling (257; 125; 333; 117). Caeliferins and lipases in grasshopper oral secretions can also activate plant defense responses (190; 122). Components of both aphids and aphid saliva induce defense responses in wheat (334; 335; 127). A heat sensitive factor, probably a peptide between 3 and 10 kD in size, in green peach aphid (GPA) saliva induces resistance to the aphid in *Arabidopsis thaliana* (47). We therefore wanted to investigate whether the perception of insects via these elicitors occurs via the same pathways as those in PTI.

There are numerous plant responses that can be used as a read-out of the activation of PTI. These include the induction of a reactive oxygen species (ROS) burst, defense gene induction, deposition of callose, and production of antimicrobial compounds such as glucosinolates and camalexin, as well as increased resistance of plants upon previous exposure to elicitors (also known as induced resistance) (328; 336). Callose is a polysaccharide that is laid down in the cell wall in response to both pathogen attack and wounding (337). Functional PENETRATION2 (PEN2) is needed for callose deposition in response to some PAMPs (205). PEN2 encodes a glycosyl hydrolase that localizes to peroxisomes (228) and is predicted to generate glucosinolates required for callose formation (205). Interestingly, glucosinolates were originally identified as important in avoiding damage by herbivores rather than pathogens (223), suggesting that these metabolites are also common between the responses to insects and pathogens. Callose is deposited in response to aphid feeding in several plant species (175; 200; 201; 338), though it is unknown whether this involves the same components as callose deposition in response to pathogens, and whether it is a response to the aphid itself or to the damage that occurs during feeding.

Several studies show that aphids induce defense genes in plants, including those involved in the salicylic and jasmonic acid pathways (339; 209; 213). Some overlap is seen between genes induced by insects and those induced by pathogens (209). However, little is known about the signaling events that take place before gene induction in the plant response to insects, or how these changes contribute to defense against herbivores. In PTI, defense genes are induced downstream of initial perception events by receptors such as BAK1 and signaling involving ROS and calcium bursts and MAP kinase cascades (328). It would be interesting to see whether signaling events such as these are also upstream of defense gene induction in response to aphids, and whether the components of signaling are shared.

To investigate whether aphids contain elicitors that can activate plant responses typical of PTI, we looked at plant innate immune responses to GPA. We used both wild type (Col-0) Arabidopsis and several defense-related mutants to identify components involved in the plant response to aphids. The majority of the results described in this chapter have been published in Prince et al. (2014) (340) on which I am a second author. I have contributed experiments to this publication or helped with executing them. In this chapter I have described the experiments I conducted or contributed to, and will refer to the published results in Prince et al. (2014) to complete the story where required. The Prince et al. (2014) paper is included as an appendix in my thesis (Appendix A). Similarly, I contributed some experiments to the Kettles et al., (2013) publication (238) on which I am also a second author. The Kettles et al. (2013) paper is also included as an appendix in this thesis (Appendix B) and I will refer to it in this chapter as needed. Finally, this chapter includes additional experiments I carried out to investigate the nature of the aphid elicitors further.

3.2 Results

3.2.1 Aphids induce defense responses in Arabidopsis

When I started my PhD research in the laboratory, David Prince had already established GPA interactions with *Arabidopsis thaliana* as an experimental system to study components of PTI. He found that GPA extract induces ROS bursts which are dramatically decreased in *bak1-5* plants, but not in mutants defective for known BAK1-interacting PRRs: *fls2* and *efr* single mutants, the *fls2/efr/cerk1* triple mutant, *pepr1-1*, *pepr1-2* and *pepr2-1* single mutants and *pepr1/pepr2* double mutants (Appendix A, Fig 5, Prince et al., 2014). David Prince also found that plants become more resistant to GPA upon treatment with GPA extract, and I aided in experiments that showed this induced resistance response is dependent on BAK1 (Appendix A, Fig. 2, Prince et al., 2014). Together these results suggest that BAK1 is required for PTI to GPA, whereas FLS2, EFR1, and PEPR1 and 2 are not. Moreover, given that the *fls2/erf/cerk1* mutant does not show a reduced ROS response to GPA extract, chitin perception may not play a role in PTI to GPA either. Hence, BAK1 is likely to associate with hitherto unknown receptors, which detect hitherto unknown GPA elicitors to trigger PTI.

It was decided to use GPA extract in the PTI assays because plants are exposed to several aphid components during colonisation, including aphid legs, outer skeleton from moults and feeding mouthparts (stylets) and excretions of the aphid (also known as honeydew). Moreover, aphid saliva contains proteins that are not only produced in the salivary glands but also elsewhere, such as in the intestines and from bacterial symbionts, such as *Buchnera aphidicola*, which are plentiful and surround aphid intestines in specialized cells called bacteriocytes (17). Aphid saliva also triggers an induced resistance response in *A. thaliana* to GPA (47) (Appendix A, Supplementary Figure S2, Prince et al., 2014), but we found that extracts from whole aphids generated more consistent results. In some experiments I also used GPA extract supernatant, which is less dense and therefore easier to infiltrate into the leaves without damaging them. To obtain the supernatant, I spun down the aphid extract to pellet the aphid exoskeleton fraction and used the aqueous phase that should contain proteins and other soluble aphid molecules.

I used whole GPA-extract to investigate the induction of three PTI marker genes: FLG22-INDUCED RECEPTOR-LIKE KINASE1 (FRK1), CYTOCHROME P450, FAMILY 81, SUBFAMILY F, POLYPEPTIDE 2 (CYP81F2) and PHYTOALEXIN DEFICIENT 3 (PAD3/CYP71B15). These genes are markers for early immune signaling, indolic glucosi-

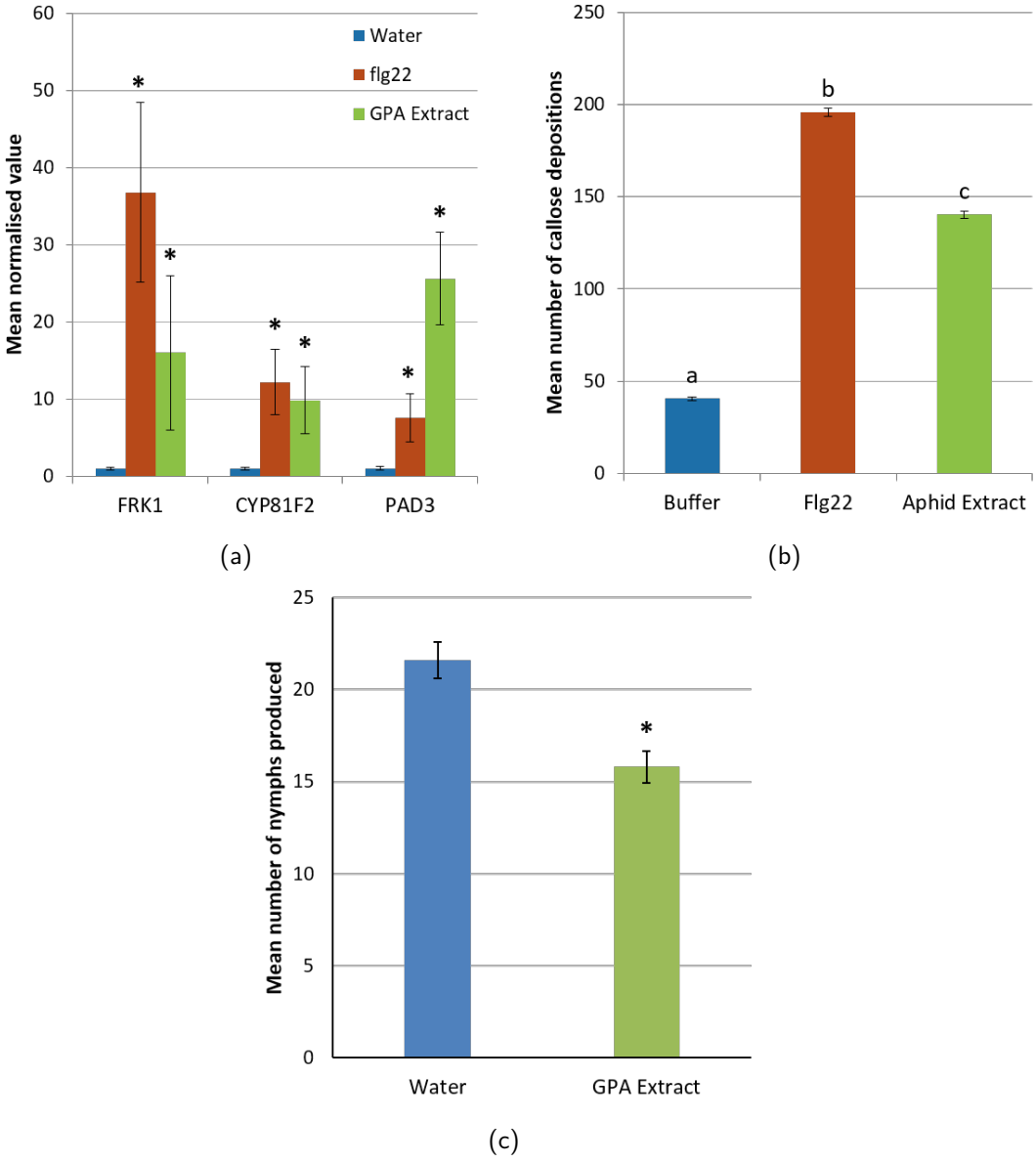


Figure 3.1: **GPA extract induces defense responses typical of PTI in Arabidopsis**. 3.1a GPA extract induces the expression of PTI marker genes. Bars show mean ± SE of target gene expression levels in 4 independent experiments (3 technical replicates per experiment). Asterisks indicate significant differences to the water treated control (Student's t-probabilities calculated within GLM at P <0.05). 3.1b Treatment with GPA extract elicits callose deposition. Bars show mean ± SE callose deposits produced per 1.34 mm^2 of leaf upon treatment with buffer, 100 nM flg22 or GPA extract supernatant in 3 independent experiments (n = 12 leaf discs per experiment). Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at P <0.001). 3.1c GPA extract causes induced resistance in Arabidopsis. Bars shows mean ± SE fecundity of aphids over 10 days in 4 independent experiments (n=6 per experiment). Asterisk indicates significant difference to water control (Student's t-probabilities calculated within GLM at P <0.05).

nolate production and camalexin biosynthesis respectively (186; 341; 235), and are known to be induced by protein and carbohydrate PAMPs (342; 343). All three genes were induced by both GPA-extract and the flg22 positive control (Figure 3.1a; Appendix A, Fig. 1a, Prince et al., 2014). FRK1 and CYP81F2 were induced to a similar level by both flg22 and GPA extract, whereas PAD3 was induced more highly by GPA extract than by flg22. These data suggest that GPA extract contains an elicitor or elicitors that induce the expression of defense genes in Arabidopsis, similarly to bacterial elicitors.

Callose deposition is commonly used to quantify plant immune activity (206), and many elicitors induce this response, including the bacterial flagellin epitope flg22 (344). I used GPA-extract supernatant in callose assays alongside buffer and flg22, as negative and positive controls respectively. The three solutions were infiltrated into Arabidopsis leaves and left for 24 hours before staining with aniline blue. Callose deposits were then visualised under magnification and UV light and quantified. I found that GPA-extract caused an increased number of callose deposits in Arabidopsis leaves compared with the buffer control, though this was not as high as that seen in flg22-treated leaves (see Figure 3.1b; Appendix A, Fig. 1b, Prince et al., 2014).

Induced resistance was also used to investigate Arabidopsis responses to GPA. This response occurs when prior exposure to an elicitor enhances plant resistance to an attacking organism (345; 140). Arabidopsis leaves were infiltrated with whole GPA-extract or a water control and then the fecundity of aphids placed on these pre-treated leaves was measured over 10 days. We found that aphids placed on leaves that had been infiltrated with GPA-extract produced less nymphs than aphids on leaves infiltrated with a water control (Figure 3.1c), suggesting that a defense response had been triggered by the pre-treatment.

A ROS burst is an early indicator of the defense response in Arabidopsis, occurring before induction of defense genes (174). To determine whether whole GPA extract was able to elicit a ROS burst, we used a luminol-based assay on leaf discs in 96-well plates, which enabled accurate luminol and PAMP application and hydrogen peroxide (H_2O_2) quantification via amount of light emitted per disc, which gives an indication of the total amount of ROS produced by the plant (346; 139). Applying whole GPA-extract to Arabidopsis leaf discs leads to production of ROS, at higher levels than in a water control (see Figures 3.2a 3.2b and 3.2c; Appendix A, Fig. 1d and 1e, Prince et al., 2014). However, the ROS burst to GPA-extract occurred after one hour. This is much later that the ROS burst to flg22, which occurs between 5 and 25 minutes after PAMP exposure (Figures 3.2a and 3.2b).

The total amount of ROS produced in this time, measured as Relative Light Units (RLU), is also less than that produced by flg22, though still statistically more than that seen in the negative control of water alone (Figure 3.2c).

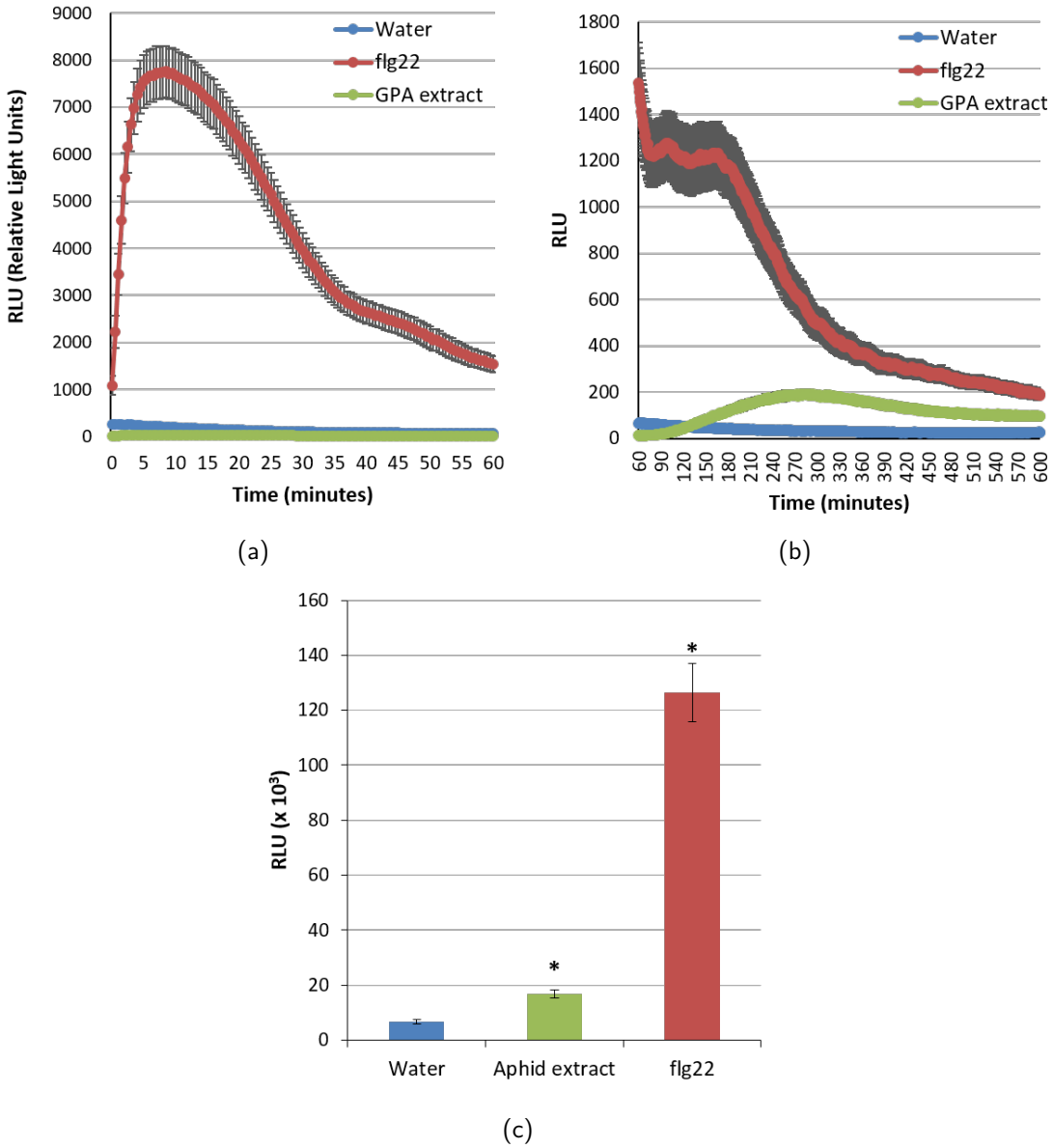


Figure 3.2: **GPA extract causes a ROS burst in Arabidopsis**. 3.2a GPA extract does not induce a ROS burst in the first hour, whereas flg22 does. 3.2b GPA extract induces a ROS burst between 1 and 10 hours. 3.2c Total ROS produced over 10 hours.
Leaf discs were treated with water, GPA-extract or 50 nM flg22 and the ROS burst measured using a luminol-based assay over 10 hours. All results show mean ± SE of ROS produced in 4 independent experiments (n=8 per experiment). Asterisks in 3.2c indicate significant differences to the water treated control (Student's t-probabilities calculated within GLM at $P < 0.05$).

3.2.2 Elicitors are conserved amongst aphid species

PAMPs/HAMPs are often conserved among related pathogens as evolution favours plant recognition of molecules that are essential for pathogen function and carry little variation

(347). I therefore looked at induction of the defense genes FRK1, CYP81F2 and PAD3 in Arabidopsis plants treated with extracts from several aphid species (Figure 3.3; Appendix A, Fig. 6, Prince et al., 2014). I used extracts from the pea aphid (*Acyrthosiphon pisum*), the cabbage aphid (*Brevicoryne brassicae*) and the English grain aphid (*Sitobion avenae*) alongside GPA extract. The expression of all three genes was induced to similar levels by the different aphid extracts, although induction of FRK1 and CYP81F2 was not statistically significant after treatment with *S. avenae*-derived extract. These results provide evidence that the aphid-derived elicitors detected by Arabidopsis are potentially conserved between different aphid species.

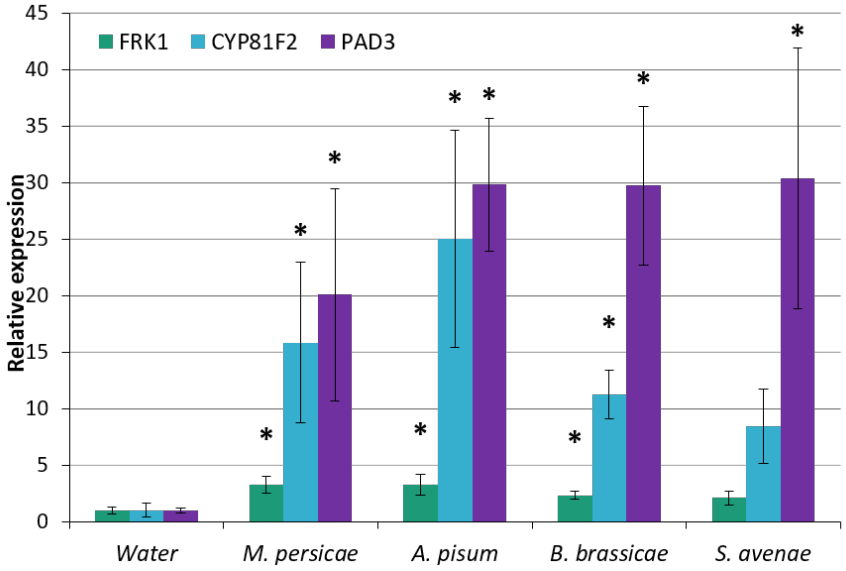


Figure 3.3: **Extracts from several aphid species induce the expression of PTI marker genes**. Bars show mean ± SE of target gene expression levels in 4 independent experiments (3 technical replicates per experiment). Asterisks indicate significant differences to the water treated control for each of the genes (Student's t-probabilities calculated within GLM at P <0.05).

3.2.3 The response to aphid elicitors involves known components of PTI in plants

As GPA extract induces the expression of genes that are involved in the PTI response of Arabidopsis, we wanted to investigate if other components known to be involved in PTI also play a role. First, it was investigated if BAK1 and the NADPH-oxidase respiratory burst oxidase homolog D (AtRBOHD) are involved in the detection of GPA elicitors. AtRBOHD is a membrane-associated NADPH-oxidase that is responsible for ROS bursts produced as a component of both PTI and ETI (178; 348). BAK1, acting as a co-receptor, is a central regulator of many PTI responses that have been characterised (349). For investigation into BAK1 involvement in GPA-induced immune responses we used the Arabidopsis *bak1-5* mu-

tant, which has a mutation in its cytoplasmic kinase domain that affects immune signaling but not brassinosteroid signaling, avoiding pleotropic effects (147). In contrast, the Arabidopsis null mutant for BAK1, *bak1-4*, shows reduced growth and a more readily inducible hypersensitive (HR) response, which together could deter aphids (350). Consistent with this, GPA shows similar fecundity on Arabidopsis Col-0 and *bak1-5* plants, but significantly reduced fecundity on Arabidopsis *bak1-4* plants (Appendix A, Supplementary Fig. S1, Prince et al., 2014). ROS burst assays showed that the GPA-extract triggered ROS burst in the *bak1-5* mutant was much reduced, and in *AtrbohD* the ROS burst was lost altogether (Figure 3.4a; Prince et al., 2014, Fig. 2a, Appendix), indicating that both BAK1 and AtRBOHD are involved in the production of the ROS burst upon detection of GPA elicitors.

CYP81F2 is induced by aphid extracts (Figure 3.3) and is required for callose biosynthesis, upstream of the myrosinase PEN2 (227). I therefore investigated whether BAK1 and PEN2 are required for GPA-triggered callose deposition. In both *bak1-5* and *pen2-1* mutants, the number of callose deposits was significantly reduced compared with that of wild type Col-0 (Figure 3.4b; Appendix A, Fig. 2b, Prince et al., 2014). BAK1 and PEN2 are therefore required for callose deposition upon perception of GPA elicitors.

I also investigated PAD3 expression, which was highly induced by GPA extract, in the *bak1-5* and *AtrbohD* mutants after GPA-extract exposure. Unexpectedly, the increase in PAD3 expression still occurred in *AtrbohD* and *bak1-5* mutants (Figure 3.4c; Appendix A, Fig. 3d, Prince et al., 2014). Induction of PAD3 expression by GPA elicitors is therefore not dependent on BAK1 and ROS induction via AtRBOHD. This indicates that other recognition components mediate PAD3 induction upon the detection of GPA elicitors (see Chapter 4 in which I have investigated this further). These data also suggest that GPA extract may have at least two elicitors, one of which activates callose deposition and ROS via BAK1 and another which triggers PAD3 expression in a BAK1-independent manner.

3.2.4 Defense responses to aphids involve the phytoalexin camalexin

Previous work in the laboratory showed that GPA fecundity increases on *cyp79b2/b3* mutant plants (Figure 3.5b; Appendix B, Fig. 3f, Kettles et al., 2013). CYP79B2 and B3 are cytochrome P540s which convert tryptophan to indole-3-acetaldoxime (IAOx) (306), indicating that IAOx, which is required for the production of auxin, indolic glucosinolates and camalexin (see Figure 3.5a; Appendix B, Fig. 3e, Kettles et al., 2013), is involved in plant resistance to GPA. Camalexin is likely mostly responsible for this resistance to GPA, because

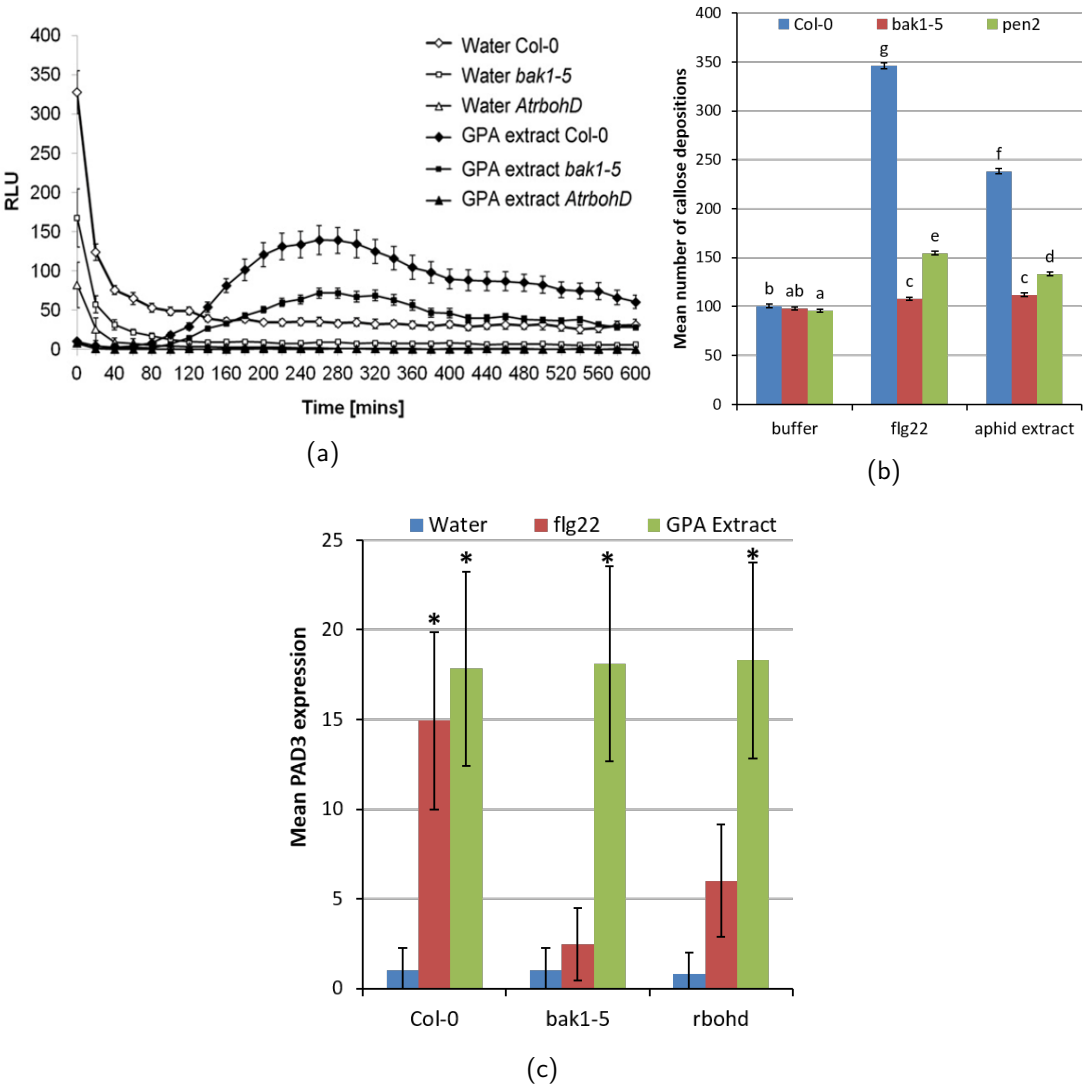


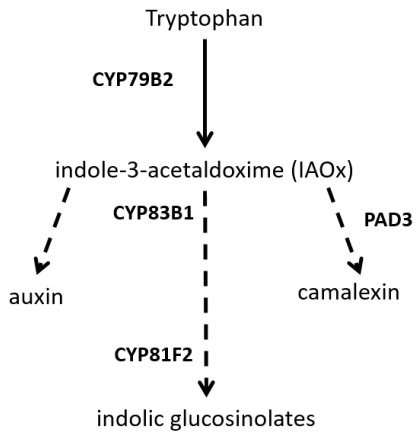
Figure 3.4: **The plant response to aphid extract involves known components of the plant immune system**. 3.4a The ROS response of Arabidopsis to GPA extract is reduced in *bak1-5* and absent in the *AtrbohD* Arabidopsis mutants. Bars show mean ± SE of ROS produced in 16 leaf discs over 600 minutes. Experiment was repeated 3 times with similar results, data from one representative experiment are shown. Asterisks indicate significant differences to the water treated control (Student's t-probabilities calculated within GLM at P <0.05). Experiment conducted by David Prince. 3.4b Callose depositions upon flg22 and GPA extract treatments are reduced in *bak1-5* and *pen2-1* mutant Arabidopsis. Bars show mean ± SE of callose deposits produced per 1.34 mm^2 of leaf upon treatment with buffer, 100 nM flg22 or GPA extract supernatant in 3 independent experiments (n = 12 leaf discs per experiment). Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at P <0.05). 3.4c The induction of PAD3 gene expression by GPA extract does not require AtRBOHD or BAK1. Bars show mean ± SE of PAD3 expression levels in 3 independent experiments (3 technical replicates per experiment). Expression levels were normalised with the water control of Col-0 set at 1. Asterisks indicate significant differences to the water control (Student's t-probabilities calculated within GLM at P <0.05).

GPA fecundity similarly increased on *pad3* mutant plants (Figure 3.5b and Appendix B, Fig. 3f, Kettles et al., 2013), but did not increase significantly on Arabidopsis mutated in CYP81F2 (Figure 3.5c and Appendix B, Fig. 3g, Kettles et al., 2013), which is required for the production of indolic glucosinolates downstream of IAOx (Figure 3.5a). I used the *cyp79b2/b3* and *pad3* mutant plants in induced resistance assays. I found that resistance induced upon treatment of the plants with GPA-extract is lost on both the *cyp79b2/b3* and *pad3* plants, whereas it occurs on Col-0 wild type plants (Figure 3.5d; Appendix A, Fig. 3c, Prince et al., 2014). PAD3, and so camalexin, are therefore involved in the Arabidopsis induced resistance response to GPA.

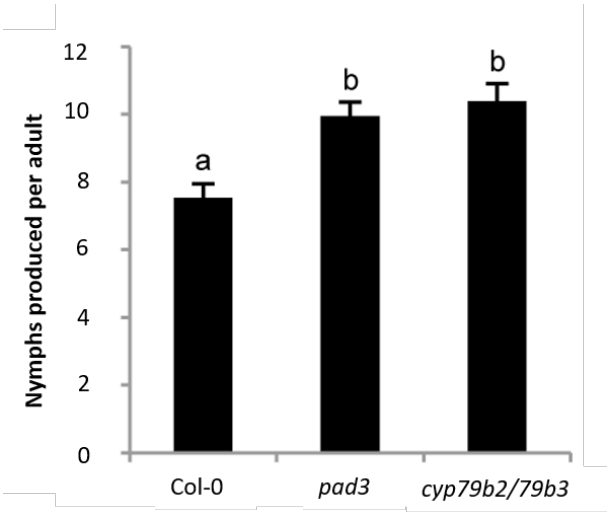
Previous experiments in the laboratory have shown GPA fecundity is reduced upon feeding the aphids purified camalexin via artificial diets in which the camalexin concentration is similar to that present in plants (Figure 3.5e; Appendix B, Fig. 5c, Kettles et al., 2013) (238). Moreover, *PAD3* is induced at the sites of aphid feeding (Appendix B, Supplementary Fig. S6, Kettles et al., 2013). Therefore it is likely that PAD3-mediated production of camalexin by Arabidopsis is directly toxic to aphids.

Prior work in the laboratory also showed that *PAD3* gene expression and camalexin production are dramatically increased in the endoribonuclease Dicer-like 1 (*dcl1*) mutant, which has a defective micro RNA (miRNA) pathway. DCL1 is involved in the production of miRNAs from their precursors by performing the first cleavage steps on pre-miRNAs (351; 352). In contrast, *PAD3* expression and camalexin production in the *dcl2/dcl3/dcl4* triple mutant, which has a functional miRNA pathway but is defective in the processing of small interfering RNA (siRNA) and other small RNA (sRNA) (353), was similar to that of Col-0 (Appendix B, Fig. 3a and Fig. 5a, Kettles et al., 2013). Aphid fecundity decreases dramatically on the *dcl1* mutant compared to Col-0 and *dcl2/3/4* triple mutant plants (Appendix B, Fig. 1, Kettles et al., 2013). Moreover, aphids raised on *dcl1* plants contain higher camalexin concentrations than those raised on Col-0 and the *pad3* mutant control (Appendix B, Fig. 5b, Kettles et al., 2013). To complete this work, I investigated aphid fecundity on *dcl1*, the *dcl1/pad3* double mutant and Col-0. I found that aphid fecundity is partly restored on the *dcl1/pad3* mutant (Figure 3.5f; Appendix B, Fig. 6, Kettles et al., 2013), showing that increased *PAD3* expression and camalexin are partly behind the reduction in GPA fecundity seen on a *dcl1* plant.

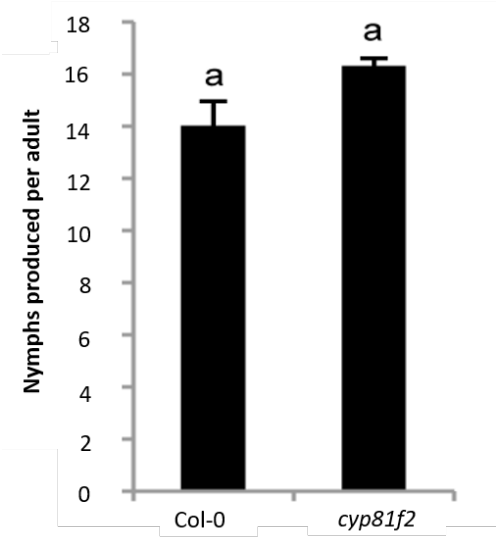
Taken together, this work demonstrated that PAD3 and camalexin are important components of the plant resistance response to GPA. It also showed that the regulation of PAD3


Tryptophan
CYP79B2
indole-3-acetaldoxime (IAOx)
CYP83B1
PAD3
auxin
camalexin
CYP81F2
indolic glucosinolates

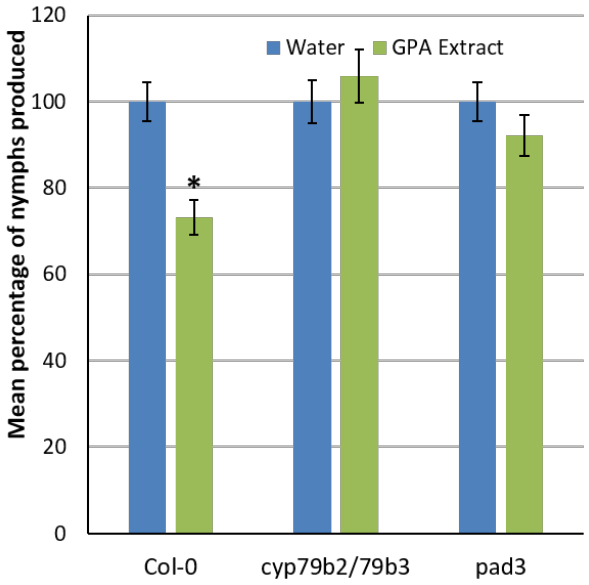
(a)


Nymphs produced per adult
12
10
8
6
4
2
0
a
b
b
Col-0
pad3
cyp79b2/79b3

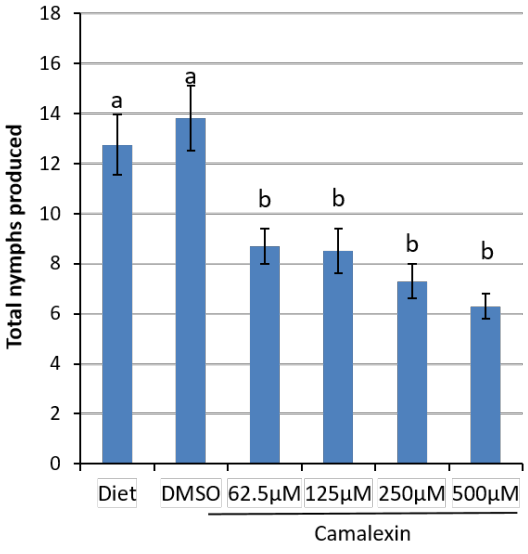
(b)


Nymphs produced per adult
18
16
14
12
10
8
6
4
2
0
a
a
Col-0
cyp81f2

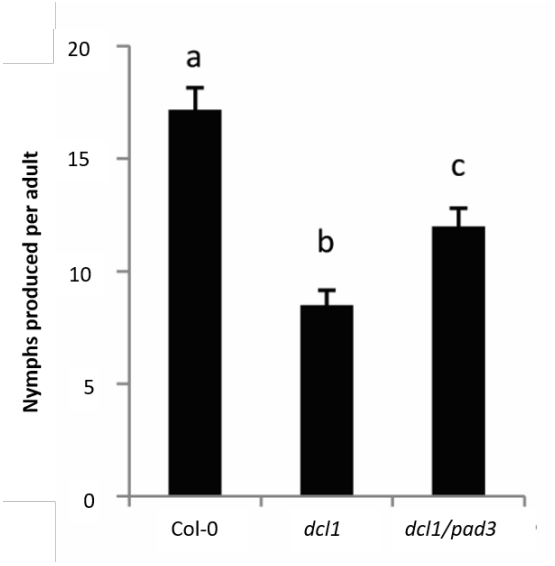
(c)


Mean percentage of nymphs produced
120
100
80
60
40
20
0
Water
GPA Extract
*
Col-0
cyp79b2/79b3
pad3

(d)


Total nymphs produced
18
16
14
12
10
8
6
4
2
0
a
a
b
b
b
b
Diet
DMSO
62.5µM
125µM
250µM
500µM
Camalexin

(e)


Nymphs produced per adult
20
15
10
5
0
a
b
c
Col-0
dcl1
dcl1/pad3

(f)

Figure 3.5 *(previous page)*: **Defense against aphids in Arabidopsis involves the phytoalexin camalexin**. 3.5a Figure showing the biosynthetic pathway of camalexin from tryptophan via the P450 enzymes CYP79B2/B3 and PAD3, from Kettles et al. (238). 3.5b Aphid fecundity is increased on *pad3* (camalexin deficient) and *cyp79b2/b3* (camalexin/indole glucosinolate deficient) mutants. Bars represent mean ± SE fecundity of aphids in 2 independent experiments (n=10 per experiment). Letters indicate significant differences between treatments at $P < 0.05$ as determined by analysis of deviance (ANODE). Experiment conducted by Graeme Kettles. 3.5c Aphid fecundity is unchanged on *cyp81f2* Arabidopsis mutants. Bars represent mean ± SE fecundity of aphids in 2 independent experiments (n=10 per experiment). Letters indicate differences at $P < 0.05$ as determined by analysis of deviance (ANODE). Experiment conducted by Graeme Kettles. 3.5d Induced resistance caused by GPA extract is lost on *pad3* and *cyp79b2/b3* mutants. Bars shows mean ± SE fecundity of aphids over 10 days in 4 independent experiments (n=6 per experiment). Nymph counts were normalised with the water control set at 100%. Asterisk indicates significant difference between water and GPA-extract treatment (Student's t-probabilities calculated within GLM at $P < 0.05$). 3.5e Camalexin reduces aphid fecundity. Camalexin was fed by artificial diet with DMSO as a negative control. Bars represent the mean number of nymphs produced ± SE in 2 independent experiments (n=5 feeders per experiment). Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at $P < 0.05$). Experiment conducted by Graeme Kettles. 3.5f Aphid fecundity is partially restored on a *dcl1/pad3* double mutant. Aphid fecundity is higher on *dcl1/pad3* than on *dcl1* single mutants, but is not fully restored to wild-type levels. Bars represent the mean (± SE) of 18 plants of each genotype from three independent experiments. Letters indicate differences at $P < 0.01$ as determined by t-probabilities within a generalized linear model (GLM).

gene expression and camalexin production involves a miRNA component, supporting my observation that their regulation includes other pathways in addition to those involving BAK1.

3.2.5 Investigation of the aphid extract elicitor

The work on BAK1 and PAD3 provided evidence that GPA extract contains at least two elicitors. I wished to investigate the biochemical properties of these elicitors. Previous work identified that a 3-10 kD fraction was responsible for the ROS burst in Arabidopsis (Appendix A, Fig. 4c, Prince et al., 2014), and that both the 3-10 kD fraction in GPA extract and GPA saliva and a larger than (>) 10 kD fraction could lead to an induced resistance response (Appendix A, Fig. 4d, Prince et al., 2014), providing additional evidence of the presence of at least two elicitors in GPA extract. To further characterise the elicitor properties, the GPA extract supernatant was boiled and proteinase K-treated before use in a ROS burst assay and infiltration of Arabidopsis leaves for use in induced resistance assays. The boiling treatment removed any eliciting activities; both the ROS burst and the induced resistance response were lost compared to the non-boiled control (Figures 3.6; Appendix A, Fig. 4a and 4b, Prince et al., 2014). Similarly, proteinase K treatment also led to the loss of the induced resistance response to GPA extract supernatant (Figure 3.6b; Appendix A, Fig. 4b, Prince et al., 2014). However, proteinase K itself induces a ROS burst in Arabidopsis (Figures 3.7b and 3.7c; Appendix A, Supplementary Fig. S3, Prince et al., 2014). I could therefore not determine if the ROS burst to proteinase-K-treated GPA extract is reduced. Interestingly, proteinase K does not generate an induced resistance response, whereas GPA extract does (3.6b; Appendix A, Fig. 4b, Prince et al., 2014). Therefore a response in-

duced by GPA extract in addition to ROS provides specificity to the Arabidopsis response to GPA. These data suggest that the elicitors present within the two fractions are likely to contain heat-sensitive proteins or peptides that are dependent upon their 3D structure for the eliciting activity.

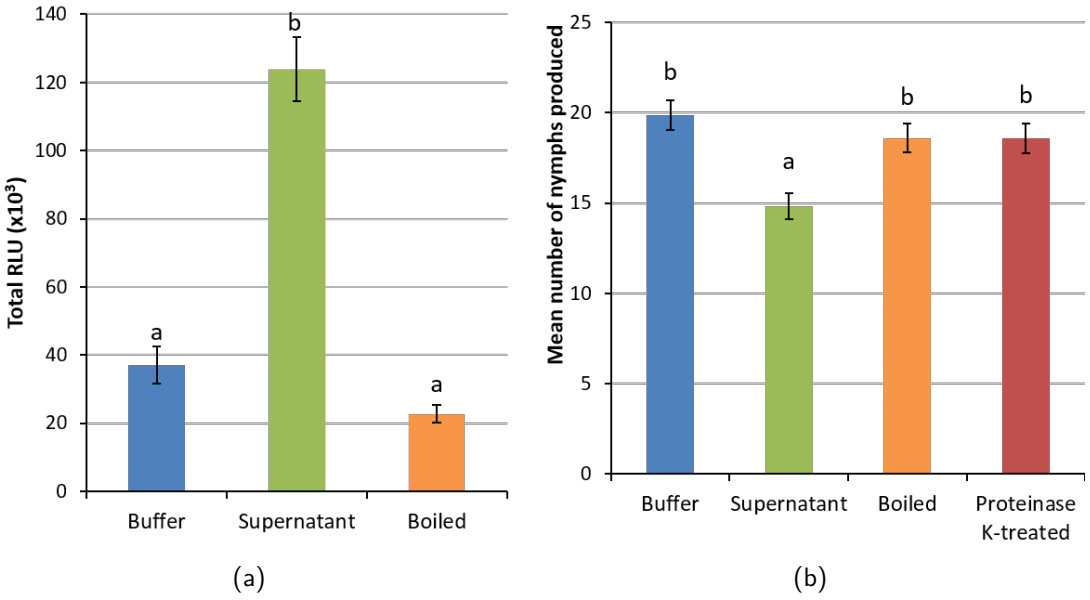


Figure 3.6: **GPA-extract eliciting activities disappear upon boiling and proteinase K treatments**. 3.6a Boiled GPA extract does not elicit a ROS burst. Leaf discs were treated with water, GPA-extract supernatant or boiled GPA extract and the ROS burst measured using a luminol-based assay. Bars show mean ± SE of ROS produced in 16 leaf discs over 600 minutes. Experiment was repeated 3 times with similar results; data from one representative experiment are shown. Asterisks indicate significant differences to the water treated control (as determined by analysis of variance (ANOVA) at P <0.001). Experiment conducted by David Prince. 3.6b Boiled and proteinase K-treated GPA extract does not elicit induced resistance. Bars show the mean ± SE fecundity of aphids in 3 independent experiments (n=10 per experiment). Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at P <0.05).

The findings that the ROS burst to GPA extract occurs over a prolonged period of time compared to that of flg22 (Figures 3.2a and 3.2b) and the sensitivity of GPA extract to heat and proteinase treatments (Figures 3.6a and 3.6b) suggest that the elicitor could be an enzyme that needs to catalyse components before recognition by the plant can occur. It is known that the activities of pathogen-secreted enzymes can release plant damage associated molecular patterns (DAMPs), which are degradation products detected by specific PRRs of the plant that induce immune responses (354) . To investigate whether GPA extract possesses the enzymatic activity of proteolysis, I carried out ROS burst assays in the presence of a protease inhibitor cocktail (Figure 3.7a). The presence of the cocktail did decrease the amount of ROS produced in response to GPA extract to that of the level seen in the water control treatment (Figure 3.7a). However, the ROS burst produced by flg22 also decreased in the presence of the cocktail, and flg22 is a peptide that does not require protease activity to induce a ROS burst. The protease inhibitor cocktail used in the ex-

periments contains EDTA and 1,10-phenanthroline, both of which are capable of chelating calcium ions (chelation of metal ions prevents the action of metalloproteases). As a calcium burst is also involved in the PTI response and occurs upstream of the ROS (185; 355; 180), calcium ion chelation may have an effect on the ROS burst itself, rather than inhibiting any potential protease action. To test this, I used EDTA as a control to account for the presence of calcium chelation activity in the protease inhibitor cocktail. Running a ROS burst assay including both the protease inhibitor cocktail and EDTA demonstrated that calcium chelation affects the ROS burst response to GPA extract and flg22 (Figure 3.7b). This experiment also demonstrates that the calcium burst plays a role in the defense response to GPA upstream of ROS induction, as it does for other PTI-elicitors.

I decided to investigate individual protease inhibitors. I ran the ROS burst assay with both phosphoramidon, which inhibits metallo-endopeptidases, and leupeptin, which inhibits cysteine, serine and threonine peptidases. Leupeptin reduced the ROS produced upon exposure of leaves to proteinase K (a serine protease) (Figure 3.7c), indicating that the experiment worked. Neither of the protease inhibitors reduced the ROS burst produced by GPA extract or flg22. These results suggest that the elicitor in GPA extract, despite being heat sensitive, is not a metallo-endopeptidase, a cysteine, serine or threonine-type protease. The elicitor may have other enzymatic activities, such as modulation of carbohydrates and lipids.

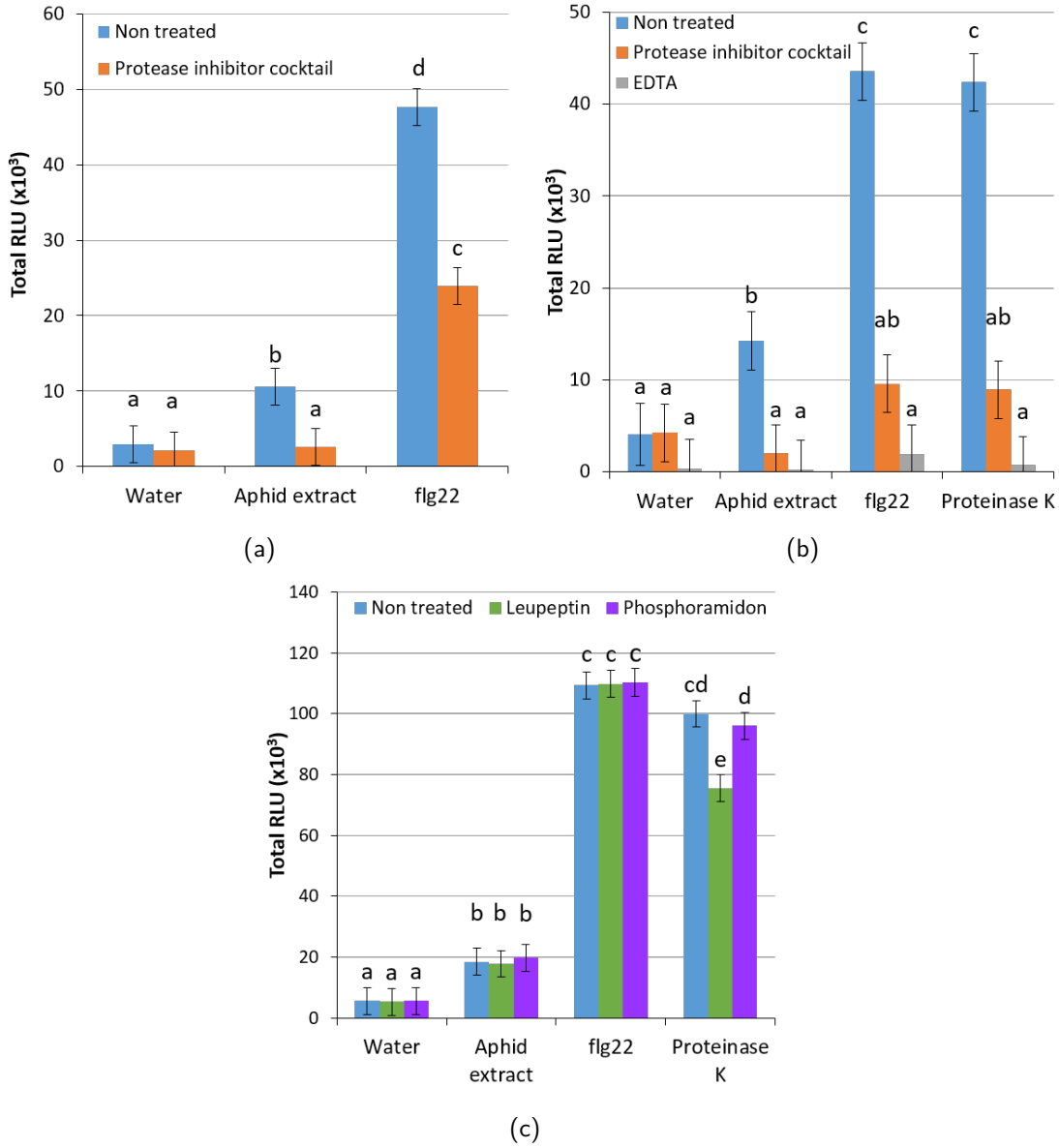


Figure 3.7: **Protease inhibitors do not inhibit GPA-extract ROS eliciting activities**. 3.7a Treatment with protease inhibitor reduces the ROS burst in response to both GPA extract and flg22. Bars show mean ± SE of ROS produced in 3 independent experiments (n=8 per experiment). Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at P <0.05). 3.7b Calcium chelation by EDTA prevents the ROS burst to GPA extract. Bars show mean ± SE of ROS produced in 1 experiment (n=8). Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at P <0.05). 3.7c The protease inhibitors phosporamidon and leupeptin have no effect on the ROS eliciting activity of GPA extract. Bars show mean ± SE of ROS produced in 4 independent experiments (n=7 per experiment). Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at P <0.05).

3.3 Discussion

This research shows that GPA-derived elicitors trigger plant immune responses characteristic of PTI, including the induction of PTI marker genes, PEN2-dependent callose deposition, an AtRBOHD-dependent ROS burst and induced resistance. The extract that was used throughout these investigations was prepared from whole aphids. Although saliva has been used previously in aphid elicitor investigations (47; 334; 335), saliva composition can vary depending on the material used to collect it and the developmental stage of the aphid, as well as aphid feeding stage (such as probing, long-term phloem feeding or xylem feeding) (30; 356; 357; 358; 359; 21; 358). Saliva also contains proteins that are not produced in the salivary glands (39; 360). GPA extract was found to induce specific defense responses against GPA; infiltration into leaves prior to aphid colonisation affected aphid fecundity (induced resistance). This is in contrast to proteinase K which, like GPA extract induces a ROS burst, but does not lead to induced resistance against aphids. GPA extract induces PAD3 expression and camalexin, both of which have been shown to have a direct effect on aphids. Using GPA extract therefore has biological relevance.

GPA extract was used in assays to directly investigate defense responses such as the ROS burst and callose deposition, instead of measuring defense responses against aphids by proxy, using aphid survival or fecundity on mutant plants. Using GPA extract in this way was useful for the identification of potential aphid receptors, identifying BAK1 as a component of aphid perception in Arabidopsis. BAK1 would not have been identified in aphid assays alone, as there is no difference in aphid fecundity between Col-0 and *bak1-5* plants. PRRs known to interact with BAK1 (FLS2, EFR and PEPR1/2) were not found to be required for the ROS burst response to GPA-extract, suggesting that they are not needed for detection of the aphid. I used GPA extract for additional receptor screening (Chapter 4), further validating the use of GPA extract as a tool to study plant responses to GPA.

Some assays, such as the callose assay, were carried out with a spun-down variant of GPA extract, which was still able to induce a response. This suggests that the insoluble fraction of GPA-extract, supposedly made up mainly of exoskeleton, does not contribute any elicitor action. The insect exoskeleton is composed mainly of chitin (361). Arabidopsis has a PRR which recognises chitin; the LysM receptor kinase CERK1 (362). However, CERK1 is not required for the ROS burst to GPA extract (340), so it does not appear that this aphid product is detected by plants, at least in Arabidopsis.

The GPA elicitor is likely to be present in aphid saliva, which comes in direct contact with the plant during aphid feeding. This is in agreement with saliva alone having the ability to generate an induced resistance response (340; 47). Others have provided evidence that GroEL, a chaperonin from the aphids primary bacterial endosymbiont *Buchnera aphidicola* is present in saliva and induces defense responses in Arabidopsis, including a ROS burst (363; 359). These defense responses are also dependent on BAK1, though it is unknown whether existing receptors of bacterial PAMPs that interact with BAK1, such as FLS2 and EFR, are also required for immune responses to GroEL.

GroEL is a 60-kDa chaperonin that is one of the most abundant proteins produced by *B. aphidicola* and can readily be detected on protein blots of whole aphid extracts (364). The 3-10 kD fraction of GPA extract induces a ROS burst in Arabidopsis, whereas the greater than 10 kD elicitor fraction does not (340), suggesting that GroEL is not present in our investigations. Both fractions do however generate an induced resistance response to GPA (340). The ROS burst to purified GroEL occurred over a much shorter timeframe than the ROS burst induced by GPA extract with a peak at 15 minutes, which is similar to the flg22-induced ROS burst (363). In contrast, the ROS burst in response to GPA extract in our investigations occurs one hour after elicitor exposure. The ROS burst induced upon application of purified GroEL was not lost upon boiling, in fact it was enhanced (363), unlike the GPA extract used in our assays and those of de Vos et al., which lost its defense eliciting activities altogether upon boiling (47). Together this indicates that GroEL may not be the elicitor present in either fraction of GPA extract in our investigations. However, we cannot exclude the possibility that the 3-10 kD fraction contains shorter peptides of GroEL, that may be generated by the action of aphid proteases.

Although peptides matching the protein sequence of GroEL are detected by mass spectrometry in aphid saliva (363; 365), it is not clear if these are derived from whole or digested fragments of GroEL protein. If shorter GroEL peptides generated by proteolytic activity in aphid saliva are detected by plant PRRs, this could explain the absence of a ROS burst upon boiling of GPA extract and saliva. Moreover, the protease K treatment could cleave the proteases or GroEL peptides, thereby removing their presence from the GPA extract. Experiments with leupeptin and phosphoramidon suggest that any potential proteolytic activity is not due to metallo-endopeptidases or cysteine, serine or threonine-type peptidases, although it cannot be excluded that these protease inhibitors were added after the cleavage of GroEL into smaller peptides by aphid-derived proteases had already occurred. It therefore

remains to be determined if GroEL or peptide-derivatives play a role in the induction of the plant defense responses we see induced by GPA extract. However, it is likely that at least two elicitors are involved, due to the eliciting activities of different fractions of GPA extract.

It is possible that the ROS burst that would be caused by GroEL present in whole GPA-extract is masked by the presence of other proteins that would act to prevent or delay the ROS burst, such as effectors like Mp10 (46). Effectors present in GPA extract would not have the stylet as a means of delivery into plant cells. Though effector action often takes place inside plant cells and PRR detection of elicitors takes place extracellularly, there are pathogen effectors that work in the apoplast (273). For example, the fungal pathogen *Cladosporium fulvum* and the oomycete *Phytophthora infestans* both secrete effectors (Avr2 and EPIC1/2B) into the apoplast that inhibit the tomato defense protease Rcr3 (366). As the GPA stylet probes intercellularly in the plant and saliva is secreted throughout the probing process (28; 21), aphid effectors may also be delivered to the apoplast, potentially preventing recognition of aphid elicitors or inhibiting extracellular defense enzymes that would attack stylets.

Our work suggests that elicitors present within aphids are sensitive to both boiling and proteinase K treatment. There are numerous ways this may affect potential elicitors to prevent perception. Boiling could disturb protein modifications or the 3D structure of an eliciting peptide, so that a receptor no longer binds to it. Proteinase K treatment could cut an active peptide into several pieces, again preventing recognition. The fact that the ROS burst induced by GPA extract occurs over a long time frame, in addition to boiling and proteinase K sensitivity suggests that enzymatic activity of an elicitor may be needed to create a product that accumulates over time, and is then detected by the plant. Products released from the action of pathogen enzyme function on plant molecules are already known to act as damage associated molecular patterns (DAMPs). An example of these are oligogalacturonides produced from the degradation of pectin, which are released by the action of cell wall degrading enzymes from pathogenic microbes (332). Something of a similar nature may occur when aphid saliva comes into contact with the extracellular environment of a plant during probing. AtPeps are well characterised DAMPs in Arabidopsis that are detected by the PRRs PEPR1 and PEPR2 (367; 368), however these do not seem to be involved in aphid detection as neither of these receptors are required for the ROS response to GPA-extract (340). Glycoproteins produced in Russian wheat aphid (*Diruaphis noxia*)-infested wheat can induce defense responses in other wheat plants that have not been exposed to aphids (131). This suggests induction of a plant-based elicitor by aphid

feeding, which can activate plant immune responses.

The results of using protease inhibitors suggests that potential DAMPs are not produced by protease action. A varied array of GPA proteins could induce the ROS burst, or lead to the production of molecules that do. For example, aphid saliva contains several different non-protease enzymes, including glucose oxidases (GOX), glucose dehyrogenases, alpha-glucosidase, alpha-amylase and pectinases (39; 363; 30; 34; 369). A potential candidate for an elicitor-producing enzyme is GOX, which acts as an elicitor in lepidopteran species (211; 125; 124) and can also act as an inhibitor of some plant defense responses (257). Beta-glucosidase also has eliciting activity; beta-glucosidase from *Pieris brassicae*, induces the release of plant volatiles that attract parasitoids of the caterpillar (370). Pectinases and cellulases present in aphid saliva may act as elicitors, because the application of these enzymes on wheat causes the release of volatiles that are attractive to an aphid parasitoid (334). In the future it would be interesting to look at whether carbohydrate digesting enzymes found within aphid saliva, such as GOX, are responsible for the eliciting activity of GPA-extract. Though these enzymes would all be larger than the 3-10 kD that we found to be able to induce a ROS burst in *A. thaliana*, the discovery that GroEL, a protein larger than 10 kD, can also induce a ROS burst suggests that the centrifugal filter columns we used may not be entirely efficient at partitioning proteins by size. It may not be the enzymes themselves, but products they make from other components of aphid saliva that are detected, which would explain the difference in ROS burst induction by the 3-10 kD and greater than 10 kD fractions. Both fractions are however able to cause induced resistance, suggesting that elicitors are present in both fractions that can be detected by the plant. Proteinase inhibitor treatments in conjunction with induced resistance assays may therefore be used in the future to look at eliciting activity of the greater than 10 kD fraction.

It seems that the GPA-elicitor is specific for aphids, as the use of other molecules that induce a ROS burst, such as proteinase K, does not generate an induced resistance response. GPA-extract therefore causes responses specific for GPA that will be most effective against this insect. For example, the induction of PAD3 by GPA and GPA extract suggests a targeted increase of camalexin biosynthesis, which is effective in reducing aphid colonization of Arabidopsis (340; 238). Others have also shown that PAD3 gene expression is upregulated upon aphid feeding (175; 47), including specifically around GPA stylet penetration sites (238). Moreover, camalexin is toxic to aphids and is found inside aphids that feed on plants with high camalexin concentrations (238). This phytoalexin is therefore not just effective against fungal and bacterial pathogens (307; 371), but plays a role in plant

resistance to aphids too. Our findings appear to contradict those of other researchers who did not find changes in fecundity of aphids on *pad3* and *cyp79b2/b3* plants (372; 230). However, these investigations used non-aged aphids that were exposed to the plants for a short period of time (2-5 days), whereas the protocol used in our lab counts the progeny of nymphs that were born and reared over a period of two weeks on the mutant plants. A similar assay using the cabbage aphid, *Brevicoryne brassicae*, on *pad3* plants also found increased fecundity, though this was after UV-B light treatment to induce camalexin accumulation in the wild-type Arabidopsis control (175). *B. brassicae* may not induce camalexin to the same degree as UV-B, as this specialist aphid is able to colonise glucosinolate producing brassicas successfully.

Camalexin is a lipophilic molecule that disrupts the integrity of bacterial membranes (373). Membrane disruption explains why camalexin is toxic to many organisms, including bacteria, fungi and plant cells. It also has antiproliferative activity against human prostate cancer and leukaemia cells, where it induces apoptosis (374; 375). The toxicity of camalexin against even plant cells (373) suggests that its biosynthesis needs to be tightly controlled to ensure that the plant itself is not damaged by camalexin accumulation. PAD3 induction after pathogen perception in Arabidopsis is due to the action of the WRKY transcription factor WRKY33 and the NAC family transcription factor ANAC042 (376; 377). ANAC042 induction after flg22 perception is suggested to be downstream of both calcium and ROS signaling, and is dependent upon ethylene signaling (376). WRKY33 however, seems to be ethylene independent, and WRKY33 is actually required upstream of ethylene induction in plant immunity (378; 379). This suggests that there may be two potential signaling pathways leading to PAD3 induction after pathogen and aphid perception.

WRKY33 is activated downstream of both MPK4 and MPK3/6 MAP kinase signaling (380; 381; 382). Interestingly, several WRKY transcription factors induced downstream of MAPK in *N. benthamiana* induce expression of the NADPH oxidase RBOHB, which is responsible for the ROS bursts in both PTI and ETI (383). The group of *RBOHD*-inducing *N. benthamiana* WRKYs includes WRKY8, which is the closest *N. benthamiana* WRKY to WRKY33. The upregulation of RBOHB by WRKYs is required for the oomycete elicitor INF1-induced ROS burst, but not for the flg22-induced ROS burst (383). Like the ROS burst in response to GPA extract, the ROS burst induced by INF1 also takes place over a greater timeframe than the flg22-induced ROS burst (144). Perhaps regulation of Arabidopsis RBOHD by WRKY33 also mediates the late ROS burst to elicitors in GPA extract. In support of this, the ROS burst to GPA extract and PAD3 induction are both seen 1 hour

after exposure to GPA extract, suggesting WRKY33 could be regulating them both. GPA fecundity also increases on *wrky33* mutants, suggesting WRKY33 regulation of successful plant defense responses against aphids (312).

The decrease of aphid fecundity on *dcl1* mutant Arabidopsis and the increased accumulation of camalexin in these plants (238) suggests that regulation of camalexin biosynthesis in Arabidopsis is at least partly achieved via a miRNA pathway. In Arabidopsis, some miRNAs do indeed play a role in secondary metabolism. This group includes miR393, which is induced following exposure to flg22 as well as *Pseudomonas syringae* (384; 385). MiR393 acts to repress auxin signaling by negatively regulating auxin receptors. This removes repression of the salicylic acid pathway by the auxin pathway and increases resistance to biotrophic pathogens (384). Through reduction of auxin signaling, activation of auxin response factor (ARF) 9 is prevented, which negatively regulates glucosinolate production and positively regulates camalexin accumulation. MiR393 therefore both increases SA-regulated immunity and decreases the level of camalexin present within tissues (386). Lack of miR393 within *dcl1* mutants may therefore be the reason for their increased camalexin levels, and so increased resistance to aphids. Removing camalexin from *dcl1* plants by crossing with *pad3* increases aphid fecundity above that seen on *dcl1* plants. However, fecundity is not totally restored to levels seen on wild-type (Col-0) plants. This suggests that though camalexin contributes to the increased resistance to of *dcl1* plants to GPA, there are other factors involved too. The balance between the phytohormones SA and JA may play a role, as GPA has been found to induce SA accumulation, preventing JA responses which are more successful against the insect (215). Multiple miRNA families are differentially regulated during insect exposure, including those involved in phytohormone signaling, so miRNAs other than miR393 may also regulate the plant response to insects independently of camalexin (387; 388).

Indolic glucosinolates may also play a role in plant-aphid interactions. CYP81F2 is induced by GPA-extract, which catalyses the production of indolic glucosinolates. In our lab *cyp81f2* mutant plants were found to have no significant effect on aphid fecundity, suggesting that indolic glucosinolates do not have as big a role as camalexin (238). This is different to Pfalz et al., who found an increase of GPA fecundity on *cyp81f2* mutant plants (341). However, the experimental set up was again different to ours, with nymphs counted over a 5-day period, rather than the two weeks used in our assays. Experiments were conducted with a different GPA genotype to the one in our lab, and it is possible that GPA genotypes differ in their susceptibilities to phytochemicals. Nevertheless, CYP81F2

produces 4-methoxy-I3G in the indole glucosinolate biosynthesis pathway and this is needed for callose deposition upstream of PEN2 (205). PEN2 is a myrosinase that hydrolyses the 4-methoxy-I3G and is required for callose deposition in response to flg22, though not to fungal chitin (206). I found that PEN2, as well as BAK1, is required for callose deposition in response to GPA extract, placing PEN-2 mediated callose deposition in the BAK1-dependent aphid elicitor perception pathway.

Callose is deposited at aphid feeding sites and in phloem sieve tubes in response to aphid feeding (175; 389). Callose deposition in sieve tubes is needed for Bph14-mediated resistance to the brown planthopper (*Nilaparvata lugens*) in rice and Vat-mediated resistance to the melon/cotton aphid (*Aphis gossypii*) in melon (201; 338). Whether the callose induced in the Arabidopsis-GPA interaction has any direct effect on the aphid has yet to be seen; callose in the phloem could form a barrier to feeding. Though PEN2 is required for GPA extract-induced callose deposition, *pen2-1* mutant Arabidopsis plants do not have decreased resistance to GPA, suggesting that callose is not needed in defense against the insect (47). Perhaps this is because GPA can tolerate callose deposition. Some insects species have developed ways of counteracting callose deposition, for example the brown planthopper activates beta-1,3-glucanase genes in rice which can act to unplug sieve tube occlusions (201).

Like callose deposition, ROS are also induced by insect feeding (175; 176; 177). The production of ROS in response to different insects has been recorded in several plant species, including a fern, which suggests that ROS production is a conserved response to herbivory by insect pests (390; 391; 246; 158). In Arabidopsis, the ROS response to PAMPs is due to the action of respiratory burst oxidase homologues AtRBOHD and AtRBOHF (178). I found that AtRBOHD was also required for the ROS burst to GPA extract. *AtrbohD* and *AtrbohF* mutant plants have increased susceptibility to GPA, indicating that NADPH-produced ROS are important in defense against aphids (179; 177). Furthermore, the candidate aphid effector Mp10 is able to block the ROS burst induced by the bacterial PAMP flg22 (46), which suggests that ROS signaling is a target for aphid effectors.

Though BAK1 and RBOHD are needed for the GPA extract-induced ROS burst, they are not required for PAD3 induction, showing that camalexin synthesis is induced independently of ROS signaling. This suggests that separate immune pathways are activated in response to aphids, one involving BAK1-dependent ROS production and callose deposition, the other involving PAD3 induction. These different immune responses to aphids may be downstream of different receptors which detect different aphid elicitors. Further study is

required to investigate the receptors that provide specificity to aphid perception that might be interacting with BAK1, as well as to identify which pathways these identified components of PTI are acting in in response to aphids. I continue this study in Chapter 4.

Chapter 4

PAD3, AGB1 and three Receptor-Like Kinases are involved in the immune response to GPA in Arabidopsis

Contributors: Claire L. Drurey, Sam Mugford, Cyril Zipfel and Saskia A. Hogenhout

4.1 Introduction

In the last chapter, I identified components involved in plant defense responses to aphids, such as BAK1, PEN2, RBOHD and PAD3. These components also play a role in plant PTI responses to microbial pathogens (239; 178; 205; 143). I wished to investigate the role of some of these defense genes further and identify other components involved in the immune response to GPA, for example cell surface receptors that specifically perceive aphid elicitors.

BAK1, also known as SERK3 (Somatic Embryogenesis Receptor-like Kinase), is an LRR-RLK required for the perception of many pathogens, including bacteria, fungi, oomycetes and viruses (392; 393; 394). BAK1 has also been implicated in the perception of herbivores, being found responsible for the regulation of jasmonic acid and secondary metabolite levels in *Nicotiana attenuata* upon *Manduca sexta* feeding (110), although in these experiments BAK1 expression levels was knocked down by RNAi, possibly inducing pleotropic defects. Another member of the SERK family, SERK1, is required for Mi-1 mediated resistance to the potato aphid, *Macrosiphum euphorbiae* (395). Mi-1 is an NB-LRR protein, indicating that members of the SERK family are also involved in effector-triggered immune (ETI) responses to insects, although the aphid effector that triggers Mi-1-mediated ETI has not yet been identified.

We previously found that the BAK1 mutant, *bak1-5*, is deficient in both the ROS and callose responses to GPA extract. Unlike other identified *bak1* mutants such as *bak1-4*, the *bak1-5* mutant is deficient solely in innate immune signaling through altered kinase activity. Brassinosteroid signaling and cell death control are not affected in *bak1-5* mutants (147). Plant defense responses to GPA therefore involve BAK1 in its immune signaling capacity. BAK1 acts as a co-receptor, mediating signaling via other pattern recognition receptors (PRRs), including leucine-rich repeat receptor-like kinases and receptor-like proteins (LRR-RLKs and LRR-RLPs), which upon perception of the pathogen/pest elicitors, directly interact with BAK1 (349). Thus, a PRR is likely to perceive aphid elicitors and then associate with BAK1 to mediate a plant defense response. It is known that receptors already identified as BAK1 interactors, such as FLS2, EFR and PEPR1 and PEPR2, are not required for aphid-induced defense responses (340). I therefore wish to identify the PRR that is responsible for the detection of aphid PAMPs/elicitors.

PRRs in Arabidopsis are usually RLKs or RLPs, with an extracellular domain that interacts with a PAMP, a transmembrane helix domain that anchors the receptor to the

membrane, and an intracellular kinase domain that carries out the signaling role (114). PRRs are sorted into several families, usually based on what the extracellular domain is composed of. The RLKs include those with LRRs, proline-rich domains, carbohydrate binding domains including lectins and lysin motifs (LysM), and domains of unknown function 26 (DUF26) (133). The DUF26 and LRR families are the largest within the RLKs (396). RLPs are proteins with similar extracellular domains to the RLKs that do not have intracellular kinase domains. Many have LRR domains (397). The most well studied PRRs, EFR and FLS2, are both LRR-RLKs that interact with BAK1 in order to signal recognition of their PAMPs, bacterial elongation factor Tu (EF-Tu) and flagellin respectively (146; 137; 329). However, not all PRRs interact with BAK1. CERK1 is a LysM-type RLK from Arabidopsis that acts alone as a homodimer to recognise fungal chitin as a PAMP (362; 398). Interestingly, CERK1 also has a role in the perception of bacterial peptidoglycans (PGN) by interacting with the LysM-containing RLPs LYM1 and LYM3 (399). Receptors can therefore be involved in signaling to different PAMPs.

Plant components besides RLKs and RLPs, such as G-proteins, are involved in the perception of pathogens by plants. In animals, heterotrimeric G-proteins form a common signaling pathway in immunity (400). Their role in plant immunity is less well known, but they are increasingly implicated in signal transduction and defense responses (401). The G-protein β subunit of Arabidopsis in particular has been found to be involved in resistance to fungal and bacterial pathogens (194; 402; 403). As G-proteins have not yet been implicated in plant responses to insects, I was interested to investigate if they also play a role in plant-aphid interactions.

Besides BAK1, PAD3 is another component of plant immunity that I found to be involved in the defense response to aphids. It is a cytochrome P450 monooxygenase responsible for the final steps in camalexin biosynthesis (235; 236; 237). Previous results indicate that PAD3 and BAK1 do not act in the same pathway, because PAD3 was still induced upon aphid elicitation in *bak1-5* plants (340). I was therefore interested to understand how PAD3 is induced in Arabidopsis, and what other roles its induction may have beyond camalexin synthesis. This chapter is therefore focused on the further elucidation of Arabidopsis components involved in the defense response to aphids.

4.2 Results

4.2.1 Arabidopis BAK1 and AGB1 are involved in the defense response to aphids

The leucine-rich repeat receptor-like kinase (LRR-RLK) BAK1 acts as a co-receptor in both brassinosteroid signaling and PTI responses. In the previous chapter we found that BAK1 was required for the PTI-like response to GPA extract, including the ROS burst, callose deposition and induced resistance responses. David Prince also found that the pea aphid, *A. pisum*, survives better on *bak1-5* plants (340; 316).

Next, I investigated if the β subunit of the Arabidopsis heterotrimeric G-protein, AGB1, is also involved in the Arabidopsis defense response to GPA. Heterotrimeric G-proteins are found to have an increasingly important role in plant immunity. As they are located on the plant plasma membrane, they are likely to play a role in perception or early signaling events (404). The β subunit, AGB1, is required for defense responses to both biotrophic and necrotrophic pathogens (194; 402; 405; 403). The AGB1 mutant, *agb1-2*, shows a reduced ROS burst upon challenge with the bacterial PAMPs flg22 and elf18 (194). I therefore analysed this mutant for the ROS burst to GPA extract.

I found that *agb1-2* had a significantly reduced ROS burst after GPA extract challenge, though this was not as low as that of the *bak1-5* mutant (Figure 4.1a). Pea aphids, which are not adapted to Arabidopsis and die quickly on this plant species, survived better on *bak1-5* plants, confirming previous results (Chapter 3; (340)). Pea aphids also survived better on the *agb1-2* plants, giving an intermediate pea aphid survival phenotype between Col-0 and *bak1-5* plants (Figure 4.1b). These results suggested that AGB1 plays a role in plant-aphid non-host interactions, though this role may not be as important as that of BAK1. I used both *bak1-5* and *agb1-2* plants in survival and fecundity assays of GPA, which colonizes Arabidopsis efficiently. GPA did equally well on Col-0 and *bak1-5* plants, as was observed previously (340), but GPA produced more progeny on the *agb1-2* plants (Figure 4.1c), suggesting AGB1 is involved in a defense pathway that is successful against GPA. These results show that both BAK1 and AGB1 are involved in the plant defence response to aphids, but may have different roles.

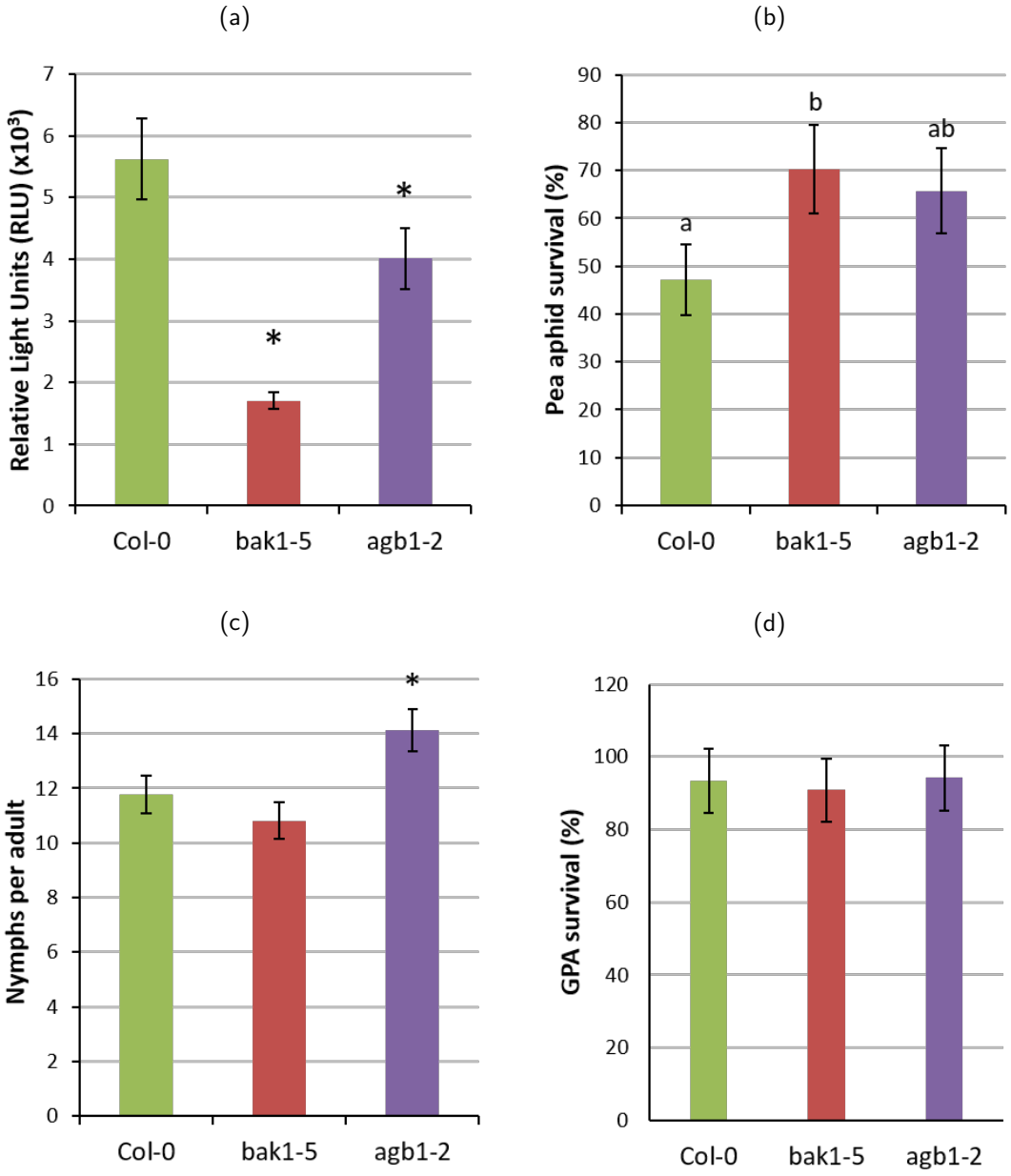


Figure 4.1: **BAK1 and AGB1 mediate plant immunity to aphids**. 4.1a The ROS burst to GPA extract is reduced in *bak1-5* and *agb1-2* plants. Leaf discs were treated with GPA-extract and ROS bursts were measured using a luminol-based assay. The bars show mean ± SE of total ROS produced over 10 hours in 3 independent experiments (n=8 per experiment). Asterisks indicate significant differences relative to the Col-0 control (Student's t-probabilities calculated within GLM at $P < 0.05$). 4.1b Pea aphid survival is increased on both *bak1-5* and *agb1-2*. Bars show the percentage of pea aphids alive on day 4 with means ± SE of 4 independent replicates (n=5 per experiment). Letters indicate significant differences between genotypes (Student's t-probabilities calculated within GLM at $P < 0.05$). 4.1c GPA fecundity is increased on *agb1-2* and not on *bak1-5* plants. Bars show mean ± SE of GPA nymphs produced over a 14-day period of 4 independent experiments (n=6 per experiment). Asterisks indicate significant differences to the Col-0 control (Student's t-probabilities calculated within GLM at $P < 0.05$). 4.1d GPA survival rates are similar on Col-0, *bak1-5* and *agb1-2* plants. Bars show mean ± SE of GPA survival over a 14-day period of 4 independent experiments (n=6 per experiment).

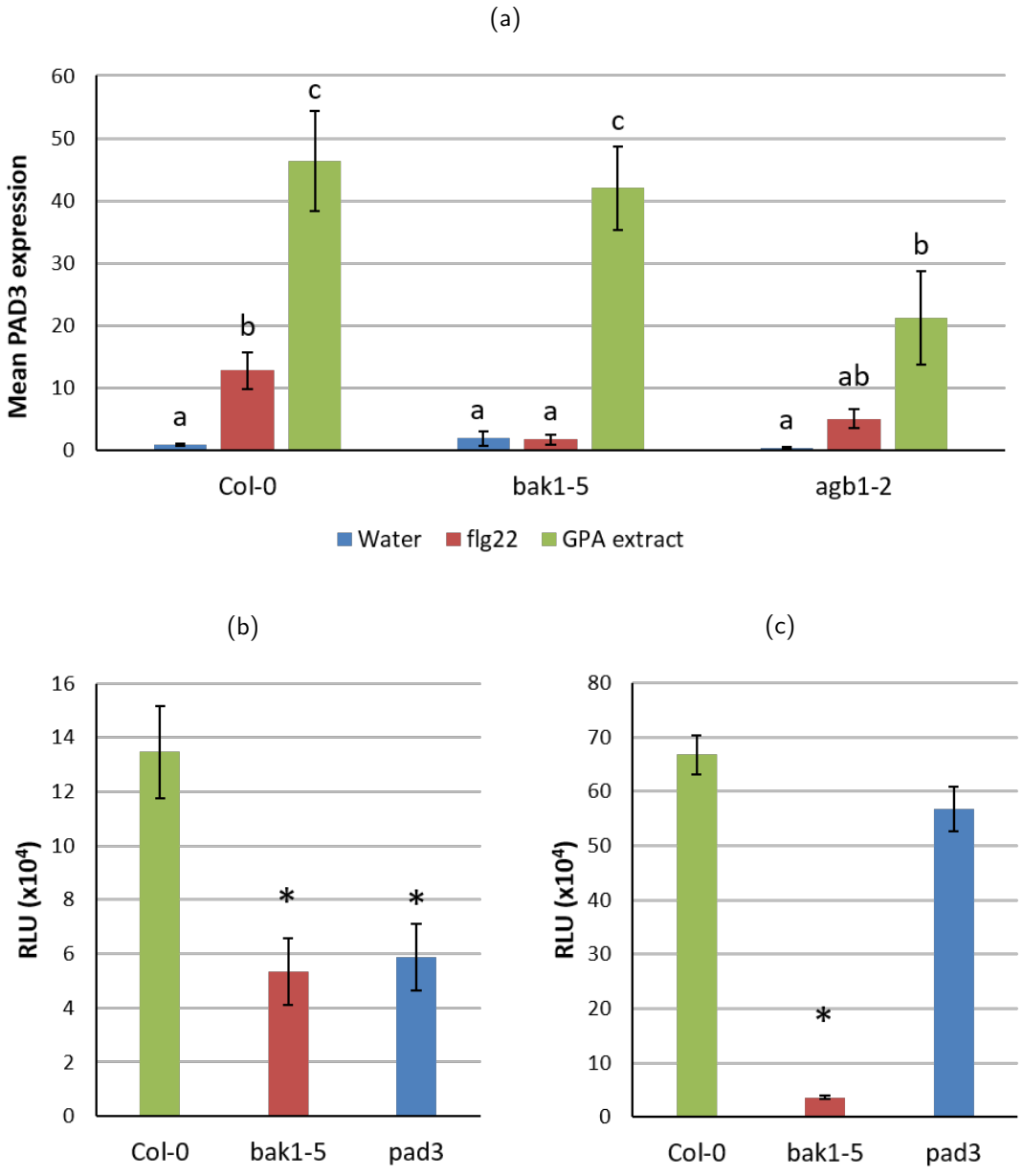


Figure 4.2: **PAD3 and AGB1 act in the same pathway to mediate plant immunity to aphids**. 4.2a AGB1 is required for full PAD3 expression upon exposure to aphid extract. Data shown are means ± SE of target gene expression levels of 3 independent experiments. Letters indicate significant differences between treatments (Student's t-probabilities calculated within GLM at P <0.05). 4.2b The ROS burst to GPA extract is reduced in *pad3* plants. Leaf discs were treated with GPA-extract and ROS bursts measured using a luminol-based assay. Bars show mean ± SE of total ROS produced over 10 hours in 4 independent experiments (n=8 per experiment). Asterisks indicate significant differences relative to the Col-0 control (Student's t-probabilities calculated within GLM at P <0.05). 4.2c The ROS burst to flg22 is unchanged in *pad3* plants. Leaf discs were treated with flg22 and ROS bursts measured using a luminol-based assay. Bars show mean ± SE of total ROS produced over 1 hour in 2 independent experiments (n=8 per experiment). Asterisks indicate significant differences to the Col-0 control (Student's t-probabilities calculated within GLM at P <0.05).

4.2.2 PAD3 acts downstream of AGB1 and impacts the ROS response to aphid elicitors

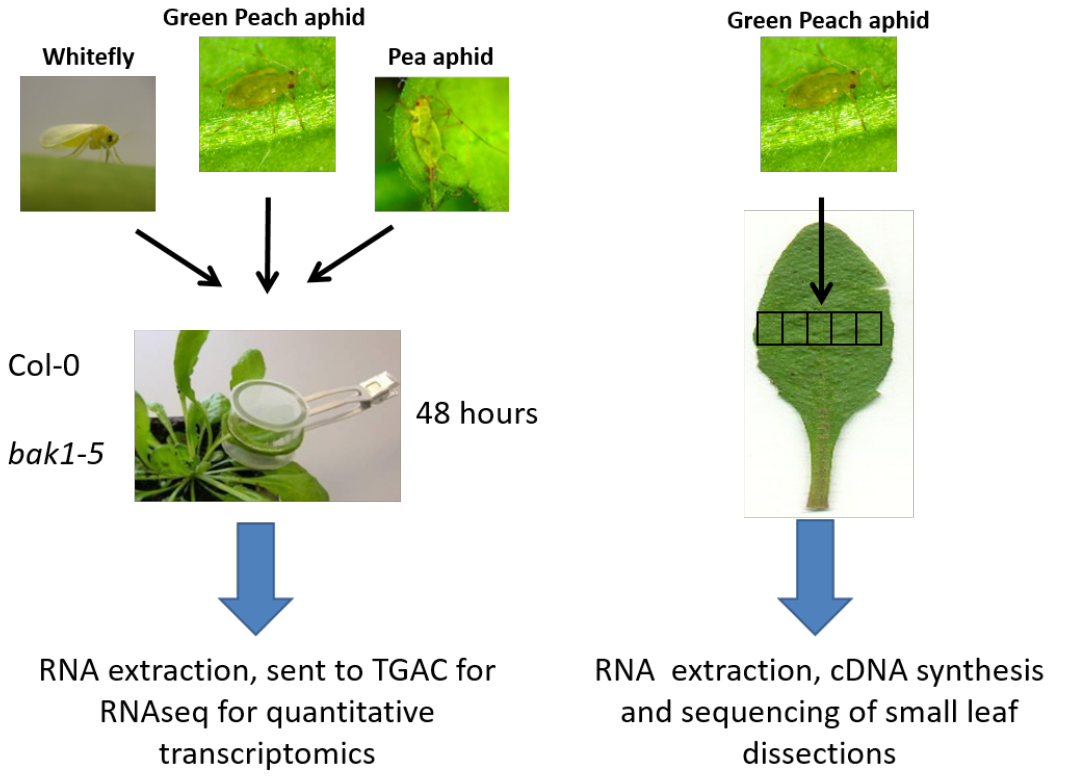
I found in the previous chapter that aphid extract induced the expression of defense gene *PAD3*, which is a cytochrome P450 monooxygenase involved in the final stages of camalexin biosynthesis (235; 236; 237). I found that neither BAK1 nor RBOHD were required for *PAD3* induction upon treatment with aphid extract, whereas BAK1 is required for callose deposition and the ROS burst response to the same treatment (Chapter 3 and (340)). To investigate if *PAD3* induction is dependent on AGB1, I carried out qRT-PCRs on leaf discs of *agb1-2* plants exposed to aphid extract. Flg22 and water treatments were included as positive and negative controls respectively. As I had found previously, both flg22 and GPA extract induced *PAD3* expression in a wild-type Col-0 plant. Also as before, *PAD3* induction was lost in response to flg22 in *bak1-5* versus Col-0 plants, whereas *PAD3* induction by GPA extract was similar between Col-0 and *bak1-5* plants. On the other hand, in the *agb1-2* mutant the *PAD3* response to both flg22 and GPA extract was reduced by half, though not down to water-treated levels (Figure 4.2a). Therefore AGB1, but not BAK1, acts upstream of PAD3 in defence responses to aphid extract, whereas BAK1 and AGB1 are both upstream of PAD3 in responses to flg22.

To investigate if PAD3 affects earlier aspects of plant immunity, such as the ROS burst, I conducted ROS burst assays on the *pad3* mutant using GPA extract as the elicitor. Surprisingly, I found that the ROS burst to this extract is reduced in the *pad3* mutant to a similar level as that of *bak1-5* plants (Figure 4.2b). Conversely, the ROS burst in response to flg22 on *pad3* plants was not reduced (Figure 4.2c). This result is at odds with the knowledge that PAD3 acts downstream in PTI. PAD3 therefore plays a specific role in response to aphid elicitors that may involve a feedback mechanism with upstream PTI signaling pathways.

4.2.3 The hunt for potential aphid receptors in Arabidopsis

PAMPs can induce the expression of PRR genes (137). Therefore, to identify candidate PRRs that perceive aphid elicitors, differentially expressed genes from RNA-seq experiments were identified. The RNA-seq data (The Genome Analysis Centre (TGAC), Norwich, UK) were generated from Col-0 and *bak1-5* Arabidopsis leaves exposed to GPA, pea aphids or whitefly in clipcages for a period of 48 hours. Empty clip cages were used as a no-insect control. Another RNA-seq data set was generated from microdissected aphid feeding sites of

(a)


Green Peach aphid
Whitefly
Pea aphid
Green Peach aphid
Col-0
bak1-5
48 hours
RNA extraction, sent to TGAC for RNAseq for quantitative transcriptomics
RNA extraction, cDNA synthesis and sequencing of small leaf dissections

(b)

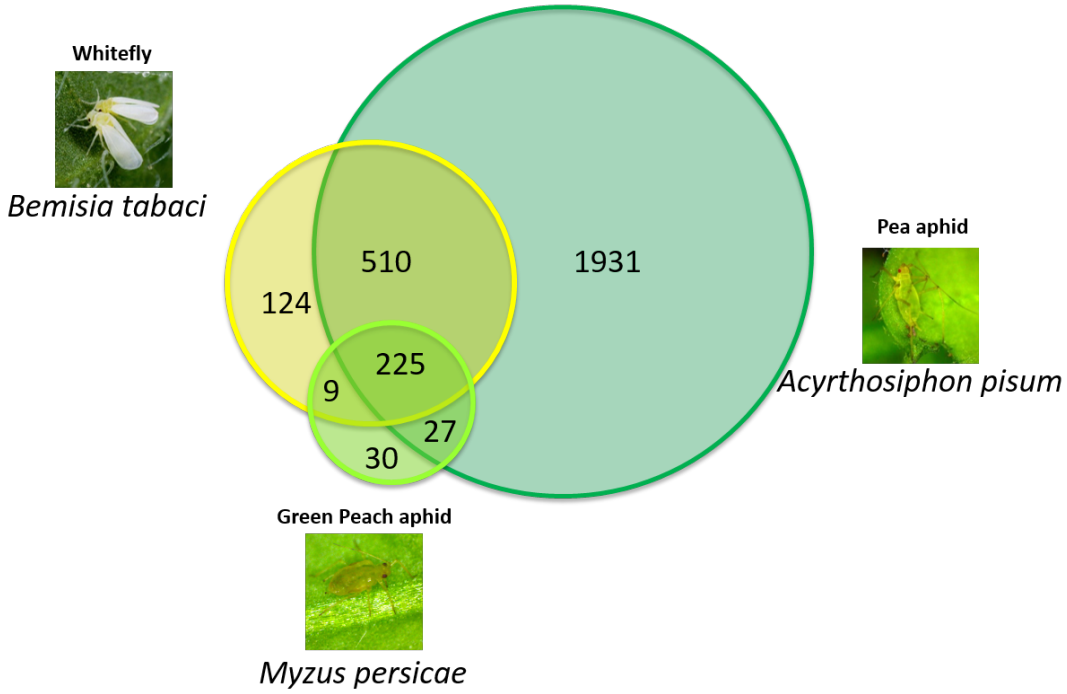

Whitefly
Bemisia tabaci
510
1931
124
225
9
27
30
Pea aphid
Acyrthosiphon pisum
Green Peach aphid
Myzus persicae

Figure 4.3 *(previous page)*: **RNAseq data collection procedure** 4.3a RNAseq sample collection. RNA was extracted from leaf samples of Col-0 and *bak1-5* Arabidopsis exposed to whitefly (*Bemisia tabaci*), GPA (*Myzus persicae*) and pea aphids (*Acyrthosiphon pisum*) as well as an empty clipcage control for two days and sent to TGAC for RNA sequencing. Leaf samples of Col-0 Arabidopsis were also taken around GPA feeding sites and used in RNA sequencing. 4.3b Overview of genes differentially regulated by insect exposure in whole Arabidopsis leaves. Diagram shows the number of genes differentially regulated in Col-0 Arabidopsis after exposure to GPA, whitefly and the pea aphid compared to a no-insect control. This does not include leaf samples around aphid feeding sites. Diagram originally generated by Sam Mugford. Credit to Anna Jordan (JIC Insectary) for the whitefly image.

Arabidopsis leaves (a collaboration of the Hogenhout lab with Walter Verweij and Matthew Clarke at TGAC). The two RNA-seq data sets were combined and mined for genes showing differential expression. After analysis and filtering using an adjusted P-value of 0.05 for a significant log2 fold change in expression levels, over 2,000 genes showed differential expression in Arabidopsis leaves that had been exposed to the insects (Figure 4.3b). Most genes (1,900 of 2,000) were differentially expressed in samples exposed to the pea aphid (*A. pisum*). Pea aphid does not colonise Arabidopsis, and the induction of many defence response genes may deter this aphid.

The two RNA-seq datasets identified 31 potential receptor candidates, including 7 LRR-RLKs, that were differentially expressed upon insect treatments of Col-0 and *bak1-5* plants (Table 4.1). All of these were upregulated in response to *A. pisum* and most were also upregulated in response to GPA on Col-0 plants. 14 receptor candidate genes were upregulated in *bak1-5* plants in response to pea aphids, indicating a BAK1-independent response. A small number of candidate receptor genes were downregulated in *bak1-5* plants compared to non-insect controls. These results indicate that PRRs may or may not depend on BAK1 for differential expression.

		Feeding Site	B. tabaci	A. pisum	M. persicae	Col-0		bak1-5	
Gene Name	Description	log2 fold change	log2 fold change	log2 fold change	log2 fold change	*M. persicae*	*A pisum*	*M. persicae*	*A. pisum*
AT1G07390 (RLP1)	RLP	n.s	n.s	n.s	n.s	4.48	2.72	-1.01	0.80
AT1G16140 (WAKL3)	WAKL, RLK	n.s	n.s	999999.00	n.s	74.67	74.90	n.s	n.s
AT1G16150 (WAKL4)	WAKL, RLK	n.s	n.s	n.s	n.s	n.s	2.17	2.41	1.63
AT1G35710	LRR-RLK	3.48	-0.19	0.95	0.49	2.83	2.39	2.41	1.51
AT1G51830	LRR-RLK	n.s	n.s	n.s	999999.00	1.06	1.29	-75.01	-75.01
AT1G55200	PK	n.s	0.63	0.74	0.16	1.13	1.34	-0.94	n.s
AT1G62090	PK	n.s	n.s	n.s	n.s	1.69	2.45	4.99958E-23	n.s
AT1G66880	PK	4.03	-0.18	0.44	0.20	1.76	1.37	1.97	1.15
AT1G72460	LRR-RLK	n.s	n.s	n.s	n.s	n.s	3.48857E+22	4.79343E+22	9.6504E+22
AT1G78940	PK	n.s	n.s	999999.00	999999.00	12.93	1.40	-11.90	-10.64
AT2G29220 (LECRK-III.1)	LECRK	n.s	n.s	999999.00	n.s	76.49	77.04	n.s	76.00
AT2G31880 (SOBIR1)	LRR-RLK	3.96	-0.28	0.01	0.64	1.55	1.34	1.66	0.81
AT2G32680 (RLP23)	RLP	3.75	-0.76	0.87	0.70	3.71	3.31	2.82	1.66
AT2G37710 (LECRK-IV.1)	LECRK	3.65	0.31	n.s	0.54	1.40	1.04	1.67	1.00
AT2G40270	PK	n.s	n.s	n.s	n.s	1.40	2.07	1.71	2.57
AT2G47060 (PTI1-4)	PTI1-like kinase	n.s	-0.31	-1.83	0.64	2.39	11.04	-0.24	0.86
AT3G20190 (PRK4)	LRR-RLK	n.s	n.s	n.s	n.s	1.96	3.79	0.60	0.72
AT3G59350	PK	n.s	-1.74	-2.36	0.06	13.27	11.29	-8.39	0.78
AT3G59730 (LECRK-V.6)	LECRK	n.s	-1.01	n.s	0.23	2.81	1.31	-1.08	n.s
AT4G11460 (CRK30)	CR-RLK	n.s	n.s	999999.00	999999.00	1.26	2.64	-1.84	0.98
AT4G23140 (CRK6)	CR-RLK	4.47	-0.13	1.71	0.76	3.58	2.86	3.04	1.95
AT4G23150 (CRK7)	CR-RLK	4.59	n.s	n.s	n.s	4.01	3.42	2.94	1.91
AT4G23210 (CRK13)	CR-RLK	n.s	n.s	n.s	n.s	3.76	3.53	2.21	1.34
AT4G25390	PK	n.s	n.s	n.s	n.s	67.49	67.15	n.s	n.s
AT4G28670	DUF26	n.s	n.s	n.s	n.s	75.05	75.08	n.s	n.s
AT4G35030	PK	n.s	n.s	n.s	n.s	9.38	9.93	-5.79	5.94
AT5G06940	LRR-RLK	n.s	n.s	n.s	n.s	1.05	1.56	-1.13	-0.62
AT5G35390 (PRK1)	LRR-RLK	n.s	n.s	n.s	n.s	3.04	2.26	-0.06	0.32
AT5G39000	M/RLK	n.s	n.s	n.s	n.s	n.s	2.06	6.05	2.85
AT5G49770	LRR-RLK	n.s	n.s	n.s	n.s	1.38	2.09	1.96	2.28
AT5G61550	UD-PK	n.s	n.s	n.s	n.s	n.s	1009.18	5.01	6.17

Table 4.1: **Potential aphid receptor genes differentially regulated in plants exposed to insects**. Table showing RLKs and RLPs identified as differentially regulated in one or more of the RNA-seq datasets. Green indicates receptor candidate was upregulated, red is downregulated. N.s.= not significant, transcript was not found to be significantly changed.

4.2.4 Screening of potential aphid receptors

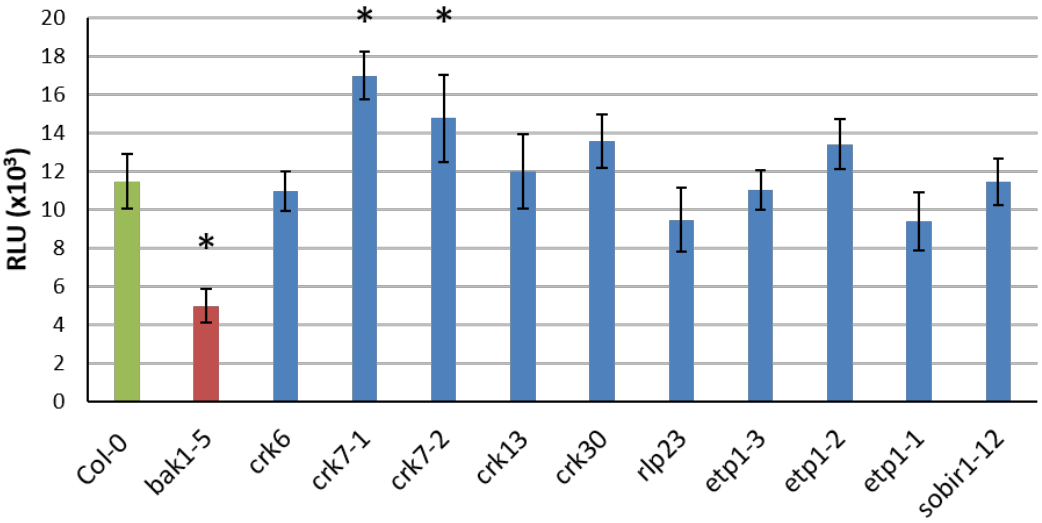
Mutants affected in several of the receptor gene candidates were available in the laboratories of Prof. Cyril Zipfel and Prof. Silke Robatzek (TSL). For some of the other candidates, T-DNA insertion lines were available in the TAIR database, and seed was retrieved for most of these. Plants that germinated and were confirmed for the T-DNA insertions were assayed for ROS responses to aphid extract. Col-0 and *bak1-5* Arabidopsis plants were included as controls.

In the first screen of 10 mutant lines (all in the Col-0 background), I found that both CYSTEINE-RICH RECEPTOR-LIKE KINASE 7 (CRK7) mutants, *crk7-1* and *crk7-2*, showed statistically significant increases in the ROS bursts compared to Col-0 (Figure 4.4a). CRK7 is a cysteine-rich receptor-like protein kinase that is linked to extracellular ROS signaling (406) and has not been implicated in pathogen responses before. Interestingly, neither *crk7* mutant showed an increased ROS burst to flg22, suggesting the response is specific for GPA extract (Figure 4.4b).

In a screen of a second set of 6 more mutant lines (all in the Col-0 background), two lines had statistically reduced responses to aphid extract compared to the Col-0 control (Figure 4.5a). For one of these lines, GK 065H10, the ROS burst was reduced to the same levels seen in *bak1-5* plants. GK 065H10 carries a mutation in the gene AT3G20190, which is annotated as POLLEN RECEPTOR LIKE KINASE 4 (PRK4), because it has sequence similarity to PRK1, a receptor-like kinase from Petunia involved in the control of pollen germination and pollen tube growth (407; 408). The other mutant line with a reduced ROS burst to aphid extract was SALK 007108, which carries a mutation in AT5G39000, encoding a malectin receptor-like protein kinase family protein. Neither PRK4 nor AT5G39000 has thus far been identified as having a specific function, or been implicated in plant defense. Neither of these lines showed a decrease in the flg22-induced ROS burst, indicating that the loss of response is specific for GPA-extract (Figure 4.5b). These RLKs could therefore be functioning as aphid elicitor receptors.

CRK7, PRK4 and AT5G3900 all contain C-terminal protein kinase domains and have transmembrane helices at their centres (see Figure 4.6). This is in agreement with having a receptor role at the plasma membrane; with the N-terminal portions predicted to be exposed to the extracellular space of the cells acting as receptors of elicitors and the C-terminal kinase domains predicted to be exposed into the cell cytoplasm having roles in

(a)



(b)

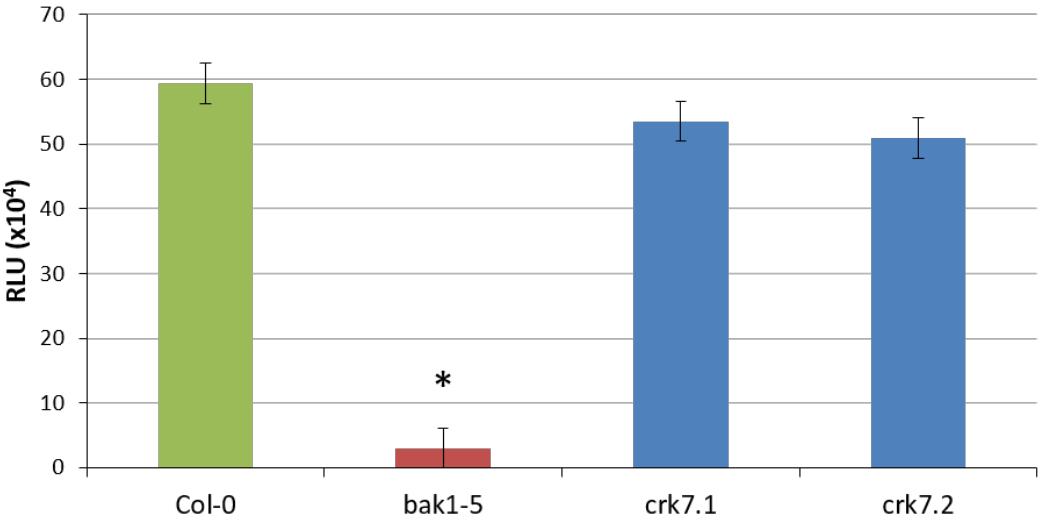


Figure 4.4: **CRK7 negatively regulates the ROS response to GPA extract**. 4.4a *crk7* mutants show an increased ROS response to GPA extract. Leaf discs were treated with GPA-extract and ROS bursts measured using a luminol-based assay. Bars show mean ± SE of total ROS produced over 10 hours in 4 independent experiments (n=8 per experiment). Asterisks indicate significant differences to the Col-0 control (Student's t-probabilities calculated within GLM at P <0.05). Mutant lines provided by the Robatzek and Zipfel labs (TSL). 4.4b *Crk7* mutants do not show increased ROS in response to flg22. Leaf discs were treated with flg22 and ROS bursts measured using a luminol-based assay. Bars show mean ± SE of total ROS produced over 1 hour in 4 independent experiments (n=8 per experiment). Asterisks indicate significant differences to the Col-0 control (Student's t probabilities calculated within GLM at P <0.05).

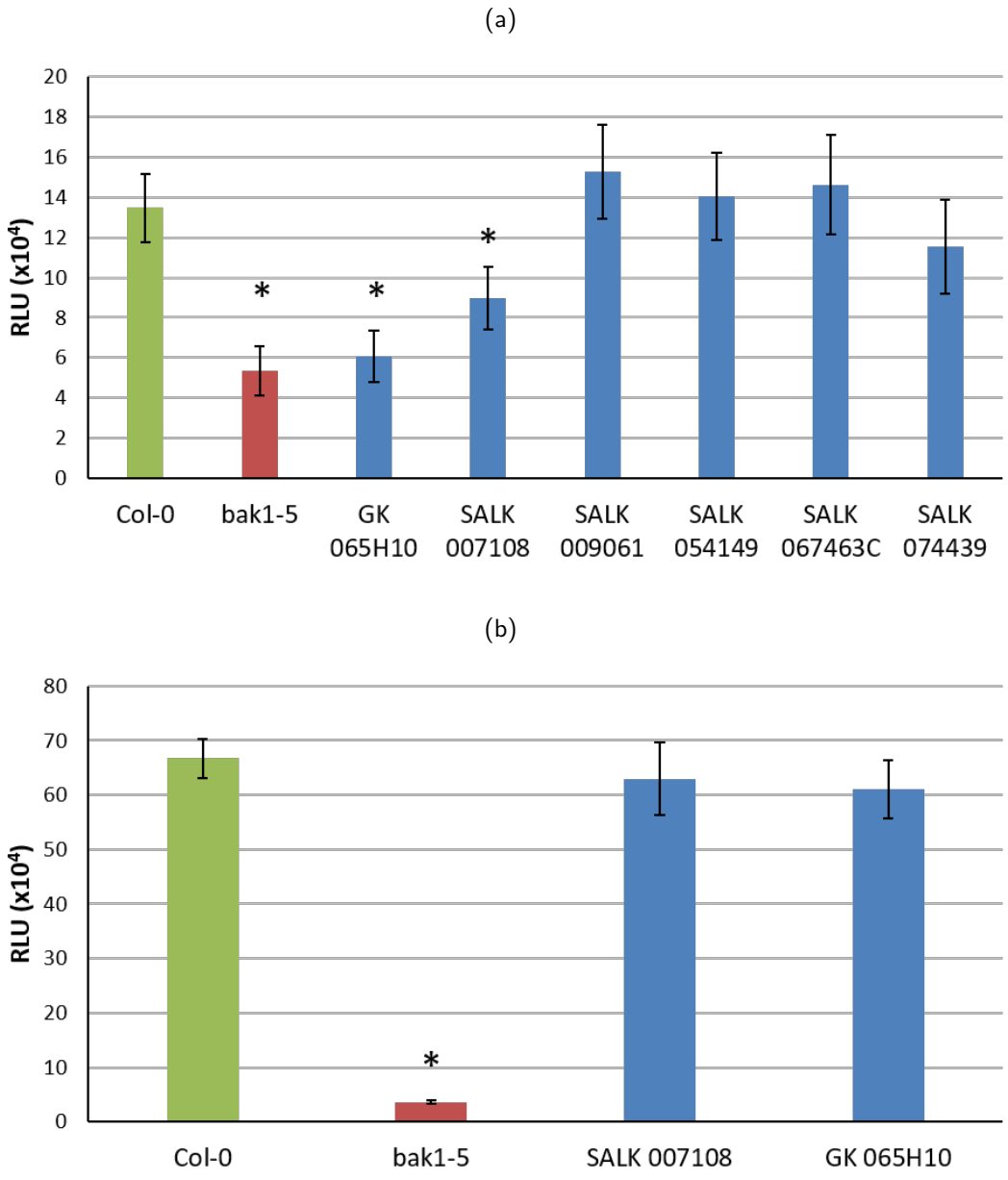


Figure 4.5: **GK065H10 and SALK 007108 T-DNA insert lines have a reduced ROS response to GPA extract but not flg22**. 4.5a GK 065H10 and SALK 007108 have a reduced ROS response to GPA extract. Leaf discs were treated with GPA-extract and ROS bursts measured using a luminol-based assay. Bars show mean ± SE of total ROS produced over 10 hours in 4 independent experiments (n=8 per experiment). Asterisks indicate significant differences to the Col-0 control (Student's t-probabilities calculated within GLM at $P < 0.05$). 4.5b GK 065H10 and SALK 007108 do not show a difference in ROS response to flg22. Leaf discs were treated with flg22 and ROS bursts measured using a luminol-based assay. Bars show mean ± SE of total ROS produced over 1 hour in 4 independent experiments (n=8 per experiment). Asterisks indicate significant differences to the Col-0 control (Student's t-probabilities calculated within GLM at $P < 0.05$).

signaling after PAMP perception has taken place. PRK4 is a LRR-RLK. It has leucine-rich repeats at the N-terminal, which are known to be involved in binding proteinaceous elicitors (409). The well-studied pattern recognition receptors (PRRs) FLS2 and EFR are both LRR-RLKs, so proteins like PRK4 are already known to be involved in perception of immune elicitors. The N-terminal portion of AT5G39000 contains a malectin-like carbohydrate binding domain, indicating that this PRR may detect polysaccharides of the aphid. The N-terminal domain of CRK7 contains regions with similarities to ginkbilobin-2 (Gnk2), which is an anti-fungal protein found in Ginkgo seeds. Proteins containing the cysteine-rich motif of Gnk2, also known as domain of unknown function 26 (DUF26), form one of the largest classes of RLKs in Arabidopsis (410). The cysteines may be involved in formation of the receptors three-dimensional structure or act as zinc binding motifs, either of which can mediate protein-protein interactions. Thus, PRK4 and CRK7 are predicted to interact with proteins, whereas AT5G39000 is predicted to interact with polysaccharides. Both CRK7 and AT5G39000 also have concanavalin A-like lectin/glucanase domains, which span the transmembrane helix region. Lectins are proteins that can bind to cell membranes, so the function of this region is probably in membrane interaction, like the transmembrane domain.

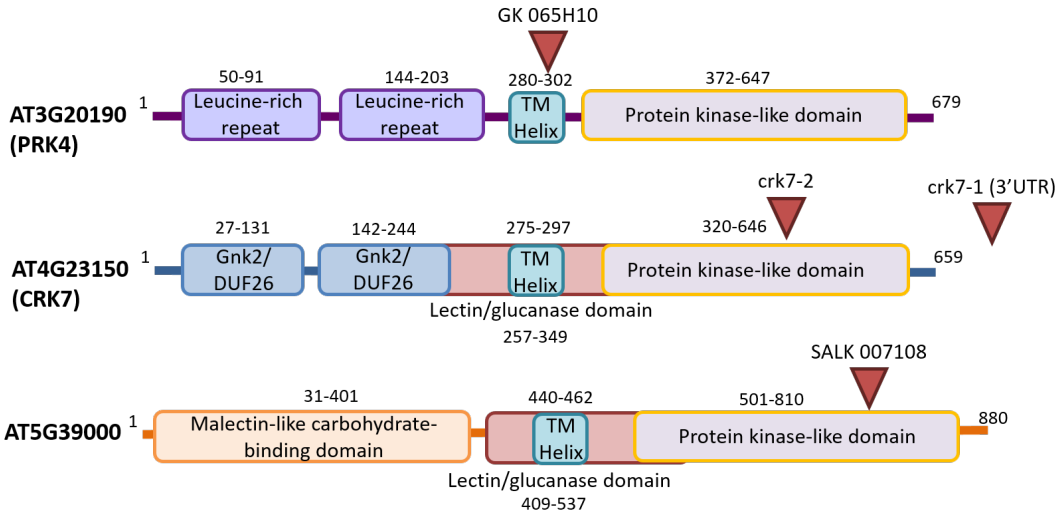


Figure 4.6: **Diagram of candidate receptor protein structure**. All three RLKs contain protein kinase domains and transmembrane helix (TM helix) domains but differ in their extracellular domains. Red arrows show region of the protein that would be affected by the T-DNA insert in the mutant indicated.

Looking at the regions where the mutations are found (Figure 4.6), the SALK 007108 T-DNA is inserted in the kinase domain of AT5G39000. This mutation may affect the phosphorylation action of the protein, or interaction with its signaling partner. The T-DNA insert of the *crk7-2* mutant is also located in the predicted kinase domain of CRK7, whereas that of *crk7-1* locates in the 3'UTR of CRK7. The ROS burst increase of the *crk7-1* mutant is similar to that of *crk7-2*, suggesting that the 3'UTR may have a regulatory function,

perhaps by influencing gene expression. The GK 065H10 insertion in PRK4, unlike the other mutants found, is in the centre of the protein sequence, in the transmembrane helix region. This may affect receptor localisation, or introduce an early stop codon to prevent receptor production entirely.

Gene Name	Common Name	Mutants Identified	Checked via ROS	Phenotype
AT1G07390	RLP1	A-NT (atrlp1-1/2)		
AT1G16140	WAKL3	A-NT		
AT1G16150	WAKL4	A-NT (wakl4-1)		
AT1G35710		SALK 067463C	yes	None
AT1G51830		SALK 009061	yes	None
AT1G55200		A-NT		
AT1G62090		A-NT		
AT1G66880		A-NT		
AT1G72460		etp1-1, etp1-2, etp1-3	yes	None
AT1G78940		A-NT		
AT2G29220	LECRK-III	A-NT (SALK 070736)		
AT2G31880	SOBIR1	sobir1-12	yes	None
AT2G32680	RLP23	rlp23	yes	None
AT2G37710	LECRK-IV	A-NT		
AT2G40270		A-NT		
AT2G47060	PTI1-4	A-NT		
AT3G20190	PRK4	GK 065H10	yes	Decreased
AT3G59350		A-NT		
AT3G59730	LECRK-V	A-NT		
AT4G11460	CRK30	crk30	yes	None
AT4G23140	CRK6	crk6	yes	None
AT4G23150	CRK7	crk7-1, crk7-2	yes	Increased
AT4G23210	CRK13	crk13	yes	None
AT4G25390		A-NT		
AT4G28670		A-NT		
AT4G35030		A-NT		
AT5G06940		A-NT		
AT5G35390	PRK1	SALK 054149, SALK 074439	yes	None
AT5G39000		SALK 007108	yes	Decreased
AT5G49770		GK 585B07	no	
AT5G61550		A-NT		

Table 4.2: **Table summarising the progress made on assaying candidate receptors identified by RNAseq**. A-NT = Mutant lines available, but not tested.

4.3 Discussion

The experiments undertaken in this chapter have identified further components involved in the plant immune response to aphids. Some of these are shared with other plant immune responses, such as BAK1, AGB1 and PAD3. I may also have identified three receptors that regulate plant immunity to aphids. BAK1 involvement in aphid detection by plants has also been verified by another lab, who found that it was required for induction of PTI responses to GroEL, a protein produced by the bacterial symbionts of aphids that is present in aphid saliva (363). BAK1 is a co-receptor that activates PTI through interactions with other PRRs, such as FLS2 and EFR. However, we previously found that FLS2, EFR and other receptors known to associate with BAK1 are not involved in the elicitation of the ROS response to aphid extract, suggesting that another, as yet unknown, BAK1-interacting PRR is involved. One of the PRRs identified in this screen may be a BAK1-interacting PRR.

My screen of mutants in receptors identified via RNAseq identified four Arabidopsis T-DNA insertion lines that had an altered ROS response to GPA extract; *crk7-1*, *crk7-2*, GK 065H10 and SALK 007108. I found that all these lines still showed the wild-type response to another elicitor, flg22. This demonstrates that the receptor candidates are involved specifically in the response to aphid elicitors, rather than PTI responses as a whole. In three of the identified lines, the T-DNA insertions are located in regions that encode crucial parts of the proteins; for *crk7-2* and SALK 007108 this is the kinase domain, and in GK 065H10 this is in the transmembrane region. These T-DNA lines are therefore likely to be null mutants of the receptors. In *crk7-1* however, the T-DNA insert is in the 3'UTR of the coding sequence. The 3'UTRs of genes are often involved in regulating expression levels or stability of transcripts. Bourdais et al. investigated expression levels of CRK7 in both *crk7-1* and *crk7-2* plants, and found that CRK7 transcript levels were reduced though still detectable in both mutants (411). *crk7-1* and *crk7-2* were found to contain 1 and 2 T-DNA inserts respectively (411), so despite the possible presence of another mutation in *crk7-2*, the only difference between *crk7-1* and the wild-type Col-0 is the change to the 3'UTR of CRK7, strengthening the evidence that it is loss of CRK7 that increases the ROS response to GPA extract. It is possible that the two other lines, GK 065H10 and SALK 007108, contain other T-DNA inserts outside of the receptor genes. Further confirmation that these PRRs play a role in detection of aphid extract is required, including the identification of additional T-DNA insert lines for PRK4 (GK065H10) and AT5G39000 (SALK 007108). Complementation assays should also be conducted, in which the affected PRRs are introduced into the T-DNA insertion lines via transformations that express the PRR

under its native promoter.

The GK 065H10 line is a mutant affected in POLLEN RECEPTOR LIKE KINASE 4 (PRK4). Interestingly, data from the RNAseq experiment showed that the induction of PRK4 was lower in *bak1-5* than wild type Col-0 plants upon exposure to GPA and pea aphid. This suggests that PRK4 induction upon insect perception is BAK1 dependent. PRK4 has a leucine-rich repeat (LRR) region at its N-terminal, making it a LRR-RLK, like PEPR1 and 2, FLS2 and EFR, which all interact with BAK1 (412). PRK4 may therefore interact with BAK1 as well. The presence of the LRR domain, which is known to mediate protein-protein interactions, in a region which is predicted to locate extracellularly suggests that PRK4 may detect a proteinaceous elicitor. This could possibly be the identified aphid elicitor protein GroEL (363).

AT5G39000, the gene disrupted in the SALK 007108 mutant, is predicted to have a carbohydrate binding extracellular domain, suggesting that it interacts with a non-proteinaceous elicitor from aphids. The main carbohydrate in aphids that could fulfill a role as an elicitor is the chitin that forms the aphid exoskeleton. However, CERK1, an Arabidopsis receptor that binds chitin (362; 413), does not appear to be involved in the ROS burst to GPA extract (340). Aphids may interfere with chitin perception by plant receptors by adding modifications to chitin molecules or by hiding their exoskeletal chitin from the receptors via interactions with proteins, such as cuticular-binding proteins which are abundant in aphids. Another carbohydrate associated with aphids is the disaccharide trehalose, which forms a major component of their hemolymph (414). Interestingly, plants also produce trehalose, and it is involved in the plant signaling response to biotic and abiotic stresses (415). Feeding of GPA induces trehalose accumulation in *Arabidopsis thaliana* (416), and this trehalose increase has been found to be required for PAD4-dependent defense against the aphid (417). It is therefore a possibility that AT5G39000 is acting as a trehalose sensor.

The potential involvement of two different receptors in aphid extract perception suggests that Arabidopsis detects more than one component in the GPA extract. This is in agreement with published studies that describe two separate fractions in GPA extract, a 3-10 kD and a greater than 10 kD fraction, both inducing immune responses in Arabidopsis (340; 47). Multiple PRRs also detect PAMPs from one bacterial species, such as flagellin, EF-Tu and peptidoglycan (114; 399). Having multiple receptors helps to ensure that a defense response will take place (418).

The cysteine-rich RLK 7 (crk7) mutants showed an increased ROS burst in response to GPA extract, suggesting that CRK7 is normally a negative regulator of the plant immune response to aphids. CRK7 gene expression is induced by extracellular ROS and the CRK is involved in mediating the responses to this ROS. CRK7 presence prevents the cellular damage that large amounts of ROS can cause (406). In the absence of CRK7, apoplastic ROS may not be detected. This absence of detection may lead to further increases in ROS, perhaps because CRK7 is not there to activate suppressors that inhibit further ROS production. However, the ROS response to flg22 was unchanged in the *crk7* mutants, confirming published data (411) and indicating that CRK7-mediated ROS suppression activity occurs specifically to aphid extract. This is in agreement with CRK7 not having an effect on plant defense against *Pseudomonas syringae* or detection of chitin (411). Other CRK proteins are integral components of plant defense (411), so perhaps different members of the CRK family respond to different pathogen types specifically.

Few receptor proteins like CRK7 have been found to have a negative effect on plant immunity; most are involved in initiating or propagating immune signaling. One example of a negative regulator is the LRR-RLK BIR2 (BAK1-interacting RLK), which negatively regulates BAK1 through direct binding, preventing interaction of BAK1 with FLS2 prior to flg22 perception (419). BIR2 has a very small extracellular LRR domain, and it is not believed to have a function in the apoplast, but in regulation of BAK1 within the cell cytoplasm. CRK7 has a large extracellular region, suggesting that in contrast to kinases like BIR2 and BAK1 it does have an extracellular function. The 44 CRKs in Arabidopsis all contain an extracellular Gnk2-like domain, but its functional role is unclear (411). The lysin motif (LysM)-containing RLK LYK3 has both a negative effect on plant immunity and a functional extracellular domain. It is believed to play a role in the cross-talk between pathogen resistance and physiological responses mediated by the phytohormone abscisic acid (ABA). LYK3 expression is repressed by fungal infection and elicitors, consistent with a role in repression of PTI responses until they are required after pathogen detection. In contrast, CRK7 is induced by aphid treatment, so would be present when PTI responses are taking place. Further investigation of CRK7 is therefore required to decipher this response, confirm that it has a role in plant-aphid interaction and how exactly it is acting in opposition to PTI.

The G-protein β subunit AGB1 regulates resistance to both bacteria and fungi by mediating hormonal defense signaling and the ROS burst (402; 194; 403; 195). I found that AGB1 also mediates the ROS burst response to GPA extract. Moreover, PAD3 expression is

reduced in *agb1-2* plants, but not in *bak1-5* plants. G-proteins therefore play a role in early, PTI-like responses to aphids and regulate an alternate, BAK1-independent response to the insects. This is in agreement with a recent publication that reports that AGB1 and other G-protein subunits are components of a novel, BAK1-independent, plant immune pathway activated by *Pseudomonas aeruginosa* secreted proteases (193). It would be interesting to identify whether aphid proteases play a role in activation of the AGB1-mediated defense response. It also remains to be investigated whether PRK4, AT5G39000 and CRK7 act in the BAK1 or AGB1 pathway, or neither.

The colonization ability of both the pea aphid and GPA is increased on *agb1-2* plants, unlike *bak1-5* mutants on which the pea aphid performs better, but not GPA. This difference may be explained by an effective suppression of BAK1-mediated PTI induction by GPA effectors and a less effective suppression of AGB1-mediated immunity. This is the first finding that a heterotrimeric G-protein subunit is involved in plant resistance to insects. It would be interesting to investigate the involvement of other G-protein subunits. Unlike animals, which have several different G-protein subunits, Arabidopsis produces only one α subunit, GPA1, one β subunit, AGB1, and two γ subunits, AGG1 and AGG2 (420). The Gα subunit has also been implicated in defense responses (401; 193). Plant G-proteins have many different functions. It is therefore likely that other G-protein components or modulators regulate the specificity of G-protein-mediated signaling. It would be interesting to investigate what the specificity components are in G protein-mediated signaling to aphids.

PAD3 is induced downstream of AGB1-mediated perception of GPA extract and is an interesting component of aphid defense. PAD3 is the final enzyme in the biosynthesis of camalexin, and previous investigations in the lab have shown that camalexin had a negative effect on aphid fecundity (236; 238). This is in agreement with GPA having increased fecundity on *pad3* mutant plants, and a reduction of the induced resistance response to aphids on *pad3* mutants (Chapter 3 and (340)). However, I found that the ROS response to GPA extract in *pad3* plants is also reduced, to a similar level as that found in *bak1-5* mutant plants. This is in disagreement with PAD3 being induced and camalexin being produced downstream of the ROS burst (421; 422). The *pad3* mutant did not show a reduction in ROS burst upon flg22 treatment, suggesting that PAD3 involvement in ROS generation is specific for aphid extract. This evidence suggests that PAD3 may regulate processes other than camalexin production in response to aphids.

Although there is no change in ROS production of *pad3* mutants in response to the

fungal pathogen *Alternaria brassicicola*, *pad3* plants do show differences in the timing of expression of many defense-related genes compared to wild type plants (423). This suggests that lack of functional PAD3 not only reduces camalexin in the plant, but is also able to disrupt various defense pathways. It is possible that PAD3 is involved in a feedback mechanism to regulate ROS, and because the ROS burst in response to GPA extract takes place over a much longer timeframe than that to flg22 (340), there is more time for PAD3 to feed back on the ROS burst. Camalexin increases ROS production in human cancer cells (375; 374), but no such role has been found in plants. Increased ROS in cancer cells is due to the increase of intracellular mitochondrial ROS production (374). In contrast, the ROS burst to GPA extract in plants is mediated via RBOHD (340) located on the plasma membrane, which produces extracellular ROS (424). Alteration of ROS dynamics may therefore not occur via the same mechanisms in plant cells as in animal cells. How PAD3 may affect the plant ROS burst to aphids requires further investigation. PAD3 in defense against pathogens is induced downstream of WRKY33 (377). The most similar *N. benthamiana* WRKY to WRKY33, WRKY8, also induces *RBOHD* transcription (383), so there may be a link between PAD3 and ROS induction via transcription factors.

This chapter has further strengthened the idea that components of PTI are shared between plant responses to biotic threats. I have also identified potential aphid elicitor receptors.

Chapter 5

The aphid effector protein Mp10 suppresses plant calcium and ROS bursts and acts in the BAK1-mediated defense response pathway

Contributors: Christine Wilson, David C. Prince, Alex Coleman, Sam Mugford, Mark Banfield, Cyril Zipfel and Saskia A. Hogenhout

5.1 Introduction

Plant-associated organisms secrete virulence proteins and other molecules, collectively known as effectors, to modulate plant defense responses and enable successful colonisation of the plant. Effectors from bacteria, fungi, oomycetes and nematodes have been studied in detail, which has led to a greater understanding of the molecular dynamics between plants and pathogens (425). Recently, investigations have begun to reveal that proteins secreted by insects also act as effectors to play a role in plant-insect interactions (24; 418). One such effector is the enzyme glucose oxidase (GOX), which is secreted by the caterpillar *Helicoverpa zea*. GOX suppresses the production of nicotine in *Nicotiana tabacum* and so prevents resistance responses to this herbivore (256). Aphids also modulate plant defenses, presumably via their saliva. For instance aphids are able to prevent sieve element occlusion before or during feeding from the phloem, though the specific salivary components responsible for this are unknown (29). When I began this research, only one aphid candidate effector was identified, namely protein C002 from the pea aphid, *Acyrthosiphon pisum*. C002 is injected into the host plant when the aphids feed and RNA interference (RNAi)-mediated knock down of the corresponding gene alters aphid feeding behaviour (41).

An effector screen conducted in the Hogenhout lab identified a suite of potential effectors that have a secretion signal and are expressed in the salivary glands of the green peach aphid (GPA), *Myzus persicae* (46). These potential effectors were further characterized for their abilities to promote aphid colonisation, cause a cell death response, or inhibit the PAMP-induced ROS burst of *Nicotiana benthamiana*. This identified the GPA homologue of C002, MpC002, and two other effectors; Mp42 and Mp10 (46). Intriguingly, the ROS burst triggered by the bacterial PAMP flg22, which requires the plant receptor-like kinases (RLKs) FLS2 and BAK1 (see Chapter 3), was suppressed upon heterologous expression of Mp10 in *N. benthamiana* (46; 316). David Prince (a former PhD student in the Hogenhout lab) and I previously found that ROS production is a component of a PTI-like plant defense response to aphids that is dependent on BAK1 but independent of FLS2 (Chapter 3, (340)). Moreover, preliminary data generated by D. Prince suggested that knock-down of Mp10 in *M. persicae* by plant-mediated RNAi reduces the colonization ability of this aphid on *N. benthamiana* (316). Taken together these results led me to the hypothesis that Mp10 suppresses plant defence responses upstream of the plant ROS burst, which is induced upon recognition of flg22 and aphid elicitors in a BAK1-dependent manner.

The action of Mp10 in plants appears more complex than simply blocking ROS re-

sponses. Mp10 also induces chlorosis in *N. benthamiana* that is reduced upon silencing of SGT1 (Suppressor of the G2 allele of *skp1*), a ubiquitin-ligase associated plant protein required for plant cell death responses, including those involved in plant immunity (426) and the plant jasmonate (JA) response (427). GPA colonization is also reduced on plants that heterologously express Mp10 (46). It is possible that Mp10 interactions with plant targets become toxic when Mp10 is present in abundance. Alternatively, the *N. benthamiana* immune system may recognize Mp10 leading to an effector-triggered immunity (ETI)-like response. The effects of Mp10 in suppressing PTI and in inducing chlorosis occur at different timepoints after Mp10 expression *in planta*, with the chlorosis response occuring later, enabling me to investigate the two roles of Mp10 in isolation.

Mp10 was previously identified as OS-D2 (Olfactory Segment D2-like protein), and this protein and homologs such as OS-D1 are chemosensory proteins (CSPs) that are present in all insect species (428; 429). CSPs are small, highly soluble proteins with a capacity for binding hydrophobic molecules in a central pocket region, which is formed within a structure of 6 alpha-helices (429; 430; 431). Their signature motif is four conserved cysteine residues which form two disulphide links (432). CSPs have been identified in the lymph of chemosensilla, where they are believed to play a role in the perireceptor events of chemoperception by binding to odorant molecules and delivering them to receptors in the cell membrane of the sensory neurones (433). In agreement with this role, some CSPs are found specifically in sensory organs, such as the antenna (434; 435). A CSP found in the antenna of the carpenter ant, *Camponotus japonicas*, is involved in binding lipophilic cuticular hydrocarbons that sense whether or not other ants are nestmates and so mediates decisions as to whether aggressive behaviour is needed (436).

Some CSPs are, however, produced in non-sensory organs, suggesting that they may have functions beyond chemoperception. These functions include delivery of pheromones and development, for instance limb regeneration in cockroaches, embryo development in honeybees and the transition from solitary to gregarious phases in locusts (437; 438; 439). These differing functions of insect CSPs may be comparable to those of animal lipocalins, which have major functions in chemoperception but also have other roles, such as pheromone release in the urine of mice (440). Members of both protein families are small, soluble and possess compact potentially stable structures, which are ideal for performing different tasks in and outside cells. Moreover, sequences of CSP and lipocalin family members suggest high variability in polypeptide sequences and folds that allow for variability in ligand binding and the fulfilment of diverse functions within organisms (433; 430). Thus,

although Mp10 is a CSP, it cannot be assumed that this protein has a role only in chemoperception of aphids; it is possible that Mp10 has adapted to modulate specific proteins or processes in plants.

Work in the Hogenhout lab detected transcripts of Mp10 (MpOS-D2) in heads and salivary glands but not the gut of GPA (46). OS-D2 and homolog OS-D1 of the vetch aphid, *Megoura vicae*, are expressed in the legs and antennae, which act as sensory organs. OS-D2 transcripts were also present in the head of *M. vicae* (441). Attempts to identify substrates for OS-D2 from *M. vicae* were unsuccessful as none of the 28 odorant compounds tested elicited responses in insects and insect cells. Hence, despite the presence of Mp10 and OS-D1 homologs in the sensory organs of aphids, it is not yet clear if they bind any odorants there. CSPs, including Mp10 and OS-D1, are conserved throughout several aphid species (441) and homologs of Mp10 from pea aphid *Acyrthosiphon pisum* and cotton aphid *Aphis gossypii* also inhibit flg22-induced ROS bursts (316), suggesting that functions of these proteins are also conserved among aphids.

Based on the finding that Mp10 plays a role in PTI apparently upstream of ROS and possibly also in ETI, I decided to examine this candidate effector further. First, I investigated whether Mp10 suppressed ROS and calcium bursts induced by aphid extract, and how these Mp10 activities compare to those of its homolog, MpOS-D1. I then helped with developing tools and conducting experiments to detect Mp10 in plants near GPA-feeding sites. Next, I investigated if Mp10 contributes to GPA fitness by knocking down Mp10 gene expression by feeding the aphids on *Arabidopsis thaliana* lines stably expressing double stranded RNA complementary to Mp10, and determined whether Mp10 acts in the BAK1 pathway. To better understand how Mp10 itself functions and to generate controls for future studies, I generated targeted mutations in Mp10 and MpOS-D1 and investigated how they affect the PTI suppression and chlorosis activities of these proteins. Together, the results provide evidence that Mp10 is an aphid effector that is required by the aphid to suppress plant calcium and ROS bursts that form part of the BAK1-mediated PTI response.

5.2 Results

5.2.1 Mp10 suppresses calcium and ROS bursts induced by the bacterial PAMP flg22 and aphid elicitors

It was previously found that Mp10 suppresses the flg22-induced ROS burst when overexpressed in *Nicotiana benthamiana* (46). To examine if Mp10 also suppresses the ROS burst induced upon perception of aphid elicitors (340), aphid extract was prepared and used to induce a ROS burst in *N. benthamiana* in the presence of vectors expressing Mp10 and the positive control AvrPtoB, which is a *Pseudomonas syringae* effector shown to suppress the ROS burst induced by flg22 by targeting BAK1 and the PRRs FLS2 and CERK1 for degradation (261; 262; 264). The empty vector was also included as a negative control. We found that Mp10 blocks the aphid extract-induced ROS burst in a similar manner to the bacterial effector AvrPtoB (Figure 5.1a).

To investigate if the ROS suppression is specific to Mp10 or is an activity shared by other CSPs, the Mp10 homolog MpOS-D1 from GPA was cloned, and I tested its suppression activity alongside Mp10 in *N. benthamiana*. Unlike Mp10, MpOS-D1 did not prevent the flg22-induced ROS burst from occurring (Figure 5.1b). Flg22 and aphid extract also elicit a burst of cytosolic calcium in *N. benthamiana* (316; 185). I used the same Mp10 and MpOS-D1 vectors in a calcium burst assay using SLJR15 *N. benthamiana* lines expressing the calcium reporter protein aequorin (185). I found that Mp10 could block the calcium burst that occurs as a result of flg22 perception in *N. benthamiana*, whereas MpOS-D1 did not (Figure 5.1c). Thus, Mp10 suppresses the calcium and ROS bursts to elicitors of PTI, suggesting that Mp10 is functional *in planta* and probably acts on components early within the PTI-pathway. As MpOS-D1 cannot function in the same way, this is not a general feature of aphid CSPs.

5.2.2 Mp10 antibodies label the cytoplasm of plant cells near aphid feeding sites

Mp10 was identified as having a secretion signal peptide and was found to be expressed in the salivary glands of GPA (46). Mp10 may therefore be delivered into plants via GPA saliva during feeding. However, direct evidence for Mp10 delivery into plants by aphids is lacking. Immunolocalization of Mp10 at or near aphid feeding sites using specific Mp10 antibodies would provide the most direct evidence of the presence of this aphid protein *in planta*. To raise antibodies, the predicted mature Mp10 and MpOS-D1 proteins (minus

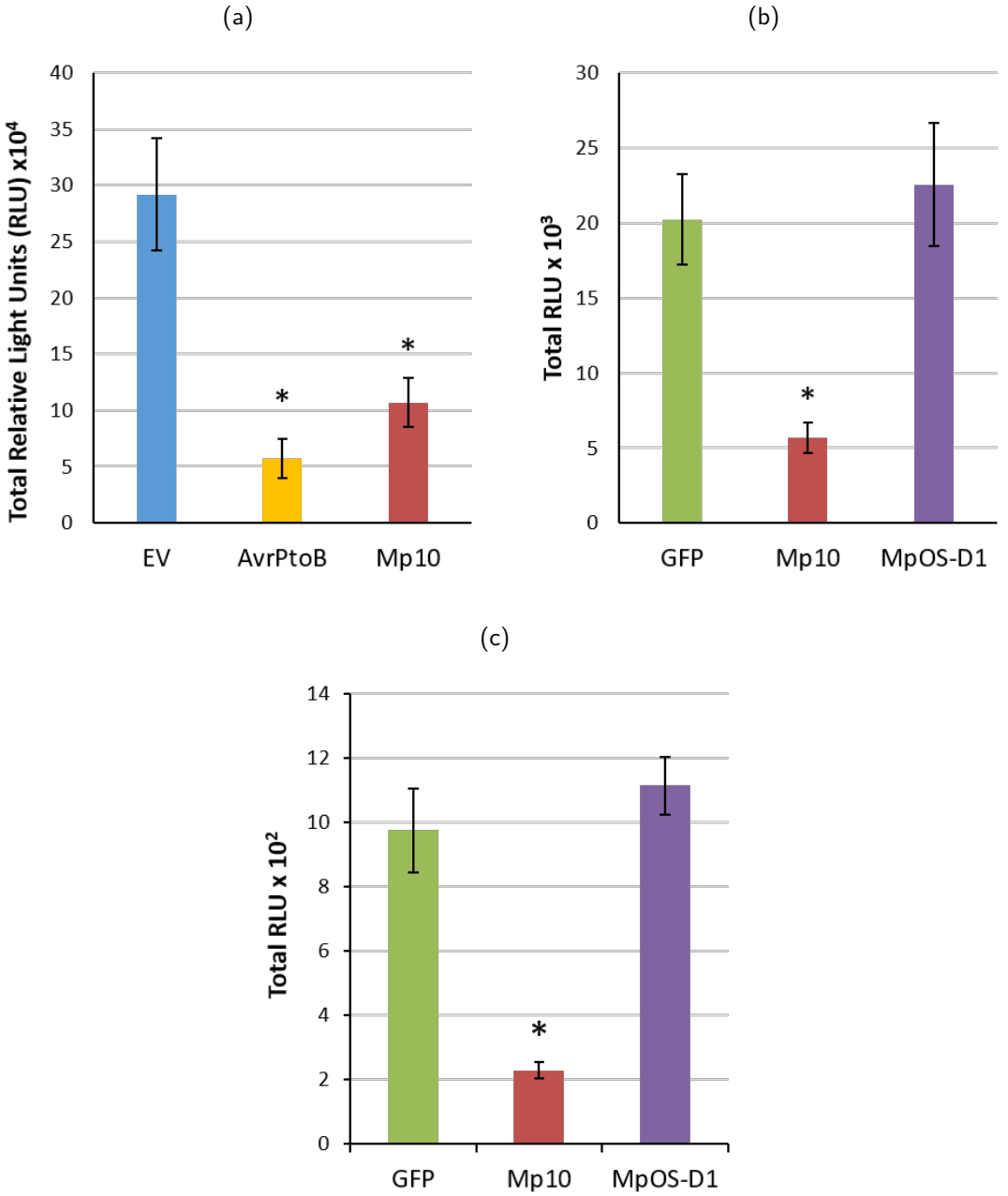


Figure 5.1: **Mp10 suppresses ROS and calcium bursts in response to the PAMP flg22 and aphid elicitors**. 5.1a Mp10 blocks the ROS burst induced by aphid extract. ROS bursts were measured over 600 minutes in *N. benthamiana* leaves transiently producing Mp10 or AvrPtoB (positive control) alongside an empty vector (EV) control. Graph shows mean ± SE of 3 independent experiments (n=8 per experiment). Asterisks (*) indicate significant differences to the EV treatment (t-probability calculated by Student's t-test at P <0.05). This experiment was conducted by David Prince. 5.1b Mp10 blocks the flg22-induced ROS burst. ROS bursts were measured over 60 minutes in *N. benthamiana* leaves transiently producing GFP-tagged Mp10 (GFP-Mp10) and GFP-MpOS-D1 alongside a GFP control. Graph shows mean ± SE of 4 independent experiments (n=8 per experiment). Asterisks indicate significant differences to the GFP treatment (Student's t-probability calculated within GLM at P <0.05). 5.1c Mp10 blocks the flg22-induced calcium burst. Calcium burst measured over 60 minutes in leaves of SLJR15 *N. benthamiana* plants transiently producing GFP-Mp10 and GFP-OSD1 alonside a GFP control. Graph shows mean ± SE of 4 independent experiments (n=8 per experiment). The asterisks indicates significant difference of the Mp10 treatment to that of the GFP control (Student's t-probability calculated within GLM at P <0.05).

signal peptides) were produced in *Escherichia coli* with N-terminal 6xHis-tags. Genscript BacPower^{TM} and FoldArt^{TM} technologies were used to express and purify the proteins, and their quality and purity was verified by SDS-PAGE and Western-blotting using anti-His-tag antibody. Anti-sera were raised in chicken (Mp10) or rabbit (MpOS-D1) by Genscript. The antisera were affinity purified using immobilized recombinant protein. Sensitivity and specificity of these antibodies were tested by Western blotting using a dilution series of the pure recombinant proteins on blots probed with a series of different dilutions of the antisera. The antibodies at 1:500 to 1:10,000 dilutions detect 5 and 25 ng of purified Mp10 or MpOS-D1 and show low background labeling of plant and aphid proteins (Figure 5.2). Moreover, the Mp10 and MpOS-D1 antibodies did not cross-react with MpOS-D1 and Mp10, respectively (Figure 5.2). Both antibodies are therefore sensitive and specific.

The antibodies were used to detect Mp10 and MpOS-D1 in aphid bodies and heads, and in leaves exposed to aphids. Whereas Western blots showed bands matching the molecular weights of Mp10 and MpOS-D1 in both whole aphids and aphid heads, these proteins were not detected in plant samples that aphids had been feeding on, or in samples of aphid saliva (Figures 5.3a and 5.3b). It is possible that Mp10 amounts are too low for detection amongst more abundant aphid and plant proteins. It was therefore decided to conduct immunolabelling of *A. thaliana* ultra-thin sections from GPA feeding sites, and visualisation of immuno-gold particles by SEM. Preliminary data revealed labeling by Mp10 antibodies in the cytoplasm of some plant cells near plant tissues that contained sheath structures typically surrounding aphid stylets in plant cells (Figure 5.4, left). The high density of staining seen in the cytosol of cells adjacent to GPA feeding sites was not seen in control samples from plants that were unexposed to aphids (the low level of binding seen in all compartments in the control samples is typical of non-specific binding) (Figure 5.4, right). This provides evidence that the GPA delivers Mp10 when feeding, likely by releasing saliva into plant cells upon puncturing by aphid stylets (28), and also that Mp10 is likely to function within the cytosol of the host plant.

5.2.3 Mp10 belongs to the distinct CSP4 cluster of the chemosensory protein family in aphids

Mp10 and MpOS-D1 belong to the family of chemosensory proteins, or CSPs, which are commonly found in insect species and are involved in olfaction and gustation. Mp10 and MpOS-D1 both have the alpha-helical structures and four conserved cysteine residues typical of CSPs (Figure 5.9a). They share 38% identity and over 50% similarity on the protein

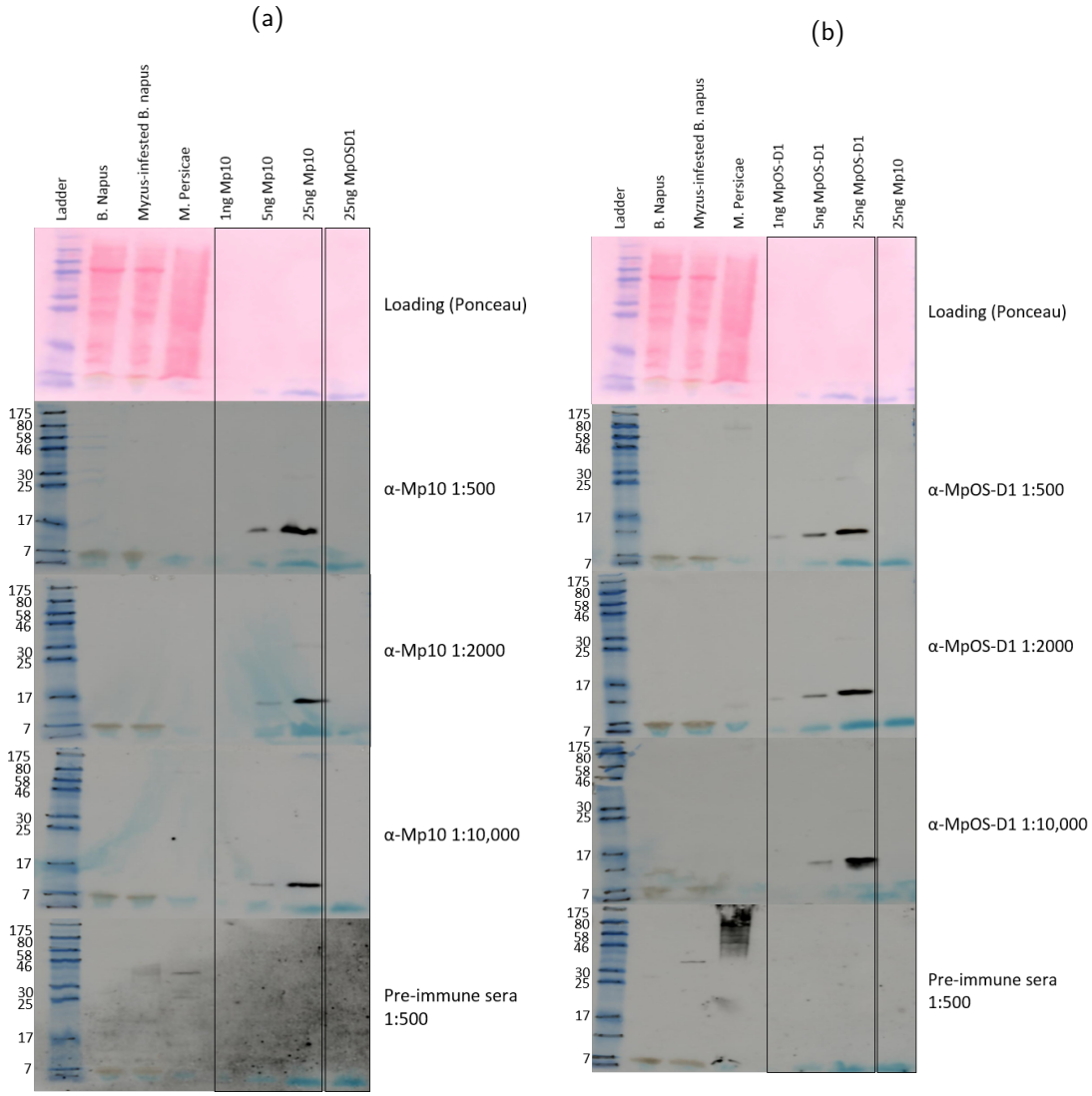


Figure 5.2: **Mp10 and MpOS-D1 antibody verification**. 5.2a Replicate membranes probed with different dilutions of affinity-purified chicken-anti-Mp10 and anti-chicken-HRP secondary. Imaged with automatic exposure. 5.2b Replicate membranes probed with different dilutions of affinity-purified rabbit-anti-MpOSD1 and anti-rabbit-HRP secondary. Imaged with automatic exposure. Experiments carried out by Sam Mugford.

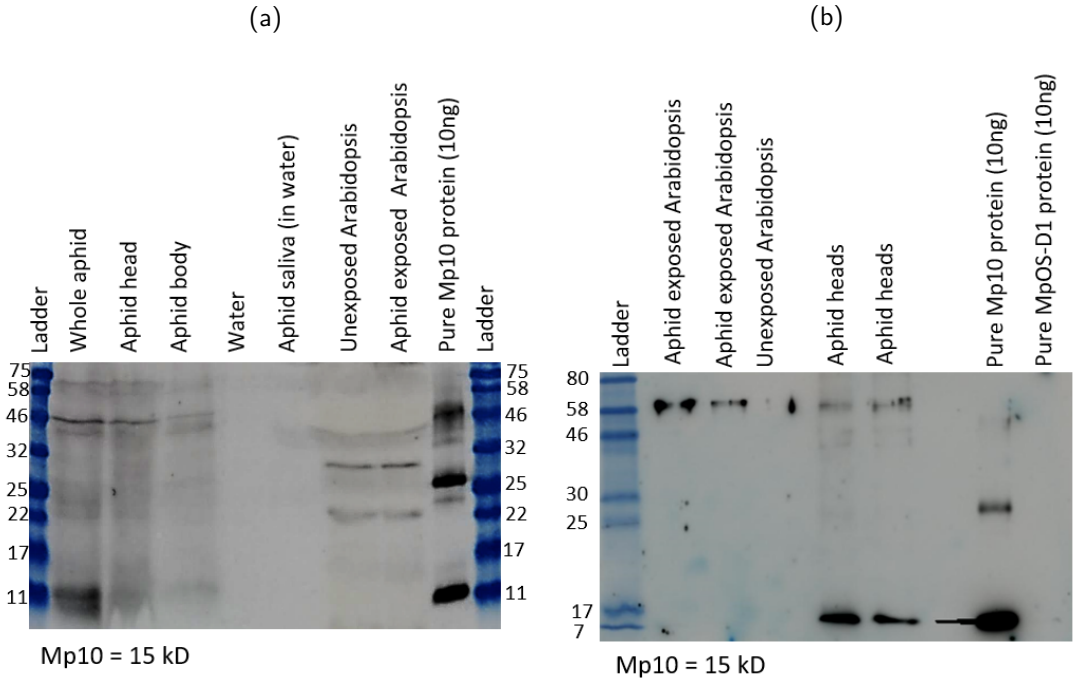


Figure 5.3: **Detection of Mp10 in aphids and in plant cells at aphid feeding sites**. 5.3a Mp10 antibody detects Mp10 protein in the aphid, but not in saliva or infested plants. Antibody was applied at a 1:1000 dilution. Samples were prepared from 10 whole aphids, from heads and bodies of 20 aphids, from saliva collected from 1000 aphids (see Methods) and from two whole leaves exposed to 50 aphids. Membrane imaged using 3 minutes exposure. 5.3b Mp10 antibody has affinity for Mp10 and not MpOS-D1. The antibody detected Mp10 in aphid heads. Antibody was applied at a 1:100 dilution. Samples were prepared from heads of 20 aphids and the plant samples from 20 pieces of $1mm^2$ at aphid feeding sites. Membrane imaged using 5 minutes exposure. Experiment was carried out by Sam Mugford.

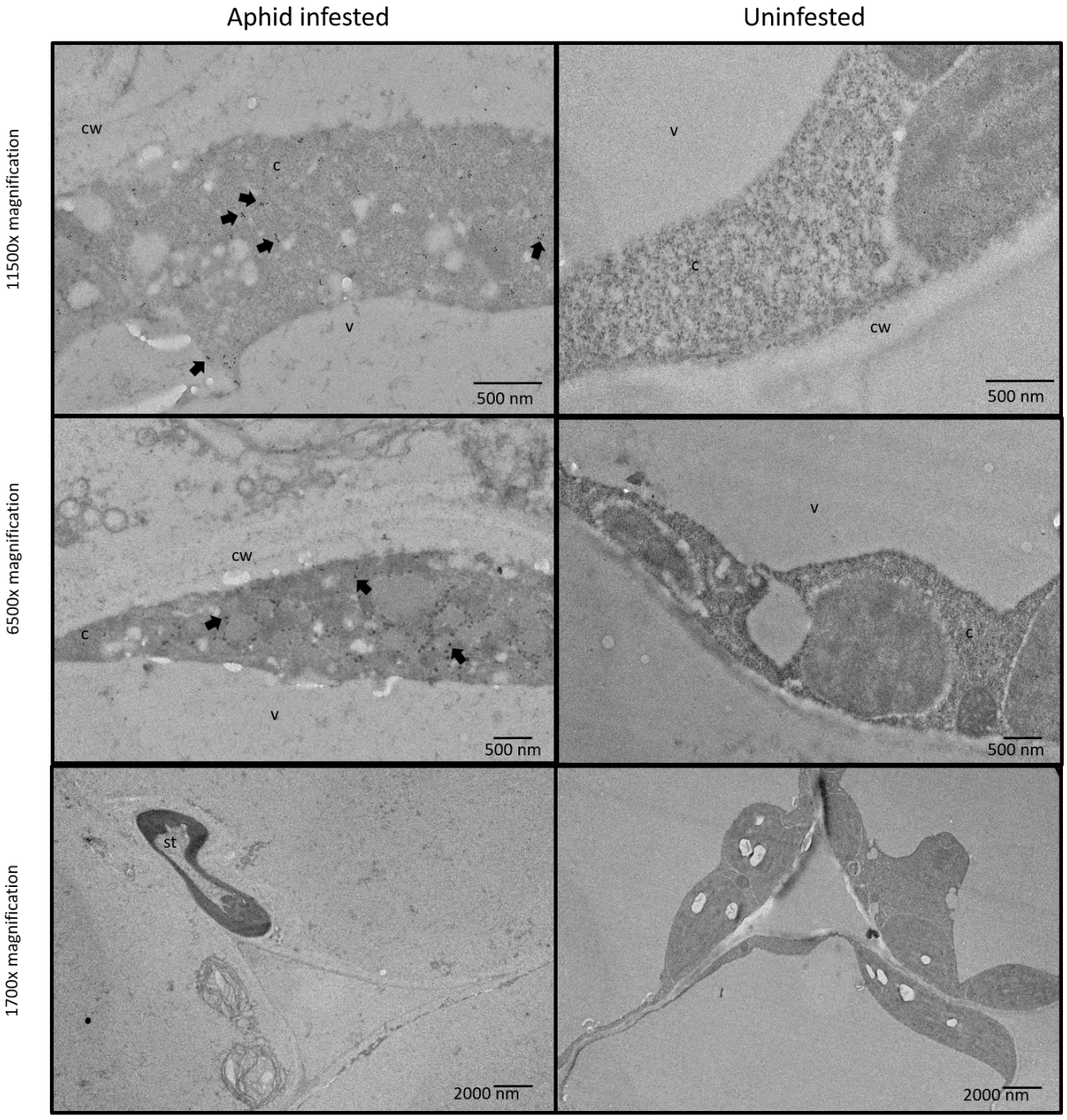


Figure 5.4: **Detection of Mp10 in aphid infested plant tissue using immunogold labelling (IGL)**. The antibody was applied at at 1:100 dilution. Images show labelling of plant mesophyll cells. Legend: c = cytosol; cw = cell wall; v = vacuole; st = stylet track; arrows indicate immunolabeling. Tissue collection carried out by Sam Mugford. Elaine Barklay conducted the sectioning and immunolabeling of plant samples.

level. To investigate if GPA has proteins that are more similar in sequence to Mp10 than MpOS-D1, protein sequence databases of the annotated whole genome sequence of GPA clone O and available sequences of the pea aphid *Acyrthosiphon pisum* and cotton/melon aphid *Aphis gossypii* were generated. The published pea and cotton/melon aphid CSPs were BLASTP searched against the clone O genome database at cut-off E-values of e^{-5}. Identified putative CSPs were reciprocally BLASTP searched against the pea aphid genome at cut-off E-value of e^{-5} to find additional CSPs and to assess if Mp10 and MpOS-D1 were identified. This interrogation identified 8 additional CSPs in GPA Clone O, 10 *A. pisum* CSPs and 9 *A. gossypii* CSPs.

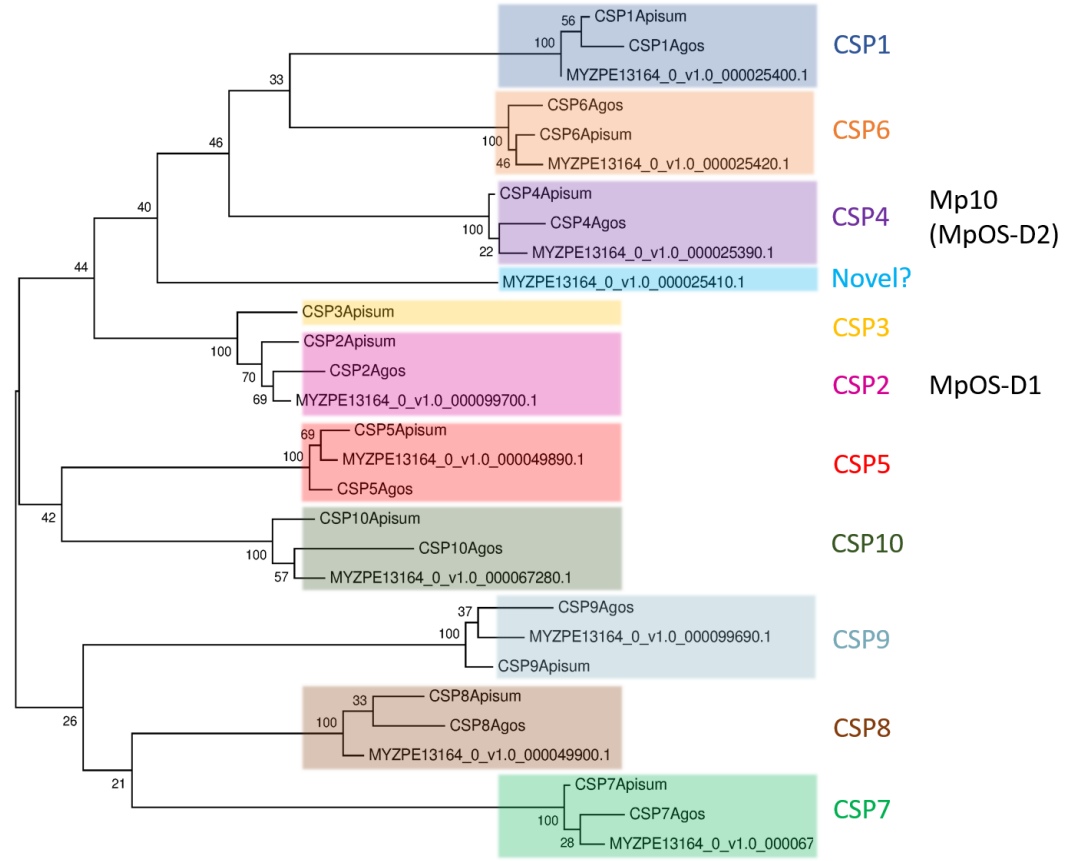


Figure 5.5: **Mp10 and OSD1 both belong to the chemosensory protein (CSP) family**. GPA contains 10 CPSs, Mp10 is homologous to CSP4 and MpOS-D1 is homologous to CSP2 from *A. pisum* and *A. gossypii*. Alignments were conducted using coding sequences (aligned using Muscle, with default values (442). Alignment can be found in Appendix D). The phylogeny was inferred from the alignment using the maximum likelihood method with 100 bootstrap replicates using MEGA (Molecular Evolutionary Genetics Analysis) software (443). Values at the base of the branches are the bootstrap values of 100 replicates. GPA BLAST database, alignments and phylogenetic tree were generated by Christine Wilson.

Alignments and phylogenetic analyses of all aphid CSPs revealed that GPA Mp10 clusters with the CSP4 and MpOS-D1 with the CSP2 of the other two aphid species (Figure 5.5). The tree reveals that nine CSPs, including Mp10 (CSP4) and MpOS-D1 (CSP2), are

present at 1:1:1 ratios in the three aphid species and these CSP clusters are well-supported by bootstrap values (Figure 5.5). This is with the exception of CSP3 that is only present in pea aphid and a novel CSP that is only present in GPA, however the bootstrap values between the CSP clusters are ill supported (Figure 5.5), so the novel CSP could be the GPA orthologue of CSP3, which might suggest that there has been some selection on and divergence of CSP3 between the aphid species.The tree shows that the CSPs present in aphids diverged before the aphid species diverged, and no subsequent duplication or diversification has occurred, with the possible exception of CSP3. The long time since diversification and the small protein size of CSPs limits the amount of usable information in the alignment, so that the bootstrap support of the more ancient branches is necessarily limited. It was previously found that Mp10 from the pea aphid and cotton/melon aphid also blocks the flg22-induced ROS burst in *N. benthamiana* (316), and their clustering in this phylogenetic tree agrees with a shared function in the suppression of plant defense. MpOS-D1 (CSP2) does not suppress calcium and ROS bursts, suggesting that other CSPs do not have the same effector role as Mp10. Despite low bootstrap values, there are CSPs that are suggested to be more similar to Mp10 in GPA that could also be tested; CSP1 and 6. However transcripts of Mp10, but not those of any of the other CSPs, are found in aphid salivary gland transcriptomes (46), making it seem likely that Mp10/CSP4 and orthologues have a unique function among the aphid CSPs.

5.2.4 Mp10 promotes aphid colonization of Arabidopsis in a BAK1-dependent manner

Next, I wanted to investigate whether Mp10 is involved in the ability of GPA to colonize plants. To test this, transgenic *A. thaliana* Col-0 lines that express dsRNA corresponding to transcripts of *Mp10* (dsMp10) and *MpOS-D1* (dsMpOS-D1) were generated and used these plants to knock down gene expression in aphids by plant-mediated RNAi (43). I found three independent transgenic Col-0 lines that successfully knock down Mp10 expression and two transgenic Col-0 lines that reduce MpOS-D1 expression in aphids feeding on those plants (Figure 5.6). To investigate if Mp10 suppresses defence responses that are induced in a BAK1-dependent manner, the dsMp10 and dsMpOS-D1 constructs were also introduced into *A. thaliana bak1-5* mutants that are compromised in the induction of plant defences to GPA, but that do not have pleotropic developmental phenotypes (340). I identified 3 independent *bak1-5* dsMp10 and *bak1-5* dsMpOS-D1 lines that knock down the expression of GPA Mp10 and MpOS-D1, respectively (Figure 5.6).

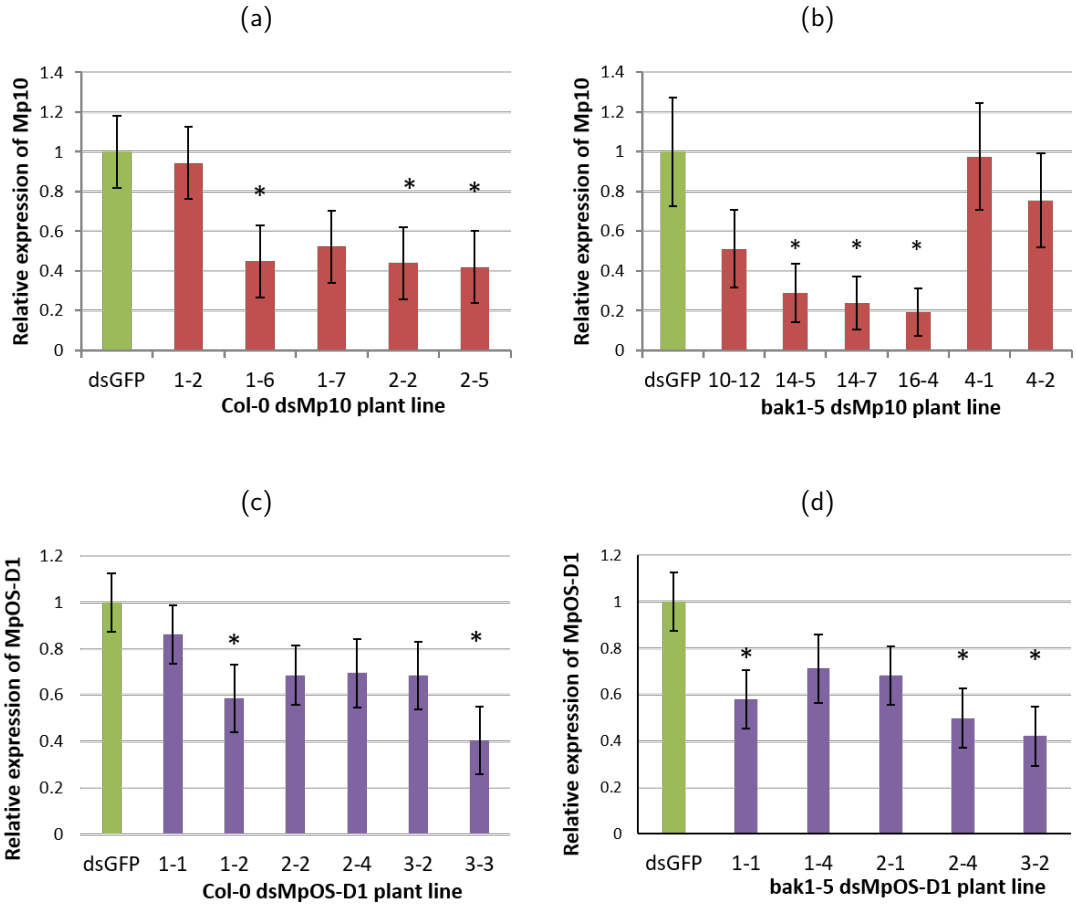


Figure 5.6: **Identification of Mp10 and MpOS-D1 silencing lines**. 5.6a Col-0 dsMp10 lines. 5.6b *bak1-5* dsMp10 lines. 5.6c Col-0 dsMpOS-D1 lines. 5.6d *bak1-5* dsMpOS-D1 lines. Bars show the means ± SE of Mp10 or MpOS-D1 expression levels in 3 independent experiments (sample of 5 aphids taken per experiment). Expression levels were normalised with the dsGFP control set at 1. Asterisks indicate significant differences to the dsGFP control (Student's t-probabilities calculated within GLM at $P < 0.05$).

I conducted aphid survival and fecundity assays on two of each of the plant lines that successfully silenced *Mp10* and *MpOS-D1*. Individual plants were seeded with 5 adult aphids, which produced nymphs over 2 days. After this time the adults were removed, and the nymphs counted to ensure there were 5 left on each plant. The nymphs were left to reach adulthood and produce nymphs over a period of 14 days. I conducted nymph counts at 11 and 14 days and measured survival at the end of the experiment, after 14 days. The results from four biological replicates were used to carry out statistical analysis and generate graphs (Figure 5.7). All biological replicates included dsGFP transgenic lines as negative controls and dsRack1 transgenic plants as positive controls. The dsGFP plants were used to assess if feeding from dsRNA in plants affects GPA. However, it has previously been shown that dsGFP does not affect aphid survival and fecundity of GPA compared to non-transgenic Col-0 plants (43). Plants expressing dsRack1 were included as positive controls, because silencing Rack1 in GPA is known to decrease fecundity of the aphid (43). Rack-1 is a scaffold protein that is involved in the regulation of cell proliferation, growth and movement in animals and does not have a known function in plant-insect interactions.

In aphids feeding on plants expressing the dsRNA, the expression levels of target genes were reduced (Figure 5.8). Importantly, *Mp10* expression levels in GPA were reduced on dsMp10 Col-0 and *bak1-5* lines, but not on the dsMpOS-D1 lines. Conversely, the dsMpOS-D1 lines knocked down *MpOS-D1* expression, but *MpOS-D1* expression was not affected on dsMp10 Arabidopsis lines, despite some shared similarity between the genes (Figure 5.8).

RNAi of Mp10, MpOS-D1 and Rack1 did not reduce aphid survival compared to the dsGFP control in both experiments (Figures 5.7b and 5.7d) confirming previous results for Rack1 RNAi aphids (43). However, GPA fecundity was reduced by about 20% upon RNAi of both Mp10 and Rack1 on Col-0 plants (Figure 5.7a and 5.7c), whereas no fecundity reduction was observed for MpOS-D1 RNAi aphids (Figure 5.7c). Interestingly, RNAi of Mp10 on *bak1-5* plants did not reduce GPA fecundity. This is in contrast to the Rack1 RNAi aphids on *bak1-5* plants which showed a reduced fecundity of about 20%, a result consistant in two independent experiments (compare Figure 5.7a and 5.7c). Mp10 knock-down therefore results in reduced aphid performance on Col-0 plants that have a functional BAK1-dependent PTI response, but not on *bak1-5* plants in which this response is not activated (147). This suggests that GPA Mp10 suppresses defence responses that are induced in a BAK1-dependent manner.

The RNAi experiments also provide evidence that aphid Mp10 is functional in the plant

host, because RNAi of Mp10 reduced aphid fecundity on Col-0 but not on *bak1-5* plants. In contrast, RNAi of Rack1 reduces aphid fecundity on both Col-0 and *bak1-5* plants, in agreement with Rack1 having important functions within the aphid unrelated to the plant. RNAi of MpOS-D1 did not affect aphid fecundity on Col-0 or *bak1-5* plants, indicating that this protein has different functions compared to Mp10. Finally, it was noticed that GPA fecundity did not improve on *bak1-5* compared to Col-0 RNAi lines (Figure 5.7), confirming previous results (340). This may be because aphid effectors, including Mp10, are highly efficient at suppressing PTI on wild type plants.

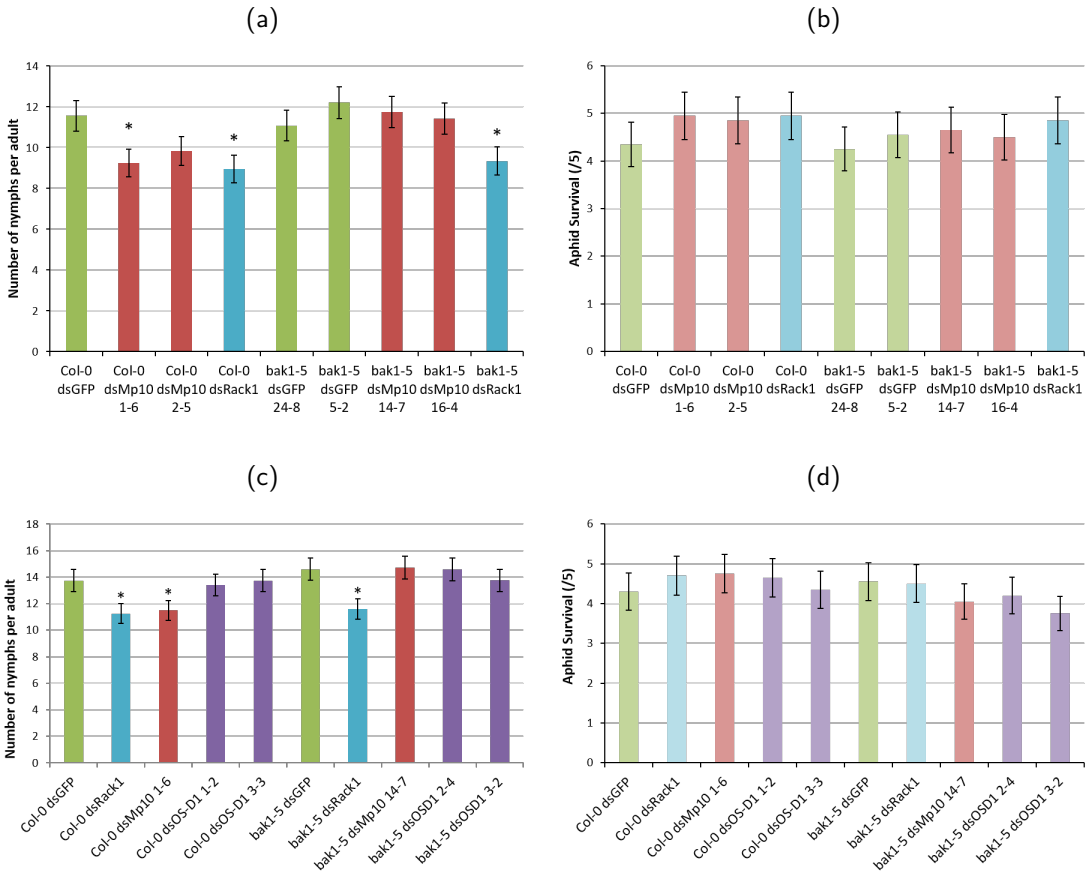
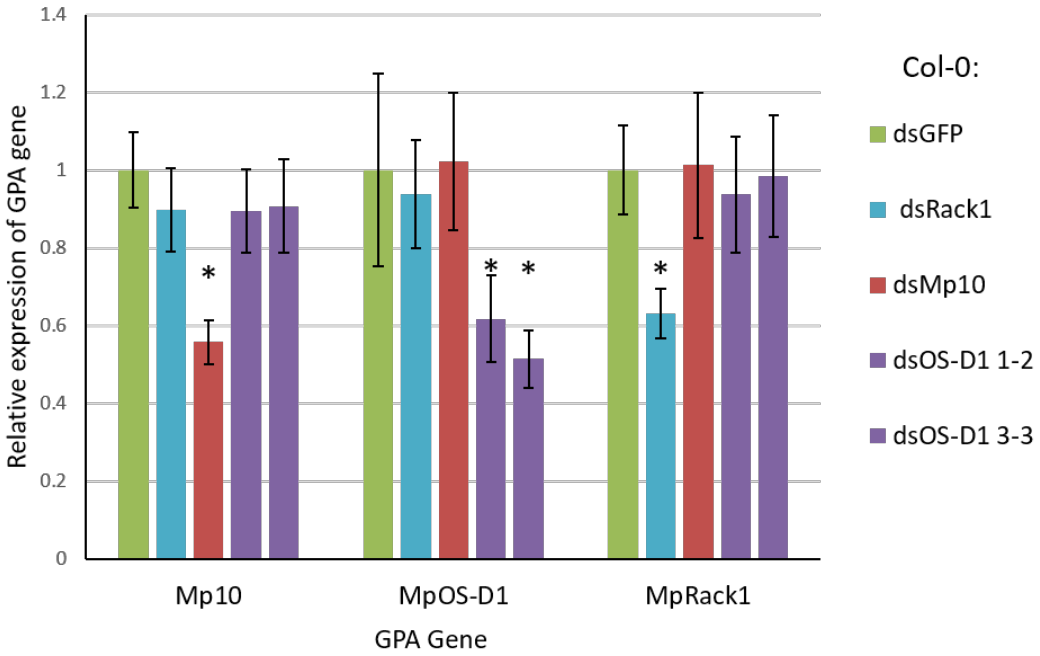
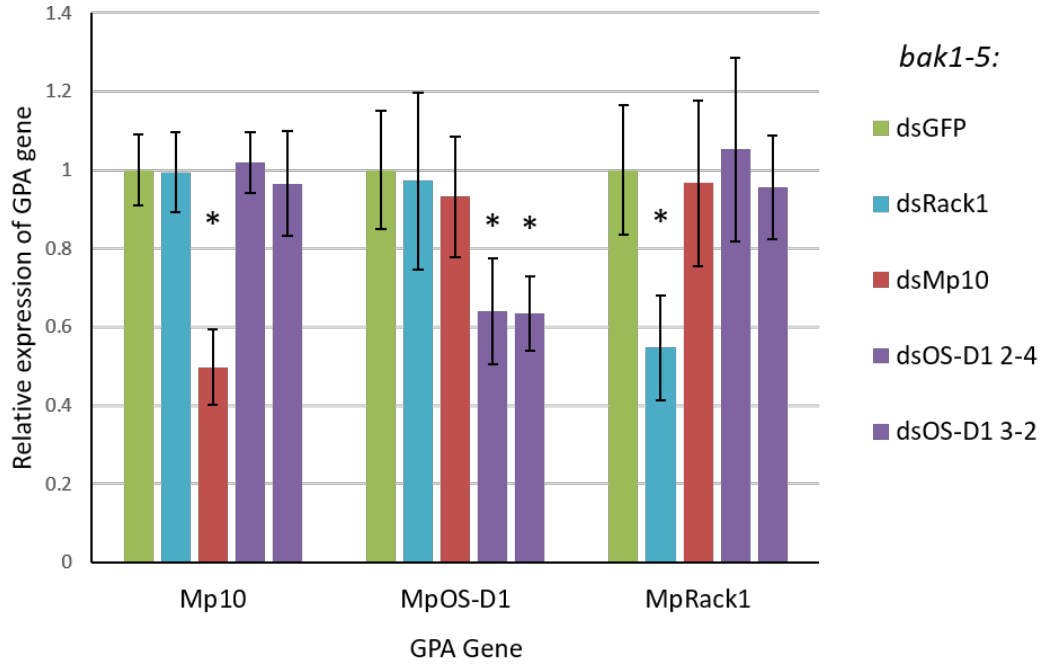


Figure 5.7: **Mp10 aids GPA colonization in a BAK1-dependent manner**. 5.7a Aphids reared on dsMp10 have reduced fecundity on wild type Col-0 plants, but not on *bak1-5* plants, whereas those reared on dsRack1 plants have reduced fecundity on both Col-0 and *bak1-5* plants. 5.7b Survival of aphids on the dsRNA Col-0 and *bak1-5* plants did not differ. 5.7c Aphids reared on dsMp10 have reduced fecundity on wild type Col-0 plants, but not on dsMpOS-D1 Col-0 and dsMp10 *bak1-5* plants, whereas those reared on dsRack1 plants have reduced fecundity on both Col-0 and *bak1-5* plants. 5.7d Survival of aphids on the dsRNA Col-0 and *bak1-5* plants did not differ.
In all graphs, bars represent the mean number of nymphs produced (5.7a, 5.7c) or number of nymphs alive (5.7b, 5.7d) at the end of the experiment $\pm$ SE in 4 independent experiments (n=5 per experiment). Asterisks indicate significant difference to control (Student's t-probabilities calculated within GLM at $P < 0.05$).



(a)



(b)

Figure 5.8: **The expression levels of Mp10, MpOS-D1 and MpRack1 were knocked down on dsMp10, dsMpOS-D1 and dsRack1 transgenic plants, respectively**. 5.8a Knock-down of aphid Mp10, MpOS-D1 or MpRack1 on dsRNA-expressing Col-0 plants. 5.8b Knock-down of aphid Mp10, MpOS-D1 or MpRack1 on dsRNA-expressing *bak1-5* plants.
Bars show the means ± SE of aphid Mp10, MpOS-D1 and MpRack1 expression levels in the different plant lines used. Results from 3 independent experiments (sample of 5 aphids taken per experiment). Expression levels were normalised against the dsGFP control set at 1. Asterisks indicate significant differences of aphid Mp10, MpOS-D1 or Rack1 expression levels of aphids reared on the dsRNA transgenic plants compared to those on the dsGFP plants (Student's t-probabilities calculated within GLM at $P < 0.05$).

5.2.5 Tryosine 40 and tryptophan 120 are required for Mp10 calcium and ROS suppression activity

Mp10 suppresses ROS and calcium bursts in the BAK1-dependent PTI pathway, and heterologous expression of Mp10 in plants results in a severe chlorosis response (46). It is possible that these phenotypes of Mp10 are related, as interference with certain components of the BAK1-dependent pathway could lead to chlorosis. To dissect this further, I decided to generate Mp10 mutants which lose calcium and ROS suppression activities.

A former PhD student in the Hogenhout lab, David Prince, had already generated Mp10 deletion mutants that showed different ROS suppression activities. He found that a truncation of the N-terminus past the tyrosine at position 40 [Tyr(40)] and a truncation at the C-terminus past the tryptophan at position 120 [Trp(120)] disrupted the Mp10 phenotype of flg22-triggered ROS suppression (316). I continued this work by generating single amino acid mutations at the tyrosine 40 and tryptophan 120 positions of Mp10. First, I swapped these residues for alanines, as alanine is a non-bulky, chemically inert amino acid that is unlikely to disrupt the secondary structure of the protein. The use of this amino acid for this function is common in molecular biology in alanine scanning (444). I also decided to exchange the equivalent residues between Mp10 and MpOS-D1 [Tyr(40) to phenylalanine and Trp(120) to Tyr in Mp10, with the opposite for MpOS-D1], to see if these residues are important in the co-option of a CSP as an effector. It would be interesting to see if I could make MpOS-D1 gain effector function by changing one or both of these residues (Figure 5.9a).

Once I had created both the single and double mutants for Mp10 and MpOS-D1, I cloned them into GFP-tagged vectors for expression in *N. benthamiana* via agrobacterium infiltration. I used leaf discs from *N. benthamiana* expressing the mutant forms alongside wild-type proteins and GFP alone in both calcium and ROS burst assays (Figure 5.9b). I found that mutating Tyr(40) and Trp(120) of Mp10 individually to either alanine or the equivalent MpOS-D1 residue (phenylalanine and tyrosine, respectively) had no effect on the ability of Mp10 to block the calcium burst in *N. benthamiana*, though changing Tyr(40) appeared to have some effect on Mp10 inhibition of the ROS burst. Mutating both residues together, either to alanine or MpOS-D1 equivalent residues (Mp10 Y40A W120A and Mp10 Y40F W120Y) led Mp10 to lose its ability to block both ROS and calcium bursts.

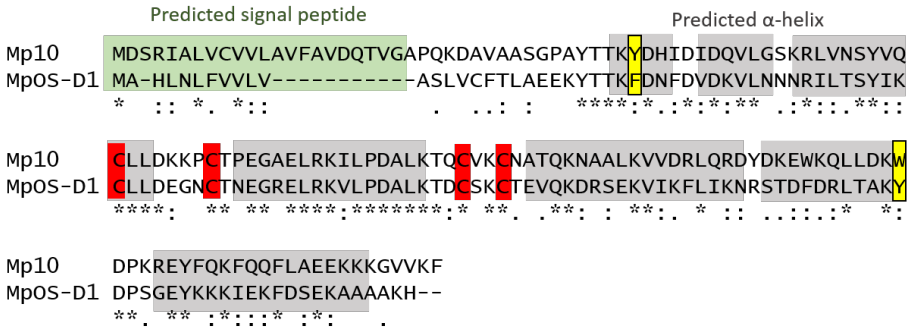
Interestingly, mutating both residues of MpOS-D1 to the equivalent residue in Mp10

(phenylalanine to tyrosine at position 28 and tyrosine to tryptophan at position 108) did not lead to gain of effector function. However, changing one of these residues at a time did; both MpOS-D1 F28Y and MpOS-D1 Y108W were able to block the flg22 induced ROS and calcium bursts. This highlights that Tyr(40) and Trp(120) play important roles in the function of Mp10 as an effector, as they can give effector activity to a similar protein, MpOS-D1.

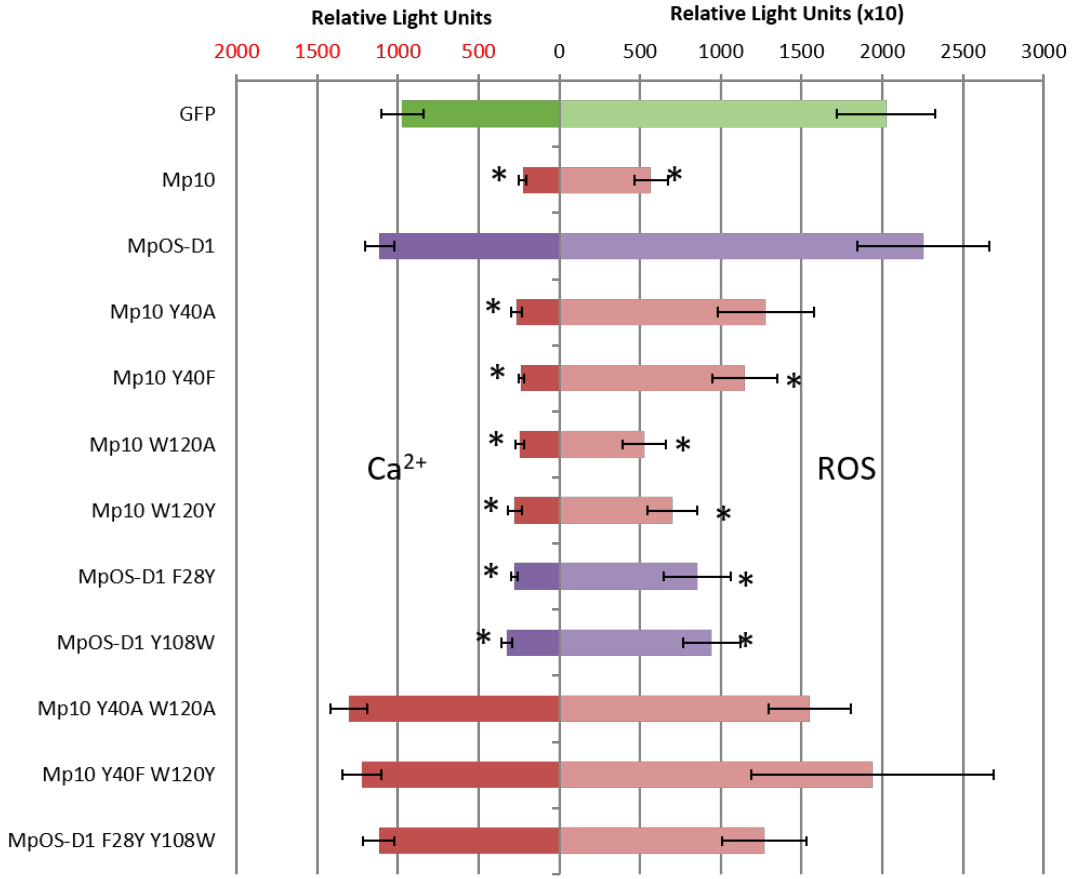
To understand why these single residue changes of Mp10 and MpOS-D1 can remove and confer effector activity, I took a more detailed look at Mp10 and MpOS-D1 protein structure. Mp10 and MpOS-D1 could both be modelled based on the existing structures of known CSPs, such as Schi-10 from the desert locust, *Schistocerca gregaria* (446). Modelling using the Phyre2 program (447) revealed that both proteins form tight alpha-helical structures held together by the disulphide bridges between the four cysteines, typical of CSPs. These alpha helices form a hollow binding pocket at the centre of the protein, which can be seen in cross-section (Figures 5.10 and 5.11). The alpha helices and hollow pocket of CSPs are involved in binding hydrophobic odorant molecules for shuttling to receptor proteins in the membranes of sensory neurones (433). In Mp10, Tyr(40) is located just outside of an alpha-helical region, near an entry site at the edge of the pocket. It is exposed to both the interior and exterior of the protein. Trp(120) is also exposed to both interior and exterior, seemingly located within a groove on Mp10's surface. It is on the edge of an alpha-helical region.

Comparing the predicted structures of WT Mp10 protein and the Mp10 Y40A W120A mutant, the largest noticeable difference is to the binding pocket in the centre of the protein. The introduction of the smaller alanine residues in the place of the bulkier tyrosine and tryptophan causes gaps in the protein, particularly noticeable near residue 40 (Figure 5.10). Tyr(40) and Trp(120) are therefore likely to have roles in maintaining the shape of the binding pocket, possibly in order for Mp10 to interact with a partner. However, when predicted structures of WT Mp10 protein and the Mp10 Y40F W120Y mutant, which also causes a loss of ROS and calcium blocking activity, were compared, no large difference in the size or shape of the binding pocket was observed. It is interesting that the switch from a tyrosine to a phenylalanine at position 40 can also, in conjunction with a change at 120, cause Mp10 to lose effector action. Tyrosine and phenylalanine are both hydrophobic amino acids, so substitution of one for the other may not affect the hydrophobic core by a large amount. However, tyrosine is more soluble (slightly less hydrophobic) that phenylalanine, which may affect interaction with a ligand in the core. From my modelling, Tyr(40) and Trp(120) could also alter the exterior structure of Mp10. Taken together, Tyr(40) and

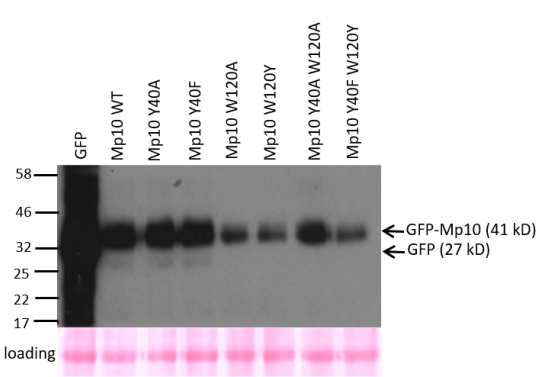
(a)


Predicted signal peptide
Predicted α-helix
Mp10 MDSRIALVCVVLAVFAVDQTVGAPQKDAVAASGPAYTTKYDHIDIDQVLGSKRLVNSYVQ
MpOS-D1 MA-HLNLFVVLV----------ASLVCFTLAEEKYTTKFDNFDVDKVLNNNRILTSYIK
Mp10 CLLDKKPCTPEGAELRKILPDALKTQCVKCNATQKNAALKVVDRLQRDYDKEWKQLLDKW
MpOS-D1 CLLDEGNCTNEGRELRKVLPDALKTDCSKCTEVQKDRSEKVIKFLIKNRSTDFDRLTAKY
Mp10 DPKREYFQKFQQFLAEEKKKGVVKF
MpOS-D1 DPSGEYKKKIEKFDSEKAAAAKH--

(b)


Relative Light Units
Relative Light Units (x10)
2000 1500 1000 500 0 500 1000 1500 2000 2500 3000
GFP
Mp10
MpOS-D1
Mp10 Y40A
Mp10 Y40F
Mp10 W120A
Mp10 W120Y
MpOS-D1 F28Y
MpOS-D1 Y108W
Mp10 Y40A W120A
Mp10 Y40F W120Y
MpOS-D1 F28Y Y108W
Ca2+
ROS

(c)


GFP
Mp10 WT
Mp10 Y40A
Mp10 Y40F
Mp10 W120A
Mp10 W120Y
Mp10 Y40A W120A
Mp10 Y40F W120Y
58
46
32
25
22
17
loading
GFP-Mp10 (41 kD)
GFP (27 kD)

(d)

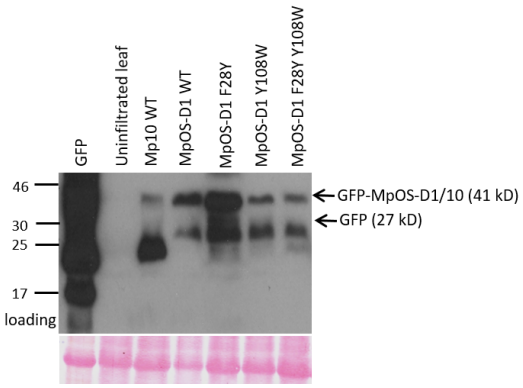

GFP
Uninfiltrated leaf
Mp10 WT
MpOS-D1 WT
MpOS-D1 F28Y
MpOS-D1 Y108W
MpOS-D1 F28Y Y108W
46
30
25
17
loading
GFP-MpOS-D1/10 (41 kD)
GFP (27 kD)

Figure 5.9 *(previous page)*: **Mp10 Tyrosine 40 and Tryptophan 120 are required for the ROS and calcium suppression activities of the effector**. 5.9a Alignment of Mp10 and MpOS-D1, showing signal peptide (in green) and areas predicted to form alpha-helices (in grey). The four cysteine residues conserved among CSPs are highlighted in red, and the Tyrosine (Y) 40 and Tryptophan (W) 120 residues of Mp10 and equivalent Phenylalanine (F) 28 and Tyrosine (YW) 108 of MpOS-D1 are highlighted in yellow. The alignment was created using Clustal Omega (445). 5.9b Y40 and W120 of Mp10 are required for Mp10 suppression of the ROS and calcium bursts. ROS bursts were measured over 60 minutes in leaves of wild type (ROS) and SLJR15 (calcium) *N. benthamiana* plants transiently producing GFP, GFP-Mp10 and GFP-MpOS-D1. Graphs show mean $\pm$ SE of 4 independent experiments (n=8 per experiment). Asterisks indicate significant differences of the GFP-Mp10 and GFP-MpOS-D1 (mutant) treatments compared to that of GFP (Student's t-probability calculated within GLM at $P < 0.05$). 5.9c Proteins corresponding to the sizes of GFP-Mp10 and GFP-fused Mp10 derivatives were detected in *N. benthamiana* leaves used for the ROS and calcium assays in 5.9b. Expression in two 10mm diameter leaf discs harvested 3 DPI was checked. Membrane imaged using 1 minute exposure. 5.9d Proteins corresponding to the sizes of GFP-MpOS-D1 and GFP-fused MpOS-D1 derivatives were detected in *N. benthamiana* leaves used for the ROS and calcium assays in 5.9b. Expression in two 10mm diameter leaf discs harvested 3 DPI was checked. Membrane imaged using 1 minute exposure.

Trp(120) could contribute to Mp10 effector function in different ways. First, they may maintain the hydrophobic core that can bind hydrophobic small molecules. Secondly, they may affect exterior interactions of Mp10 with other proteins.

Similarly to Tyr(40) and Trp(120) of Mp10, residues Phe(28) and Tyr(108) of MpOS-D1 both also form part of the hydrophobic binding pocket and are exposed to the exterior of the MpOS-D1 protein (Figure 5.11). The F28Y and Y108W mutations appear to make no noticeable physical alteration to the binding pocket. The altered groups in MpOS-D1 both point outwards, to the protein surface, rather than inwards to the binding pocket. Because both MpOS-D1 F28Y and MpOS-D1 Y108W were able to block the flg22-induced ROS and calcium bursts, the modelling predictions suggest that changes to the exterior of MpOS-D1, rather than changes in the hydrophobic binding pocket may allow this protein to block ROS and calcium bursts, possibly by binding to plant targets.

I inspected the surface of Mp10 and MpOS-D1, and their mutants, to see if there were obvious differences between those that showed effector action and those that did not. I looked at the hydrophobicity and electrostatic charges of the protein exteriors (Figure 5.12) and could not find any clear correlations between these physical attributes of the proteins and ROS and calcium burst suppression activities. Therefore, this protein structure modelling exercise did not result in the identification of an obvious binding site of Mp10 or MpOS-D1 to plant targets. One possibility is that Mp10 may need two partners for its ROS and calcium suppression function. One partner (perhaps a hydrophobic lipid-like molecule) could dock inside the hydrophobic pocket that may then induce a conformational change, as has been shown to occur in CSPs (448). The other partner may bind to the exterior of Mp10 before/after this conformational change. Thus, Mp10 function may be complex.

5.2.6 Chlorosis induction by Mp10 is an independent process to calcium/ROS burst suppression activity

To investigate whether Mp10 mutations that block calcium and ROS suppression activities can induce chlorosis, I cloned Mp10 Y40A W120A and Mp10 Y40F W120Y into a *Potato virus X*(PVX)-based expression vector and introduced these vectors into *N. benthamiana* plants using agroinfiltration of one leaf. After 10 days, systemic PVX symptoms were visible and at this time plants inoculated with the constructs containing wild type Mp10 started to show chlorosis throughout the plant, in contrast to the empty vector control construct (see Figure 5.13a). Curiously, the chlorosis occurred for both wild type Mp10 and the two Mp10 double mutants (see Figure 5.13a), which did not block calcium and ROS bursts (Figure 5.9b). Thus, the chlorosis response occurs independently of the calcium/ROS burst suppression activities of Mp10.

The Mp10-mediated chlorosis response was also visible on single infiltrated leaves with the GFP-tagged agrobacterium vectors used for the calcium and ROS burst assays at 2 to 3 days after the calcium and ROS burst measurements were taken (Figure 5.13b). In order to quantify this, I used a SPADmeter, which measures the chlorophyll content, or "greenness" of leaf tissues by comparing leaf absorbance in the red and infrared wavelength ranges, giving an indexed chlorophyll content reading. I used the SPADmeter to look at chlorosis in Mp10, MpOS-D1 and Mp10 and MpOS-D1 double mutant expressing tissues and compared these to leaves treated with GFP controls at 3, 5 and 7 days post infiltration. I found that all constructs induced chlorosis in *N. benthamiana*. This confirms that chlorosis induction occurs independently of Mp10 calcium and ROS suppression activities. Mp10 and MpOS-D1 may both activate other processes in plants or may induce ETI-like processes leading to chlorosis.

5.2.7 The chlorosis response to Mp10 in *Nicotiana benthamiana* occurs independently of salicylic acid accumulation

Previous data showed that SGT1 is required for the Mp10-induced chlorosis response, suggesting that Mp10 may induce effector-triggered immunity (ETI) caused by R-protein activation (46). The phytohormone salicylic acid (SA) plays a role in the induction of the cell death response associated with ETI and is required for the function of many R-genes (450). To investigate whether SA is involved in the chlorosis response to Mp10, I introduced Agrobacterium-based and PVX-based constructs carrying (GFP-)Mp10 and MpOS-D1 in

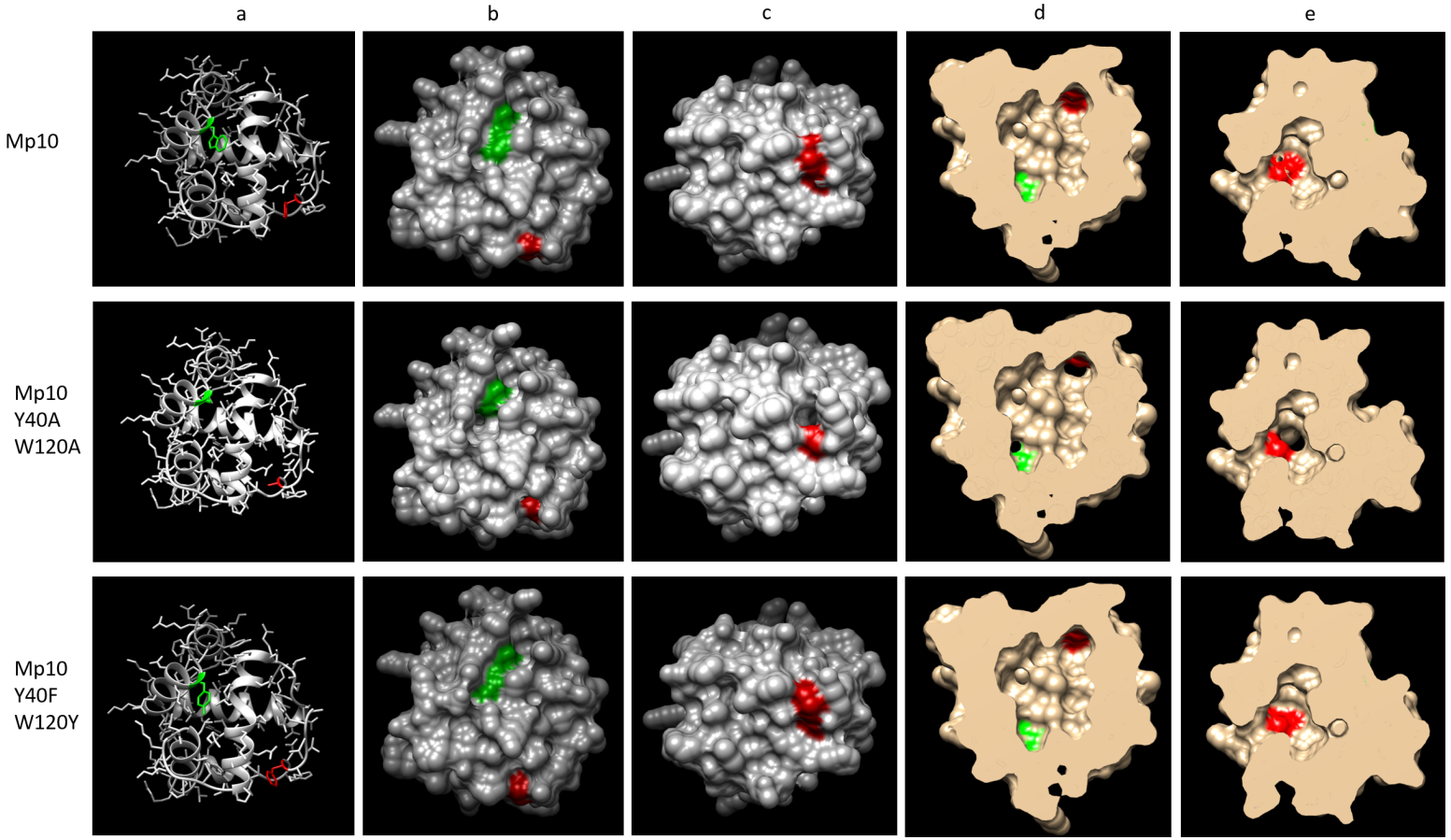


Figure 5.10: **Mp10 Tyrosine 40 and Tryptophan 120 are present in both the interior binding pocket and the exterior of the protein**. Residue 40 is shown in red and residue 140 in green. a) Model without surface showing stick atoms. b) Surface of model showing external location of residues 40 and 120 c) Surface of model showing external location of residue 40 d) Cross-section of model showing internal location of residues 40 and 120 inside the binding pocket e) Cross-section of model showing internal location of residue 40 near the mouth of the binding pocket Proteins modelled using Phyre2 (Protein Homology/analogY Recognition Engine V 2.0) with 100% confidence and 40-43% identity (447). Data exported and Molecular graphics and analyses were performed with the UCSF Chimera package (449).

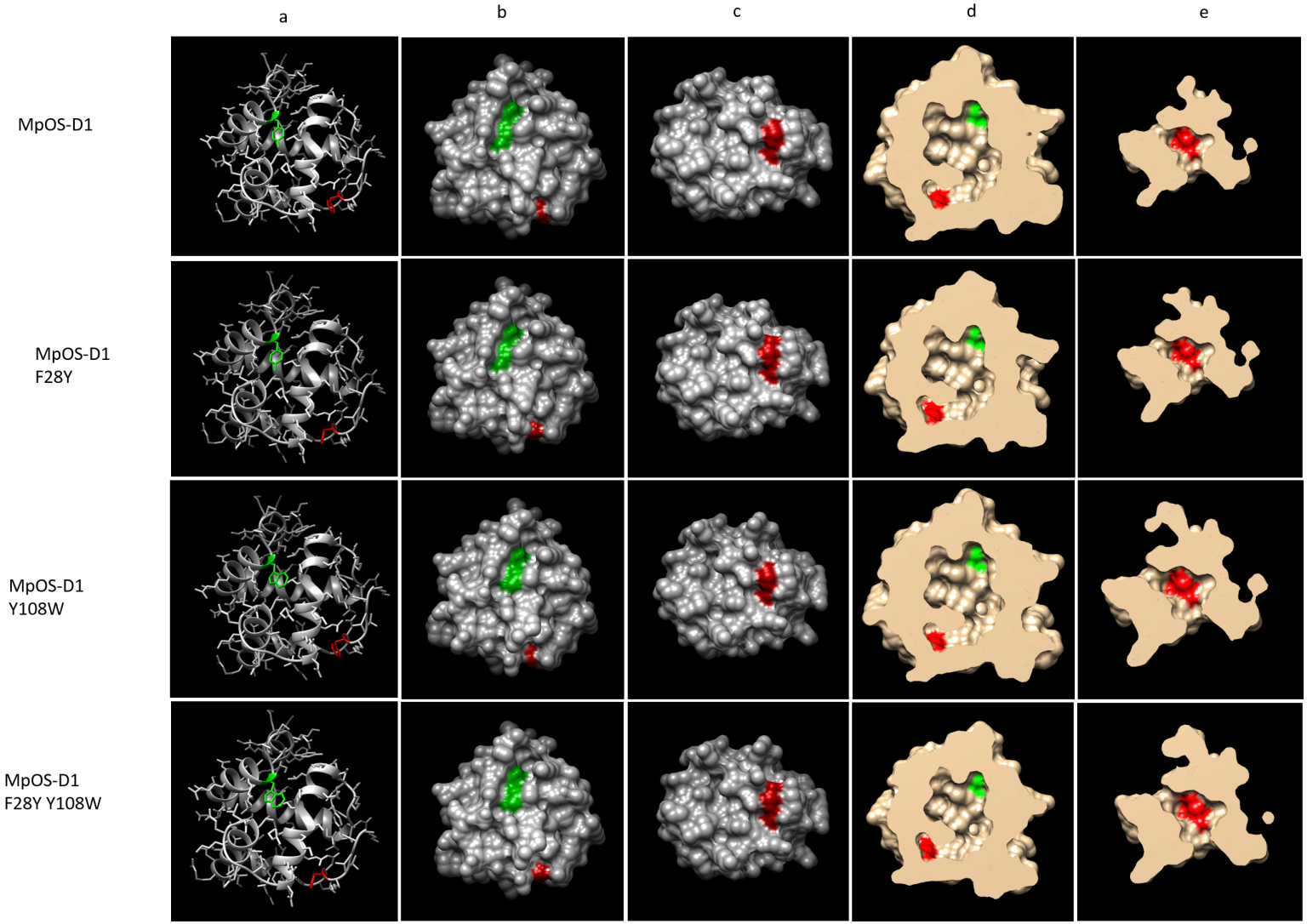


Figure 5.11: **Mutating MpOS-D1 Phenylalanine 28 and Tyrosine 108 residues to the Tyr and Trp found in Mp10 does not change the inner binding pocket**. Residue 28 is shown in red and residue 108 in green. a) Model without surface showing stick atoms. b) Surface of model showing external location of residues 28 and 108 c) Surface of model showing external location of residue 28 d) Cross-section of model showing internal location of residues 28 and 108 e) Cross-section of model showing internal location of residue 28 Proteins modelled using Phyre2 (Protein Homology/analogY Recognition Engine V 2.0) with 100% confidence and 52-54% identity (447). Data exported and Molecular graphics and analyses were performed with the UCSF Chimera package (449).

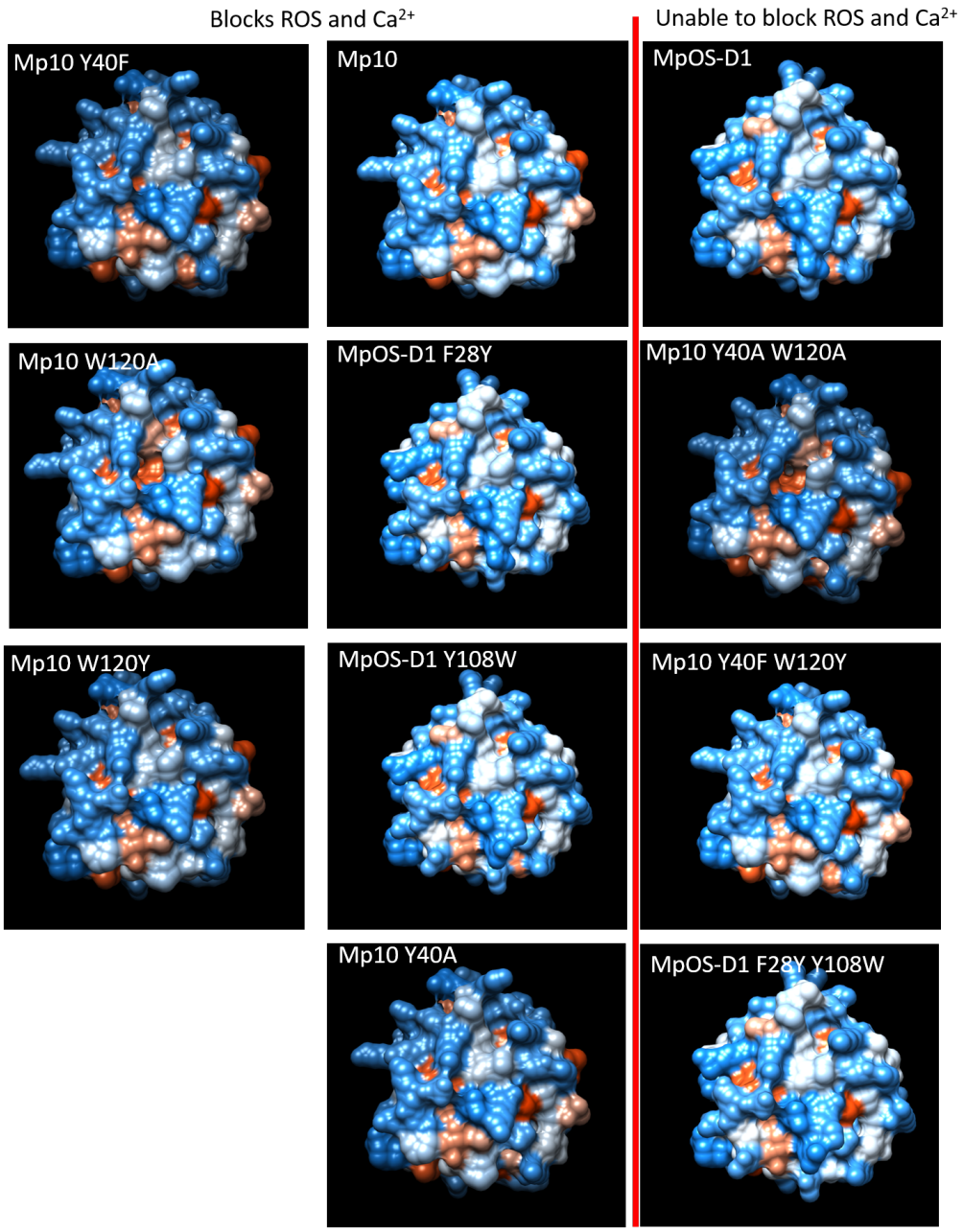


Figure 5.12: **Altering Mp10 Tyrosine (40) and Tryptophan (120), and MpOS-D1 Phenylalanine (28) and Tyrosine (108) does not cause obvious hydrophobicity changes to the surface of the proteins**. Hydrophobicity surface models of Mp10, MpOS-D1 and mutants. From blue for the most hydrophilic to orange red for the most hydrophobic. White is neutral. Proteins modelled using Phyre2 (Protein Homology/analogY Recognition Engine V 2.0) with 100% confidence and 52-54% identity (447). Data exported and Molecular graphics and analyses were performed with the UCSF Chimera package (449).

(a)

(b)

1. Mp10
2. MpOS-D1
3. Mp10 Y40A W120A
4. Mp10 Y40F W120Y
5. MpOS-D1 F28Y Y108W
6. GFP

(c)

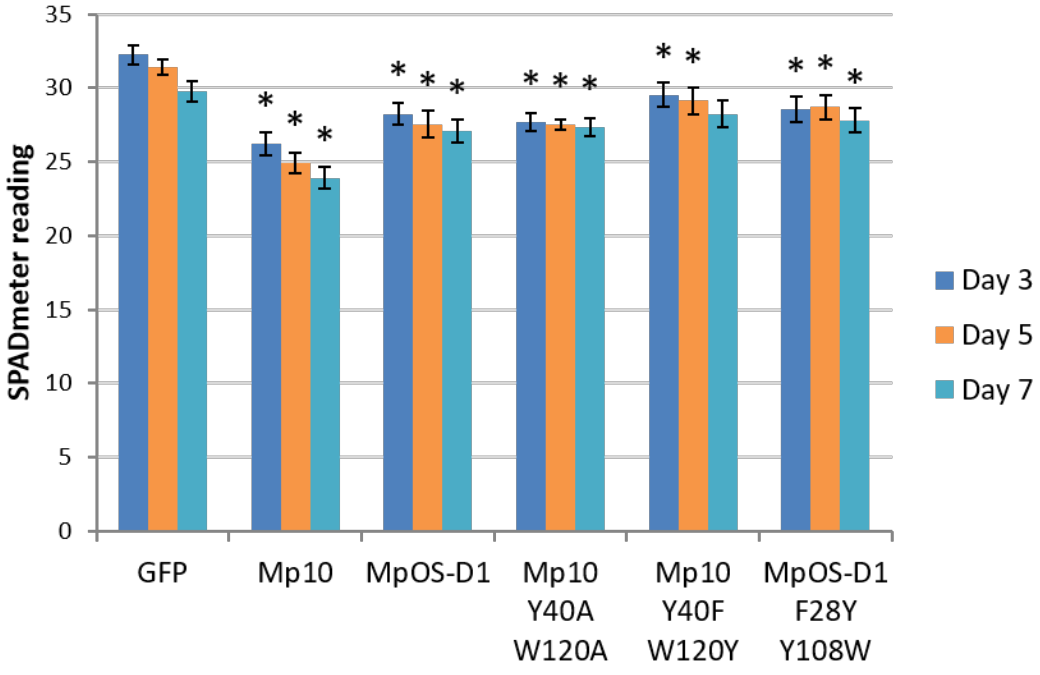


Figure 5.13 *(previous page)*: **Chlorosis induction by Mp10 is independent of calcium and ROS burst suppression activity**. 5.13a Mp10 and Mp10 double mutants (Y40A W120A and Y40F W120Y) cause chlorosis when expressed systemically via PVX in *N. benthamiana*. Plants 2.5 weeks old at time of infiltration and picture taken two weeks after infiltration. From left to right: GFP, Mp10, Mp10 Y40A W120A and Mp10 Y40F W120Y. Picture representative of 3 independent experiments. 5.13b Transient production of Mp10, MpOS-D1 and derivatives leads to chlorosis in *N. benthamiana* infiltrated areas. Photograph taken 5 DPI. 5.13c Mp10, OSD1 and double mutants all cause chlorosis when transiently produced in *N. benthamiana* via agrobacterium infiltration. Graph shows mean $\pm$ SE of 5 independent experiments (n=4 per experiment). Asterisk indicates significant difference to GFP control at that time point (Student's t probability calculated within GLM at P $<$0.05).

wild type *N. benthamiana* and *N. benthamiana* lines expressing the NahG transgene. The *NahG* gene encodes a salicylate hydroxylase that catalyses the conversion of SA to catechol, and so prevents SA accumulation in the plant (451).

I found that systemic spread of Mp10 via PVX-based expression in NahG plants also led to chlorosis at a similar level to that of wild type *N. benthamiana* (Figure 5.14a). Moreover, both GFP-Mp10 and GFP-MpOS-D1 caused chlorosis when expressed via Agrobacterium-mediated expression in leaves of NahG plants (Figure 5.14b). The accumulation of SA is therefore not required for the chlorosis responses induced by Mp10 and MpOS-D1 in *N. benthamiana*, indicating that Mp10 and MpOS-D1 may not trigger ETI.

The chlorosis response occurs upon heterologous expression of Mp10 and MpOS-D1 and derivatives in plants leading to high abundance of these proteins in plant cells. In contrast, aphids introduce only low amounts of Mp10 into plant cells when feeding (Figure 5.3). Nevertheless, chlorosis is a common symptom of aphid-exposed plants and occurs in high aphid infestations (7). The Mp10-induced chlorosis may therefore be biologically relevant outside of its action as an effector.

(a)

(b)

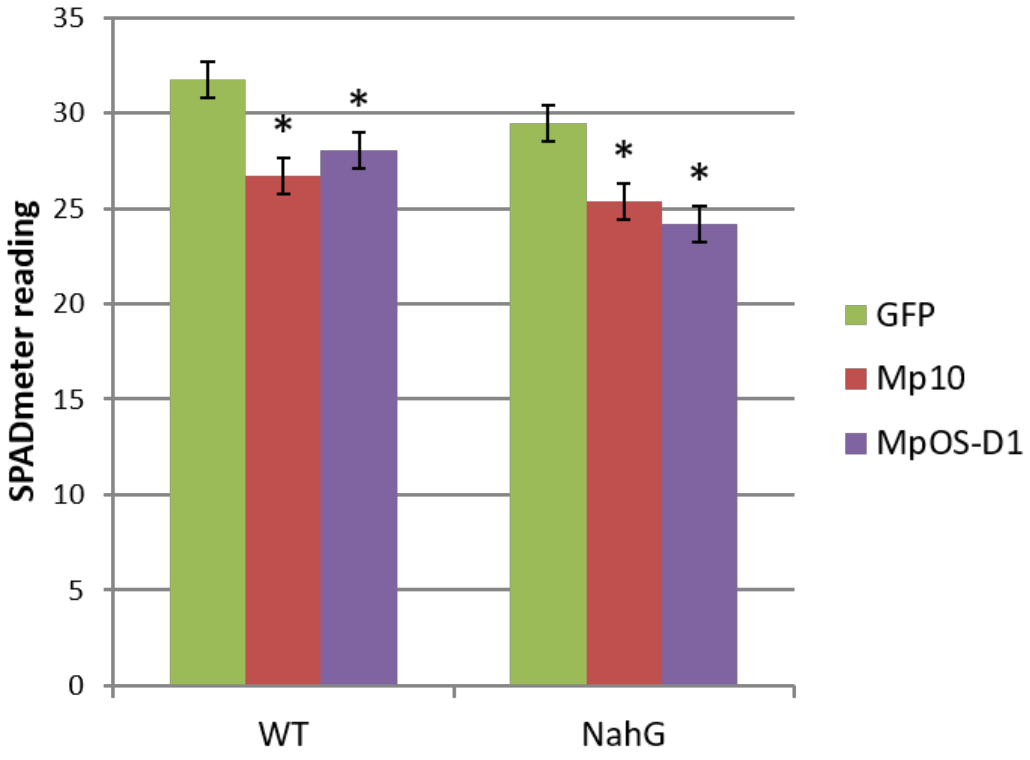


Figure 5.14: **The chlorosis response in *N. benthamiana* occurs independently of salicylic acid accumulation**. 5.14a Mp10 causes chlorosis when expressed systemically via PVX in NahG *N. benthamiana*. GFP-PVX on the left, Mp10-PVX on the right. Plants 3 weeks old at infiltration, picture taken two weeks after infiltration. Pictures are representative of 3 independent experiments. 5.14b Mp10 and MpOS-D1 cause chlorosis when transiently produced via agrobacterium infiltration in NahG *N. benthamiana*. SPADmeter readings taken at 7 DPI. Graph shows mean $\pm$ SE of 3 independent experiments (n=4 per experiment). Asterisk indicates significant difference to GFP control (Student's t probability calculated within GLM at P $<$0.05).

5.3 Discussion

Though insect effectors have been conjectured by many to exist, and there is some evidence for their use in plant-insect interactions (256; 259; 24), the molecular function of an insect effector protein has yet to be reported. The results described in this chapter demonstrate that Mp10 is an effector with a specific role in suppression of early plant defense responses. This can be seen in Mp10 suppression of the calcium and ROS bursts that form a part of PTI, which a related CSP (MpOS-D1) does not do. Silencing Mp10 expression in aphids impairs aphid performance on Arabidopsis. The reduction in fecundity seen in GPA on plants expressing dsMp10 is dependent upon BAK1, a major component of PTI. Consistent with a role in the plant, Mp10 is found in the cytoplasm of plant cells near aphid feeding sites.

Mp10 was initially found in a screen for GPA candidate effector proteins where it was identified as suppressing the oxidative burst induced by flg22 in *N. benthamiana* (46). I confirmed this finding, and also demonstrated that Mp10 suppresses the calcium burst to flg22. The finding that Mp10 suppresses both the calcium and ROS bursts, which are early components of PTI (328), suggests that Mp10 acts upstream of these responses. Mp10 can therefore be predicted to interact with components that are directly downstream of, or involved in, recognition of elicitors. In flg22 perception, binding of flg22 to the LRR-RLK FLS2 leads to association with BAK1, which initiates calcium and ROS bursts (146). Mp10 may therefore disrupt FLS2-BAK1 association. Mp10 is unlikely to act upon FLS2 directly as Mp10 also suppresses the ROS burst induced by aphid elicitors, which does not require FLS2 or other LRR-RLKs known to require BAK1 for signaling (340). Therefore, I hypothesise that Mp10 acts on BAK1 or BAK1-associated processes to suppress the calcium and ROS bursts that form a part of PTI, causing effector-triggered susceptibility, as it is BAK1 that is a shared component of flg22 and aphid elicitor perception (340; 143).

Targeting early components of PTI would be beneficial for the aphid as it would act to stop the maximum amount of immune responses, before the signaling pathways branch off into different ones, such as further ROS production and MAP kinase signaling (185). Preventing early defence responses is a strategy employed by microbial pathogens as well, for example effector AvrPtoB of *Pseudomonas syringae* DC3000 targets LRR-RLK receptors for degradation (261; 262; 264). AvrPtoB therefore suppresses calcium and ROS bursts upon flg22 perception, similarly to Mp10. AvrPtoB contains a kinase-interacting domain and an E3 ligase domain which are required for receptor binding and degradation (452; 453).

Mp10 contains no such domains, so it is unlikely to act in a similar manner to AvrPtoB. BAK1 is known to be a target of other bacterial effectors, for example HopF2 from *P. syringae* directly interacts with BAK1 to suppress PTI (261; 263). However, no effectors that have been found to interfere with BAK1-mediated PTI signaling resemble Mp10 in size and structure, so there are no clues as to how Mp10 is acting.

For Mp10 to act as an effector, it must be secreted into the plant when feeding. Previous investigations have found that Mp10 is expressed in the salivary gland of GPA (46), though homologs of Mp10 in other aphid species have also been reported to have expression in legs and antennae, as well as heads (441; 454; 455). These experiments were only looking at gene transcript levels, which might not necessarily correlate with areas where Mp10 protein is present. The development of an antibody specific for Mp10 allowed us to probe its protein localisation further. We were unable to detect the presence of Mp10 via Western blot in aphid saliva or plant tissue that had been exposed to aphid feeding. However, it was detected in whole aphids and aphid heads. It is possible that our approaches for Mp10 detection in plants and aphid saliva were not sensitive enough to detect small amounts of Mp10. The pea aphid (*A. psium*) effector C002 has been detected in plants after aphid feeding by experiments involving leaves exposed to 500 aphids (41). This is a much greater number of aphids than we used in our experiments for detecting Mp10. Perhaps if we scaled up aphid numbers to the same amount, Mp10 would also be detectable in plant samples by Western blot.

We did however detect Mp10 presence in Arabidopsis by immunogold labeling using specific antibodies for Mp10. Regions of signal for Mp10 were found in the cytoplasm of plant cells adjacent to plant tissues that show evidence of aphid feeding due to the presence of aphid stylet tracks. Some labeling was detected in plant tissues that had not been exposed to aphids, but this labeling was less dense than that seen in the aphid-exposed plant samples. The next step is to repeat this experiment for several aphid feeding sites and controls and conduct statistical analyses of the density of labeling. Despite the discovery of several potential effectors from aphids, such as Me10 and Me23 from the potato aphid, *Macrosiphum euphorbiae* and Mp1, Mp2 and C002 in GPA (456; 44), there is no direct evidence of these effectors being present in specific compartments of plant cells. The immunogold labelling results for detection of Mp10 in plant cells is therefore highly novel, and supports the role of Mp10 as an effector in the plant.

The reduction in GPA fecundity after knock-down of Mp10 also suggests that the effec-

tor has a function in the plant. Consistent with previous results published by the Hogenhout lab (43; 44), my RNAi experiments showed reduced expression of target genes of maximally 40-60% and about 20% reduction of fecundity. A larger reduction in aphid fecundity may be achieved over more aphid generations on dsMp10 plants. A 40-60% reduction in fecundity has been obtained upon rearing aphid populations on dsMp2 and dsMpC002 plants over 4 weeks (about 4 aphid generations, double the length of my fecundity experiments) (45). GPA had increased fecundity when reared on *A. thaliana* transgenic lines that express Mp2 and MpC002 (44). Conducting a similar experiment with Mp10 is challenging, because this effector also induces chlorosis that could affect aphid performance. In fact, transient expression of Mp10 in *N. benthamiana* leaf discs leads to decreased fecundity of the GPA reared on them, despite the ability of Mp10 to block PTI signaling (46). Obtaining transgenic Mp10 lines has not been successful, perhaps because Mp10 present at high levels is lethal to *A. thaliana*. Homozygous transgenic *A. thaliana* lines that express Mp10 under an inducible promoter have become available and initial studies show that some of these lines become chlorotic upon induction of Mp10. However it will be challenging to design an experimental set up in which the effect of expressing Mp10 *in planta* on aphid suppression of plant defense can be separated from the deleterious effects of Mp10 on the plant. Mp10 (or MpOS-D1) mutants that do not induce chlorosis have not yet been identified, unlike mutants that can no longer block the calcium and ROS bursts.

My comparative structure analysis highlighted two particular residues that are required for Mp10 calcium and ROS burst suppression: the tyrosine and tryptophan residues at position 40 and 120 respectively. Comparing the structure of these mutants of Mp10 with the wild type protein, I found that these residues could cause changes to both the interior pocket of the CSP and the exterior protein surface. The mutation of tyrosine to phenylalanine at position 120 and subsequent loss of effector activity suggests phosphorylation may have a role in Mp10 ROS and calcium burst suppression. This is as the only difference between the two amino acids is the presence of a hydroxyl group in tyrosine which can be phosphorylated by protein kinases. The location of the residues important for Mp10 function suggests that Mp10 action may be complex, possibly involving two plant components. For instance, a hydrophobic plant molecule that docks into the core of Mp10 and a plant protein that interacts with Mp10s exterior. CSPs are known to undergo conformational changes upon the docking of hydrophobic components (448), so Mp10 action may also involve structural changes in which one conformation interacts with plant components and another does not.

Hydrophobic molecules that play a role in plant immunity and could potentially bind to the pocket of Mp10 include PAMPs and DAMPs (457). Mp10 may act by binding to aphid HAMPs or DAMPs produced upon feeding to prevent aphid perception, in a similar manner to the fungal effector Ecp6, which binds chitin oligosaccharide PAMPs released from the cell walls of invading hyphae to prevent detection (458). However, specific PAMP-binding would not explain the Mp10-mediated suppression of the calcium and ROS bursts induced by flg22. Mp10 must have a target common to both flg22- and aphid-triggered PTI pathways. A set of hydrophobic molecules downstream of perception that Mp10 could interact with may be oxylipins; oxidised fatty acids which form an important class of signaling molecule in plant stress responses and innate immunity (459; 460). The oxylipin pathway has been found to be induced by insect feeding, including that of GPA (461; 190; 462), and plant responses involving oxylipins have been found to both deter (463) and facilitate (461) aphid colonisation of plants. Oxylipins have also been identified to prime plants for enhanced resistance against pathogen attack (464). LOX1, a lipoxygenase, and the oxylipins it produces are required for stomatal closure in response to both bacteria and flg22 downstream of the MAP kinases MPK3 and MPK6 (465). Oxylipins can therefore be seen as immune components shared in plant responses to both insects and pathogens, so could be possible targets of Mp10. However, oxylipins do not appear to be involved at the early stages in immune response. Action in priming and below MPK3 and 6 places them downstream of ROS and calcium bursts that form the very early components of the immune response that Mp10 can block. Whether Mp10 binds to hydrophobic molecules in plants has yet to be seen.

I found that mutations in Mp10 that prevented the inhibition of calcium and ROS bursts to flg22 still induced a chlorosis response in *N. benthamiana* when expressed systemically via PVX, or in smaller areas by agroinfiltration. This suggests that it is not the calcium and ROS burst blocking activity of Mp10 that leads to chlorosis. The plant may detect Mp10 itself or other activities mediated by Mp10. Chlorosis is a common symptom of aphid infestation, though its adaptive significance and whether it benefits aphid or host is uncertain (7). For greenbugs and Russian wheat aphids, inducing chlorosis and cell death has been associated with aphid success. The chlorosis is linked to changes induced in the plants metabolism by the aphid to manipulate the plants nutritional quality (466; 467). On the other hand, premature leaf senescence mediated by PAD4 is associated with defense against GPA in Arabidopsis, so GPA-induced chlorosis plays a role in basal resistance to the aphid (372).The chlorosis response to aphids, and its heightened form as localised cell death, is similar to the hypersensitive response seen in pathogen resistance, which is in-

duced as a part of effector-triggered immunity (ETI) (468). The induction of chlorosis by Mp10 may therefore be seen as an ETI response. This is in agreement with the finding that the Mp10-induced chlorosis response is dependent on SGT1, a ubiquitin-ligase associated protein that is required for ETI (46; 426). To induce ETI, effectors are recognised in the plant by R-genes. There may therefore be an R-gene in *N. benthamiana* that recognises Mp10.

The phytohormone salicylic acid (SA) is also an important signaling component of ETI; being required for the function of many R-genes, the hypersensitive response (HR) and systemic acquired resistance (SAR) (450; 469; 470). I therefore investigated the chlorosis response in NahG *N. benthamiana* plants, which do not show SA accumulation upon pathogen challenge (451). Surprisingly, the chlorosis response still occurred, showing that despite a requirement for SGT1, the chlorosis response is SA independent. This does not mean that resistance to aphids in *N. benthamiana* is totally SA independent, for example resistance to late blight in *N. benthamiana* requires both SGT1 and SA, but SA is not required for HR (451). In tomato, both SA and SGT1 have been found to be required for Mi-1 mediated resistance to potato aphids (471; 285), so both these components of immunity are implicated in R-protein based immunity to aphids. SGT1 also regulates plant resistance to *Manduca sexta*, but in this case it is required for herbivory induced jasmonic acid (JA) accumulation and negatively regulates SA levels (427). Similarly, despite the involvement of PAD4 in SA signaling, the participation of PAD4 in plant defense against GPA does not appear to involve its SA signaling role (372). The requirements for both SGT1 and SA may therefore be different in plant-herbivore interactions than those in plant-pathogen interactions, as well as different between separate herbivores, with different hormones, such as JA, playing a larger role in defense.

Our investigations showed that there are 10 full CSPs encoded within the GPA genome; the same number as that found for the pea aphid (472). The cotton aphid, *Aphis gossypii* was also found to have a similar number, with 9 CSPs, though this is based on transcriptomic rather than genomic data, and the cotton aphid may actually have 10 CSPs (455). David Prince found that Mp10/CSP4 homologs from pea aphid and the cotton aphid could also block the flg22-induced ROS burst in *N. benthamiana* (316), although these aphids do not colonize this plant species. Suppressing the BAK1-dependent PTI-like immune response alone is therefore not sufficient to allow aphid colonisation. Other effectors may confer host specificity, including MpC002, Mp1 and Mp2, which promote GPA colonization upon heterologous production in *A. thaliana*, whereas the pea aphid homologs of these

effectors do not (44).

CSPs are also present in other insect species, with 4 CSPs in *Drosophila melanogaster*, 6 CSPs in the honeybee *Apis mellifera* and 19 CSPs in both the silk worm, *Bombyx mori* and the red flour beetle *Tribolium castaneum* (473). Interestingly, CSPs are one of the most abundant proteins in the saliva produced by mandibular glands of the caterpillar *Vanessa gonerilla*, with possible functions in host plant recognition, detection of microorganisms and communication with conspecifics (474). Moreover, silencing of odorant binding proteins (OBPs), which are related to CSPs, in the mosquito *Aedes aegypti* compromised blood feeding (475). These studies support our finding of a CSP, Mp10, having adapted to have a fundamental role in aphid interactions with plant hosts. Further investigation will shed light into exactly how the CSP fulfils this function.

Chapter 6

Mp10 targets AMSH2 to interfere with plant immunity

Contributors: Claire L. Drurey, Christine Wilson, Carlos Caceres, Friederike Bernsdorff, Sam Mugford and Saskia A. Hogenhout

6.1 Introduction

In the previous chapter I investigated Mp10, an effector produced by the green peach aphid (GPA). I found that Mp10 is introduced into plants during aphid feeding, contributes to successful GPA colonisation of Arabidopsis and inhibits plant PAMP-triggered immune (PTI) responses. On the other hand, Mp10 also triggers chlorosis, which requires plant proteins involved in effector-triggered immunity (ETI), though not SA. Though this is evidence that Mp10 is an effector, its molecular function has not yet been found. Identification of Mp10 interaction with plant components would provide further evidence that this protein is delivered by the aphid into plants and has a role as an effector there. Therefore, the next step is to further investigate what plant factor(s) Mp10 interacts with.

Other insect effectors have been identified; one of the first is salivary glucose oxidases (GOX) from caterpillar species (256). GOX suppresses nicotine production in *Nicotiana tabacum*, but also induces defense responses in tomato (125; 257). Similarly, Mp10 suppresses PTI responses and induces chlorosis, which may be part of a plant defense response. How GOX suppresses or induces plant defence responses is not yet clear. The same is true of other potential insect effectors, with no specific targets found to date. This includes MIFs (macrophage migration inhibitory factors) from aphid saliva, which have been found to inhibit immune responses including callose deposition and defense gene induction by an as-yet unknown mechanism (259). There is therefore no clear understanding of how insects modulate plant defences. Mp10 blocks the ROS and calcium bursts to both flg22 and aphid extract (46). These responses occur early in PTI (328; 114), and involve the leucine-rich repeat receptor-like kinase (LRR-RLK) BAK1 (340; 363; 143). BAK1 or other components with roles early in PTI may therefore be Mp10 targets.

ROS and calcium induction appear to occur via independent parallel processes that together enforce the reactivity and specificity of the immune response (170; 180). Components involved in these aspects of immune signaling may be targeted by Mp10. BOTRYTIS-INDUCED KINASE-1 (BIK1) is a key player in the ROS response mediated via FLS2 and BAK1. BIK1 directly associates with FLS2, BAK1 and other RLKs to positively regulate several PTI responses (171; 180; 476; 266). In flg22 perception, binding of flg22 to FLS2 triggers the interaction of FLS2 and BAK1, and the phosphorylation and activation of BIK1 (477; 147). Activated BIK1 has an increased affinity for the plasma membrane associated NADPH oxidase RBOHD, which it then phosphorylates (180; 171). Phosphorylation of RBOHD increases its calcium binding affinity and allows access of calcium-dependent pro-

tein kinases (CPKs) that also phosphorylate RBOHD (183). This primes RBOHD to induce a full ROS burst upon calcium induction and CPK phosphorylation. RBOHD and another NADPH oxidase, RBOHF, are both involved in the aphid-induced ROS burst and defence responses (340; 177).Their inhibition may therefore aid aphid success.

The calcium burst of PTI involves different components depending on the immune receptor involved. The calcium burst mediated by FLS2 is dependent on a functional phosphoinositide-specific phospholipase C (PI-PLC) signaling pathway, which activates intracellular calcium stores (478). This is in contrast to the Pep/PEPR system involving the receptors PEPR1 and PEPR2 that bind the DAMP Pep1 in *A. thaliana* (170). Calcium signaling of the PEPR system requires extracellular calcium stores only and a functional version of the cyclic GMP-activated calcium channel CNGC2 (170). This is despite both PEPR1/PEPR2 and FLS2 activity being dependent on BAK1 (142). Nevertheless, maximal calcium signaling via FLS2 requires a functional Pep/PEPR system, and loss of function of FLS2 impairs Pep-mediated calcium signaling. Whether the calcium response to aphids requires PI-PLC, CNGC2 or both is unknown. Interestingly, FLS2 and PEPR1/2 are also required for a full hypersensitive response (HR) to a virulent strain of bacterium, indicating a role for PTI in effector-triggered immunity (ETI) (170). Given that GPA Mp10 suppresses both ROS and calcium responses of FLS2, it is therefore possible that this effector also suppresses ETI.

BAK1 is a central regulator of many PTI responses that have been characterised (349). It therefore provides a valid target for pathogen effectors, and several that target BAK1 have been identified. Both AvrPtoB and HopF2 from *Pseudomonas syringae* suppress PTI via direct interaction with BAK1 (261; 263). The two kinase domains of AvrPtoB bind to BAK1 and this prevents kinase activity of BAK1 in a manner that does not require kinase activity of AvrPtoB (452). HopF2 also interacts directly with BAK1; this effector binds to the BAK1 transmembrane and kinase domains that are exposed in the cytoplasm of the plant cell, where the effector is deposited via the bacterial type III secretion system (263). By interacting with immune receptors, both HopF2 and AvrPtoB block the ROS and calcium bursts early in PTI. Mp10 may therefore act similarly to HopF2 and AvrPtoB. Interestingly, HopF2 and AvrPtoB have additional targets besides BAK1; AvrPtoB also targets CERK1 and FLS2 for degradation via ubiquitinating them, and HopF2 disrupts MAP kinase cascades by targeting MKK5 and suppressing MKK5 phosphorylation of downstream MPK3/6. HopF2 is also able to perturb ETI via RIN4 (265; 264; 479; 480). This shows that some effectors have multiple targets in the plant cell. Mp10 may also interact with

multiple components of the plant immune system in order to have full effector action.

Other pathogen effectors also block the calcium and ROS bursts of PTI. Some bacterial pathogens produce exopolysaccharides that can chelate calcium in order to overcome PTI (481), and the root-knot nematode secretes the effector Mi-CRT, a calreticulin, which may suppress plant defense by virtue of its calcium binding activities (482). There is some evidence to suggest that calcium-binding proteins found in aphid saliva may fulfil a similar role (29; 32). The green rice leafhopper protein NcSP84 contains calcium binding EF-hand domains and is secreted into the phloem during feeding, suggesting that calcium binding may be a widespread mechanism of interfering with plant defense responses in insects (483). Mp10 however is not predicted to have any calcium binding domains, so direct suppression of the calcium burst seems unlikely. The effector would also have to be present at large concentrations in order to collect enough calcium to prevent a burst occurring. Alternatively, Mp10 may target calcium channels and NADPH oxidases directly or via their regulators. For instance, the *P. syringae* effector AvrPphB can target and cleave BIK1, preventing activation of RBOHD and so ROS induction (171; 266).

Approaches used to find plant targets of pathogen effector proteins include: the investigation of effector localisation within the host; genetic screens using different ecotypes of Arabidopsis; effector pulldowns from plant tissue and subsequent analysis of pulled down proteins by mass spectroscopy; and yeast two-hybrid screens of the effector genes against libraries of plant genes (484; 485). Rodriguez et al. found that Mp10 is present throughout the cell, predominantly in the cytoplasm and nuclei, which did not narrow down possible targets of Mp10 (486). Genetic screening also does not appear to be a fruitful avenue for further Mp10 research; different Arabidopsis ecotypes show little variation in GPA susceptibility (based on GPA fecundity measurements) (487). GPA probably has multiple effectors and each of these could be differentially active depending on the ecotype, masking possible variations in Mp10-target interactions among the Arabidopsis ecotypes.

This leaves the approaches of pull-down and mass spectroscopy, and a yeast two-hybrid screen. Heterologous expression of Mp10 in plants leads to a chlorosis response in the plant, which eventually causes cell death and may be lethal in stable transgenic lines. This may frustrate pull-down and mass spectrometry approaches. After considering the options, a yeast two-hybrid screen against an Arabidopsis transcript library was decided to be the most effective way of identifying a plant target of Mp10. In addition to Mp10, a screen carried out prior to my arrival identified other GPA effector candidates (46), and plant targets for

these effectors may also be identified via a yeast two-hybrid screen against the Arabidopsis transcript library. Upon identifying potential plant targets in the yeast two-hybrid screen, the biological significance of the targets can then be further investigated by *in planta* studies.

6.2 Results

6.2.1 AMSH2 was identified as potential plant target of Mp10 in a yeast-two-hybrid library screen

In order to identify potential Mp10 targets from plants, we screened Mp10 against a plant yeast two-hybrid library generated from RNA pooled from *Arabidopsis thaliana* leaves exposed to a variety of insect pests, as well as phloem sieve element RNA (see Figure 6.1). This yeast two-hybrid library was constructed by Dualsystems Biotech (now Hybrigenics). The library contained over 500,000 independent clones in the plasmid pGAD-HA (containing the activation domain). Mp10 was screened against these as bait, in the pLex-AN plasmid (binding domain).

In the initial screen 6 different colonies grew on media lacking tryptophan (W), leucine (L) and histidine (H) with 1mM 3-Amino-1,2,4-triazole (3-AT) 4 days after plating out. The pGAD-HA plasmids were isolated from these 6 colonies and introduced into *E. coli* for amplification and purification. Inserts were subsequently sequenced using pGAD specific primers. Inserts of three clones contained different fragments of sequence encoding the *Arabidopsis thaliana* protein ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM 2 (AMSH2). Inserts of the other three other clones were found to encode SERINE HYDROXYMETHYLTRANSFERASE 3 (SHM3), LIGHT-HARVESTING CHLOROPHYLL B-BINDING PROTEIN 3 (LHCB3) and RUBISCO SMALL SUBUNIT 1B (RBCS1B) (Table 6.1).

The 6 clones were co-transformed into yeast with Mp10 to confirm previous results and with an empty vector control to assess self-activation. The second screen did not confirm SHM3, LHCB3 and RBCS1B as interactors of Mp10, whereas yeast cells with the three clones containing AMSH2 sequences and Mp10 grew on both -WLH and -WLHA media, the latter suggesting a strong interaction of AMSH2 and Mp10 (Figure 6.2). There was no growth on the same media when the yeast was transformed with the empty vector control (EV), indicating that the AMSH2 sequences were not self-activating. Given that three independent clones contain AMSH2 sequences, this provides confidence that AtAMSH2 is a true interactor of Mp10 in yeast.

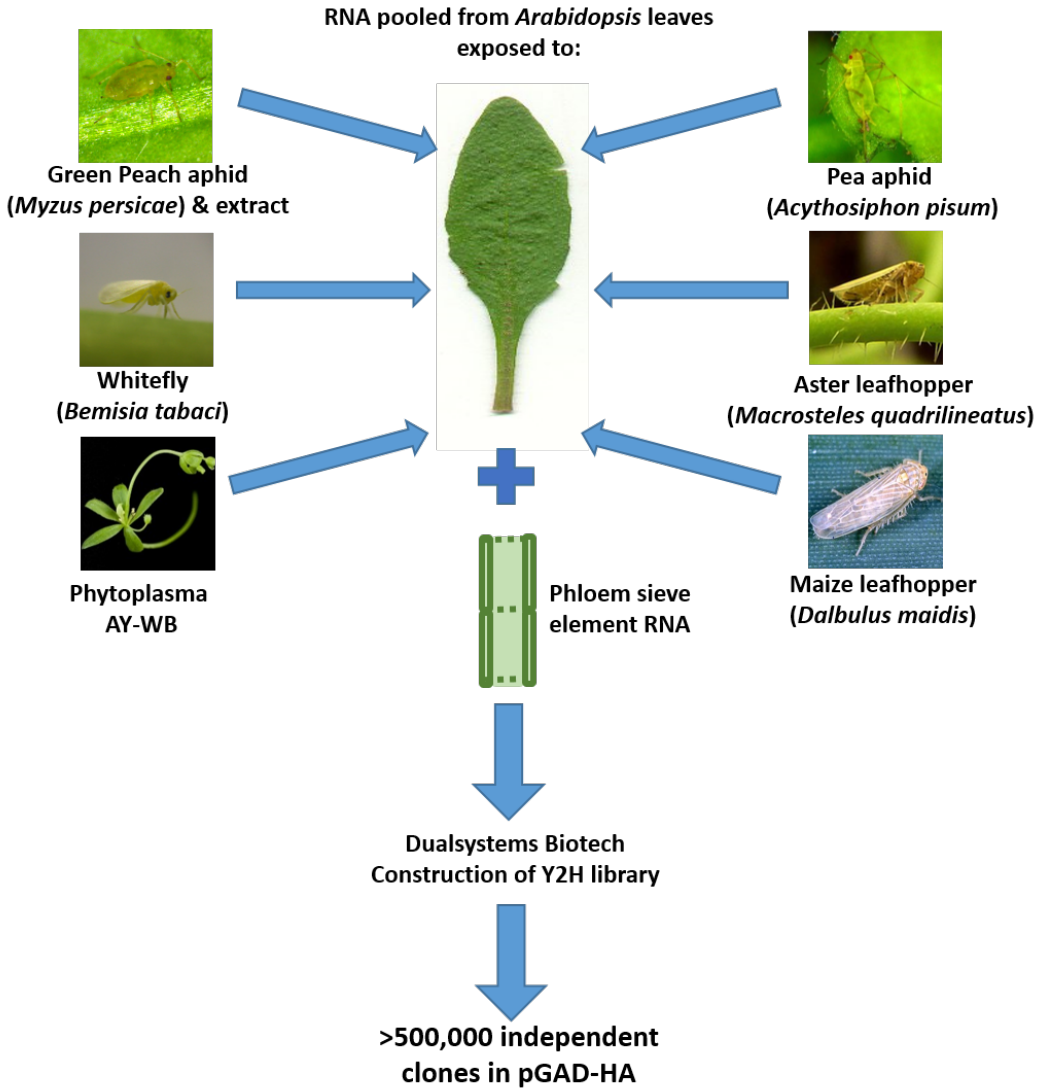


Figure 6.1: **Plant yeast-two-hybrid library construction**. RNA was pooled from Arabidopsis leaves exposed to several insect species, phytoplasma and aphid extract alongside phloem sieve element RNA. This was sent to Dualsystems Biotech (Switzerland) (now Hybrigenics (France)) for construction of a yeast two-hybrid library, which resulted in the creation of over 500,000 independent clones in the vector pGAD-HA. Maize leafhopper image from the American Phytopathological Society website http://www.apsnet.org/edcenter/intropp/PathogenGroups/Pages/Fastidious.aspx . Credit to Zigmunds Orlovskis, Anna Jordan (JIC Insectary) and Andrew Davis for the Aster leafhopper, whitefly and phytoplasma infected Arabidopsis images respectively.

Colony	Insert length (nt)	Top Hit	Identity	E-value	Description
1	495	AT1G10600	99%	0	AMSH2 (Associated molecule with the SH3 domain of STAM)
2	672	AT1G10600	99%	0	AMSH2 (Associated molecule with the SH3 domain of STAM)
3	550	AT1G10600	99%	e^{-167}	AMSH2 (Associated molecule with the SH3 domain of STAM)
4	757	AT4G32520	99%	0	SHM3 (SERINE HYDROXYMETHYLTRANSFERASE 3)
5	835	AT5G54270	99%	0	LHCB3 (LIGHT-HARVESTING CHLOROPHYLL B-BINDING PROTEIN 3)
6	697	AT5G38430	99%	0	RBCS1B (RUBISCO SMALL SUBUNIT 1B)

Table 6.1: **Table showing the BLASTN outputs for the 6 sequenced inserts**. pGAD-HA plasmids from the 6 identified yeast colonies were amplified in *E. coli* and then sequenced using pGAD specific primers, which amplified the insert region. These sequences were then used in a nucleotide BLAST 2.2.8 (488) against the TAIR 10 Arabidopsis transcript database (489).

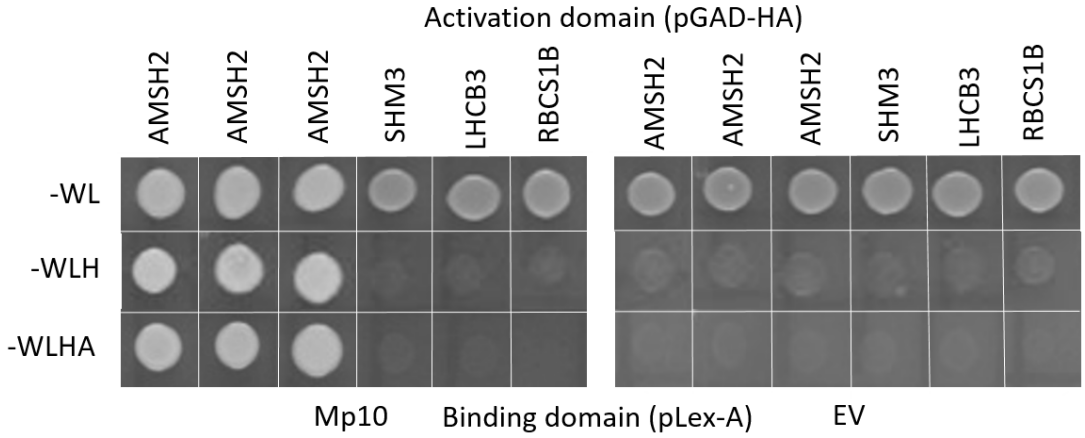


Figure 6.2: **AMSH2 was identified as a potential plant target of Mp10 in the yeast-two-hybrid library screen**. Initial screen of Mp10 against the plant Y2H library found 6 clones, 3 of which were confirmed to interact with Mp10, shown here. None were self activating, as shown in the empty vector (EV) control. Images show growth after 4 days W = tryptophan, L = leucine, H = histidine, A = alanine. Experiment conducted by Christine Wilson.

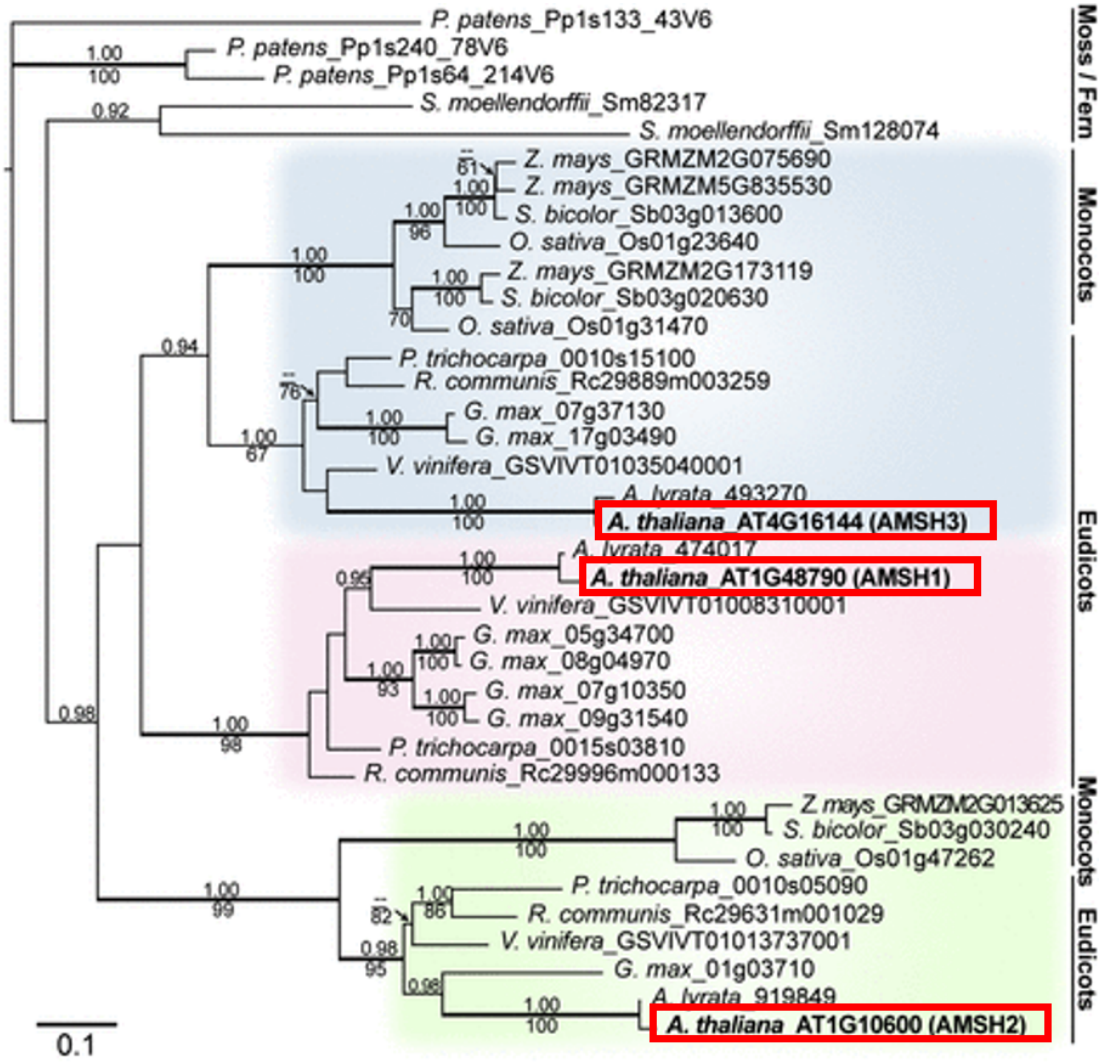
6.2.2 Mp10 interaction with AMSH2 is specific amongst AMSH family members

Through its similarity to the human AMSH protein, AMSH2 is predicted to be a deubiquitinating enzyme (DUB) that cleaves Lys-48 and Lys-63-linked polyubiquitin chains. In addition to AMSH2, *A. thaliana* has two other AMSH homologs; AMSH1 and AMSH3 (See Figure 6.3a). The three *A. thaliana* AMSH proteins are metalloprotease-type DUBs, with MPN (Mpr1p and PAD1p N-terminal) domains that contain a conserved JAMM (Jab1/MPN/Mov34) motif, which binds zinc ions. However, unlike AMSH2, AMSH1 and 3 contain UPS8 dimerisation domains at their N-termini (see Figure 6.3b). This makes AMSH2 under half the size of both AMSH1 and 3.

The AMSH2 gene model lists three potential splice variants, named AMSH2.1, AMSH2.2 and AMSH2.3. Relative to AMSH2.1, both AMSH2.2 and AMSH2.3 have longer 5' UTRs. AMSH2.2 has an alternative start codon compared to AMSH2.3, whereas AMSH2.3 has an alternative splicing site within the centre of the transcript resulting in a different and shorter third exon compared to AMSH2.1 and AMSH2.2 (see Figure 6.4a). Two out of the three inserts of the three clones that interacted with Mp10 in the yeast two-hybrid screen were most similar to full length or fragments of AMSH2.1 (Figure 6.5), the third could not be distinguished between AMSH2.1 and AMSH2.2. Mp10 is therefore most likely to interact with AMSH2.1. It is also possible that the other splice variants of AMSH2 are misannotations that are not expressed in Arabidopsis.

The initial yeast two-hybrid screen was conducted with the DUALhybrid yeast two-hybrid system (Dualsystems Biotech, Switzerland). To further confirm that Mp10 interacts with AMSH2.1, we wished to conduct the yeast two-hybrid analyses in another system, choosing the Gateway ProQuest (Invitrogen) yeast two-hybrid system. We also wished to examine Mp10 interactions with AMSH2.2, AMSH2.3, AMSH1 and AMSH3 in this system. Coding sequences for AMSH2.1, 2.2 and 2.3, AMSH1 and AMSH3 were amplified and cloned into the Gateway vector pDEST32, producing the AMSH proteins as N-terminal fusions with the DNA binding domain (BD). The Mp10 sequence was cloned into pDEST22, producing Mp10 as a N-terminal fusion with the activation domain (AD). We also cloned the coding sequence of GPA MpOS-D1, a homolog of Mp10, into pDEST22. A pDEST22 construct for the Mp10 Y40F W120Y mutant, which lost calcium and ROS repression activity (see Chapter 5), was also generated. To control for AMSH1 and AMSH3 binding activity in the yeast two-hybrid analyses, pDEST22 constructs were generated for the coding sequences of

(a)



(b)

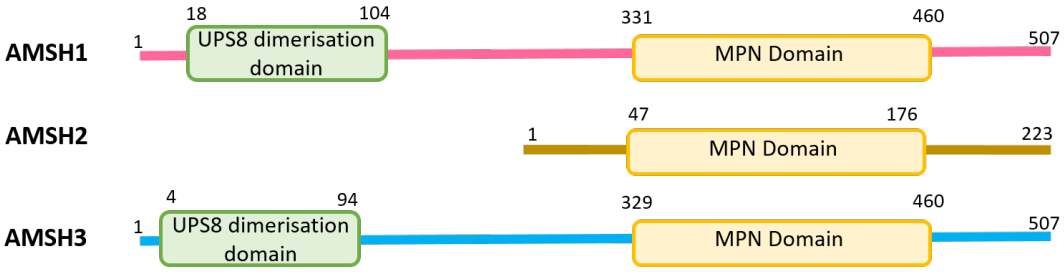
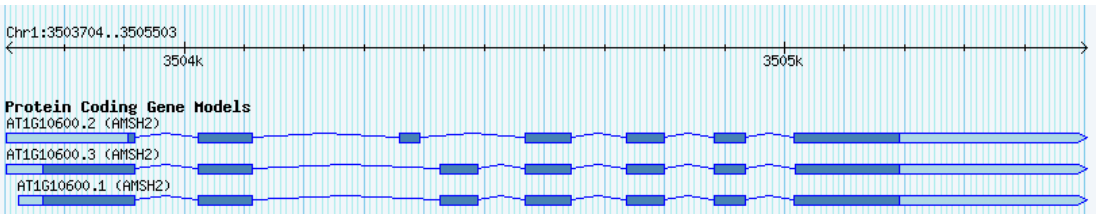


Figure 6.3: **AMSH2 is a deubiquitinating enzyme (DUB) with two homologs in *Arabidopsis thaliana*.** 6.3a There are three AMSH proteins in *A. thaliana*; AMSH1, AMSH2 and AMSH3. AMSH1 and AMSH3 are more closely related than AMSH2. AtAMSH1, 2 and 3 are highlighted using red boxes. Tree taken from Katsiarimpa et al (490). 6.3b Comparison of domains found in *A. thaliana* AMSH1, 2 and 3. AMSH2 is a smaller protein than AMSH1 and 3 and does not contain the UPS8 dimerisation domain. Domains predicted using InterPro (491).

(a)



(b)

```
AMSH2.2     ----------------------------------------------MFISQK---GYW  9
AMSH2.1     MVTLSSPSPSLSCVENVTCKSSHVSRVLISGTDNINHGESSEAKILRDVHISERLLEDFT  60
AMSH2.3     MVTLSSPSPSLSCVENVTCKSSHVSRVLISGTDNINHGESSEAKILRDVHISERLLEDFT  60
                                                        :.**::    :

AMSH2.2     RISLSLQERTLRRTSRLVG------LSLPFLVLRFSSFMNLMCQAMNEVEVFSIQNEREL  63
AMSH2.1     ELARENTEKDLETCGTLAAFLERGIFYVTTLIIPKQESTSNSCQAMNEVEVFSIQNEREL  120
AMSH2.3     ELARENTEKDLETCGTLAAFLERGIFYVTTLIIPKQESTSNSCQAMNEVEVFSIQNEREL  120
            .:: .  *: *.  . *..      : :  *::  ..  .  ******************

AMSH2.2     YPVGWIHTHPSQGCFMSSVDLHTHYSYQVMVPEAFAIVVAPTDSSKSYGIFKLTDPGGME  123
AMSH2.1     YPVGWIHTHPSQGCFMSSVDLHTHYSYQVMVPEAFAIVVAPTDSSKSYGIFKLTDPGGME  180
AMSH2.3     YPVGWIHTHPSQGCFMSSVDLHTHYSYQVMVPEAFAIVVAPTDSS-NYGIFKLTDPGGME  179
            ****************************************** .*************

AMSH2.2     VLRGCSETGFHPHKEPEDGNPVYEHCSNVYKNSNLRFEIFDLR          166
AMSH2.1     VLRGCSETGFHPHKEPEDGNPVYEHCSNVYKNSNLRFEIFDLR          223
AMSH2.3     VLRGCSETGFHPHKEPEDGNPVYEHCSNVYKNSNLRFEIFDLR          222
            *****************************************
```

Figure 6.4: **There are three potential splice variants of AMSH2 in *Arabidopsis thaliana***. 6.4a Gene models for the three splice variants of AMSH2. Both AMSH2.2 and AMSH2.3 have longer 5'UTRs than AMSH2.1. Alternative splicing of AMSH2.2 also creates a different third exon. Gene models taken from The Arabidopsis Information Resource (TAIR) on www.arabidopsis.org. 6.4b Protein sequences of Arabidopsis AMSH2 splice variants. The MPN domain is highlighted in yellow. Protein sequence taken from The Arabidopsis Information Resource (TAIR) on www.arabidopsis.org, aligned using Clustal Omega (445).

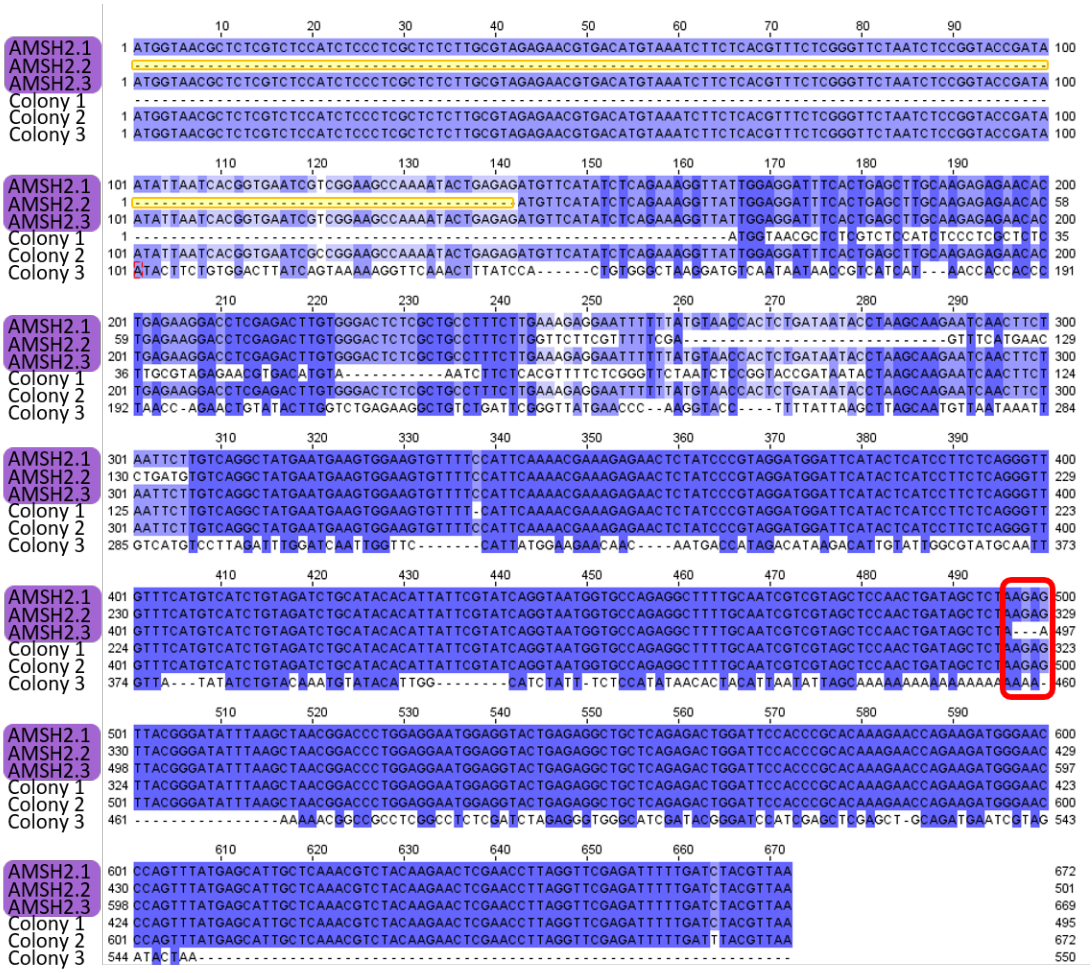


Figure 6.5: **The sequence of clones 1 and 2 from the yeast two-hybrid screen reveal that they correspond to portions of AMSH2.1**. Alignment shows sequence gained from colonies 1, 2 and 3 aligned to the coding sequence of AMSH2.1, 2.2 and 2.3. Sequence missing in AMSH2.2 that is diagnostic for this splice variant is highlighted in yellow. A region of 4 nucleotide difference between AMSH2.1 and AMSH2.3 diagnostic for these splice variants is highlighted using a red box. Coding sequences of AMSH2 splice variants taken from The Arabidopsis Information Resource (TAIR) on www.arabidopsis.org. Alignment produced using Clustal Omega (445).

the two vacuolar protein sorting (VPS) proteins VPS2.1, which interacts with both AMSH1 and AMSH3, and VPS24.1, which interacts with AMSH3 (492; 490). Finally, a pDEST22 construct with another GPA effector, Mp2, was included.

Colonies were plated out on media lacking tryptophan (W), leucine (L) and histidine (H), with differing concentrations of 3-AT. Based on growth of the yeast colonies in the presence of 100 mM 3AT, Mp10 and Mp10 Y40F W120Y interacted with both AMSH2.1 and 2.3, but not with AMSH2.2 and AMSH1 (Figure 6.6a). MpOS-D1 and Mp2 did not interact with any of the AMSH proteins and Mp2 appears to interact with itself. These data confirmed that Mp10 interacts with AMSH2. AMSH2.1 and AMSH2.3 sequences are more similar to each other than to AMSH2.2 resulting in the identification of a region of about 100 amino acids at the N-terminus of AMSH2.1/2.3 that could be responsible for the interaction with Mp10; however AMSH2.2 may not have expressed or folded correctly in yeast (see Figure 6.4b). No growth of yeast colonies containing AMSH1 and VPS2.1 was observed indicating that AMSH1 may not be active in yeast. We did not include positive controls for AMSH2.2 and MpOS-D1 activities in this experiment. Therefore, further experimentation is required to completely rule out absence of interactions of Mp10 and AMSH1/AMSH2.2, and MpOS-D1 and AMSH proteins.

Yeast cells producing BD-AMSH3 grew even in the presence of unfused AD (produced by the empty vector (EV) of pDEST22) indicating that BD-AMSH3 is self-activating, even at high (150mM) 3-AT concentrations when yeast cells containing AD-Mp10 and BD-AMSH2.1/2.3 did not show growth any longer (Figure 6.6a). To prevent self activation of BD-AMSH3, the AMSH sequences were cloned into pDEST22, producing AD-AMSH3, and Mp10 was cloned into pDEST32, producing BD-Mp10. Unlike BD-AMSH3, AD-AMSH3 did not self activate and interacted with the positive control BD-VPS24.1, but not with BD-Mp10 in the presence of 50mM 3AT (Figure 6.6b). This indicates that Mp10 and AMSH3 do not interact. However, in these conditions BD-Mp10 did not interact with AD-AMSH2.1 either. Mp10 may only interact with AMSH2 when fused to the activation domain (in pDEST22) in the Gateway system, although Mp10 fused to binding domain interacted with AMSH2 in the original yeast two-hybrid screen carried out using the DUALhybrid system (Figure 6.2) . Thus, it remains to be confirmed whether Mp10 does not interact with AMSH3.

Taken together these results indicate that Mp10 interacts with AMSH2.1 and AMSH2.3. Whereas yeast two-hybrid analyses were useful to identify AMSH2 as a potential target of

Mp10, they did not generate conclusive data for some of the interactions. Therefore, other experiments are required to further investigate the specificity of Mp10 interactions with AMSH2.

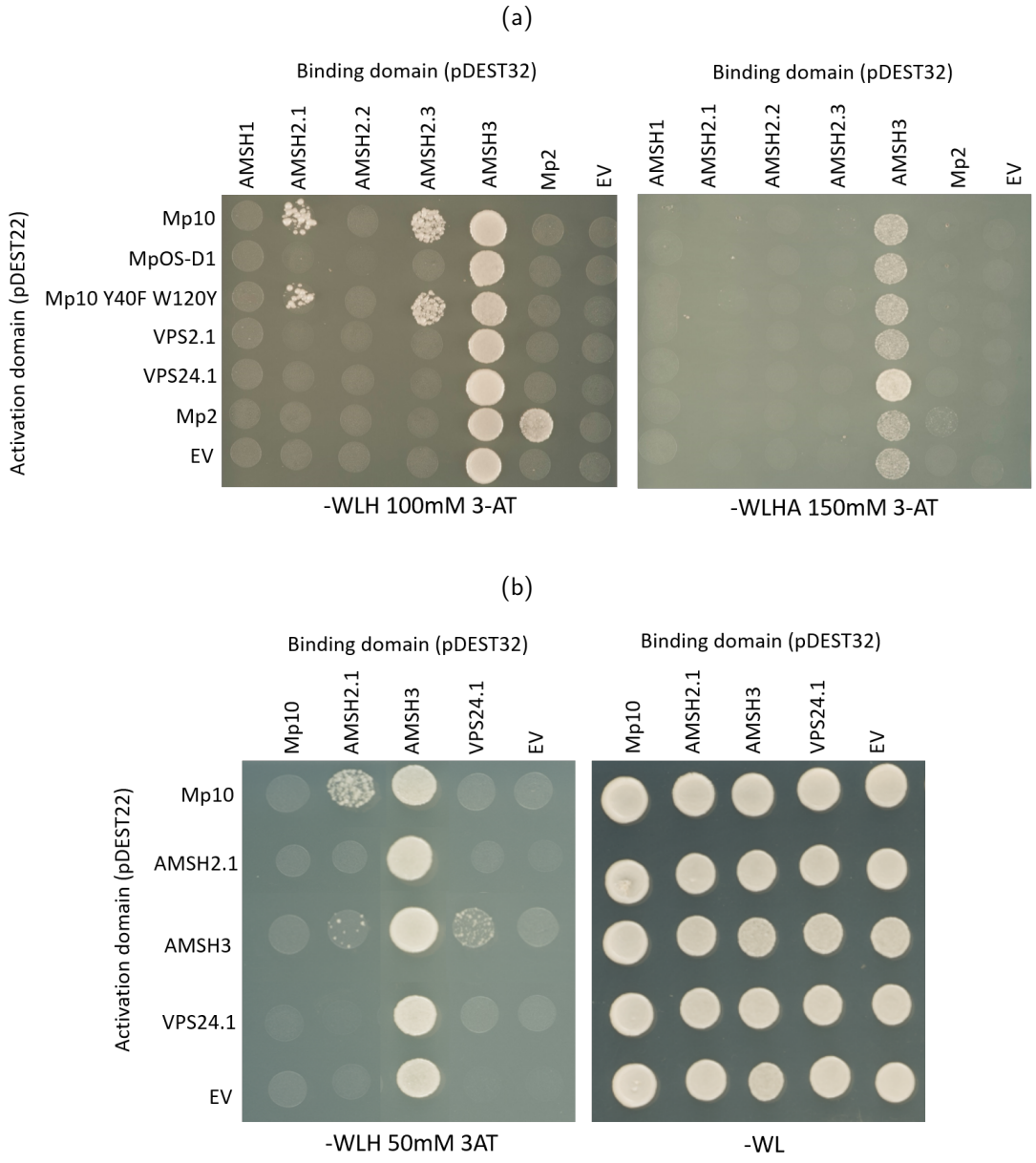


Figure 6.6: **The Mp10-AMSH2 interaction could be confirmed in the Gateway yeast two-hybrid system**. 6.6a Mp10 interacts with AMSH2.1 and AMSH2.3 but not AMSH2.2 in yeast two-hybrid. Mp10 Y40F W120Y also interacts with AMSH2.1 and AMSH2.3. AMSH3 self-activates when in the binding domain. MpOS-D1 did not interact with any AMSH proteins. Repeated 3 times with the same results. Images show growth after 5 days. Experiment conducted by Carlos Caceres. 6.6b AMSH3 is not self-activating when in the activation domain and does not interact with Mp10. AMSH2-Mp10 interaction was not seen in this orientation either. Repeated 3 times with the same results. Images show growth after 4 days. Experiment conducted by Friederike Bernsdorff.
W= tryptophan L= leucine H=histidine A= alanine EV=empty vector

6.2.3 AMSH2 affects the flg22-triggered ROS burst

As Mp10 affects the flg22-triggered ROS burst in *N. benthamiana* and AMSH2 was identified as a potential plant target of Mp10, I wished to investigate whether AMSH2 also has an effect on the flg22-induced ROS burst. I expressed AMSH1, 2 and 3 from *A. thaliana* as well as the *N. benthamiana* AMSH2, NbAMSH2, in *N. benthamiana* with N-terminal GFP tags alongside a GFP control. I used the infiltrated leaf tissue in ROS burst assays and found that expression of GFP-AtAMSH2 increased the ROS burst response to flg22 compared to the GFP control, whereas GFP-AtAMSH1 and 3 did not (Figure 6.7a). NbAMSH2 appeared to also increase the ROS response, but this increase was not significant compared to the GFP control (see Figure 6.7a). Protein products corresponding to the sizes of GFP and GFP-fusions of AMSH1, AMSH2 and AMSH3 were detected on a Western blot with GFP antibody (Figure 6.7b), indicating that all the proteins were produced during the ROS burst assays.

I also used a silencing construct to knock down AMSH2 expression transiently in *N. benthamiana*. When these leaves were used in a flg22-induced ROS burst assay, I found that the ROS burst was about 40% lower than that induced in a control (Figure 6.7c). Leaves of this experiment have been harvested and will be processed for RNA extraction to ensure that AMSH2 expression was downregulated in the RNAi plants (data not yet available). Stable transgenic *A. thaliana* that express AMSH2 RNAi under an inducible promoter will become available soon and will also be analysed for suppression of ROS upon induction of RNAi expression and subsequent flg22 treatment.

So far these data show that knock-down of AMSH2 in *N. benthamiana* phenocopies the Mp10-mediated suppression of ROS. Moreover, heterologous expression of AMSH2 increases the flg22-induced ROS response. In contrast, expression of AMSH1 and AMSH3 did not increase ROS. Together with the yeast two-hybrid experiments showing that Mp10 interacts with AMSH2, these data suggest that Mp10 interferes with AMSH2 function, possibly by degrading AMSH2 or blocking its action.

6.2.4 Investigations into Mp10-AMSH2 interaction *in planta*

I wished to investigate if Mp10 also interacts with AMSH2 in planta. To do this I carried out co-immunoprecipitation (CoIP) experiments upon production of various proteins in *N. benthamiana* leaves. *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens*

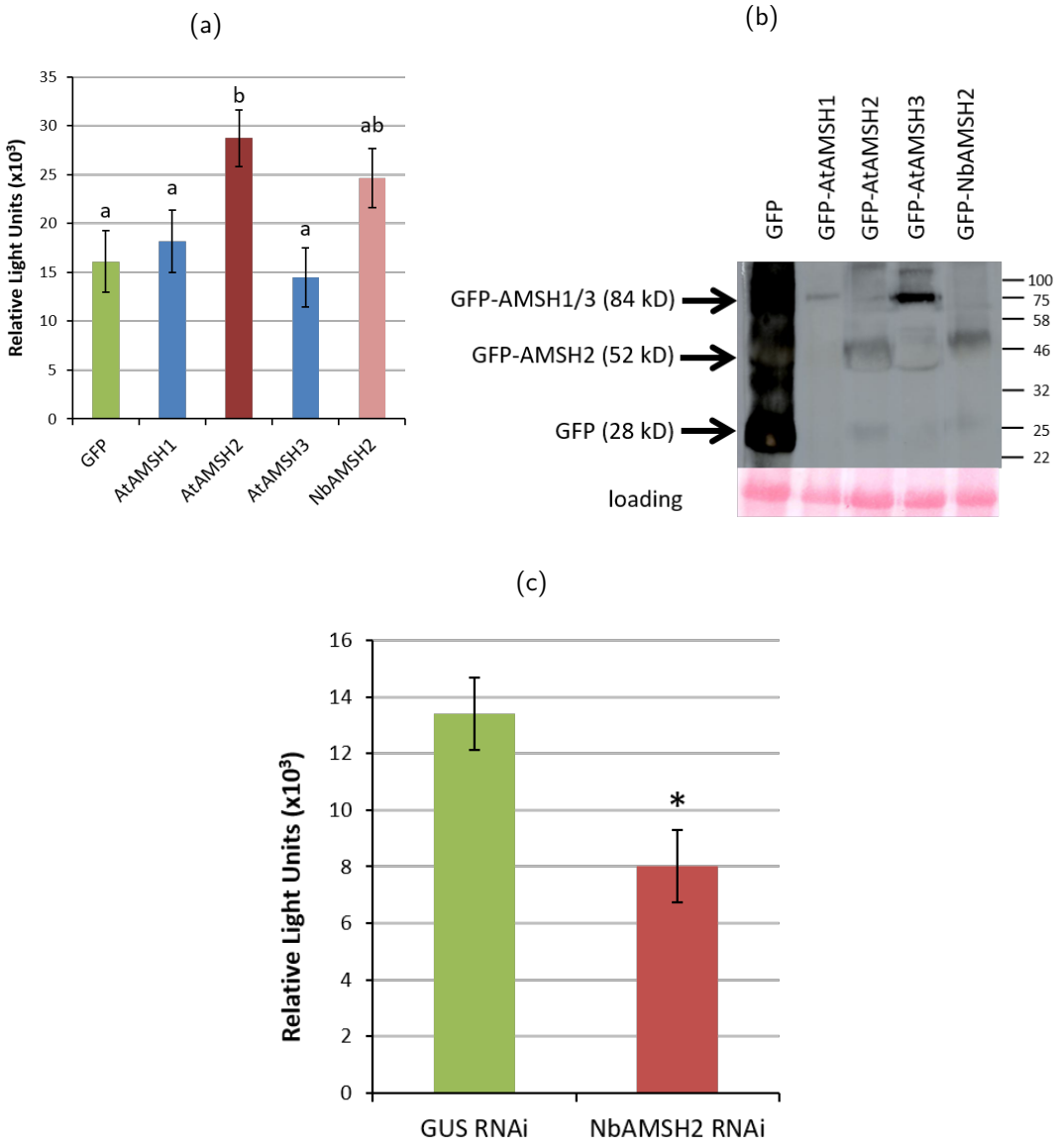


Figure 6.7: **AMSH2 affects the flg22 triggered ROS burst**. 6.7a Overexpression of AMSH2, but not AMSH1 or 3 increases the flg22-induced ROS burst. ROS burst measured over 60 minutes in *N. benthamiana* leaves transiently producing GFP-tagged AtAMSH1, 2 and 3 and NbAMSH2 alongside a GFP control. Graph shows mean ± SE of 3 independent experiments (n=8 per experiment). Letters indicates significant differences between treatments (Student's t-probability calculated within GLM at P <0.05). 6.7b Proteins corresponding to the sizes of GFP-AtAMSH1, 2 and 3, GFP-NbAMSH2 and GFP were detected in *N. benthamiana* leaves used for the ROS assays in 6.7a. Expression in two 10mm diameter leaf discs harvested 2 DPI was checked. Membrane imaged using 2 minute exposure. 6.7c Silencing of NbAMSH2 reduces the flg22-induced ROS burst. ROS burst measured over 60 minutes in *N. benthamiana* leaves transiently producing dsGUS or dsNbAMSH2. Graph shows mean ± SE of 3 independent experiments (n=8 per experiment). Asterisks indicate significant differences to the GFP treatment (Student's t-probability calculated within GLM at P <0.05).

cultures to transiently deliver constructs for the production of GFP-AMSH1, GFP-AMSH2 and GFP-AMSH3 alongside FLAG-tagged Mp10 (FLAG-Mp10) and FLAG-MpOS-D1 in the leaves. The GFP-tagged proteins were pulled down using GFP-Trap beads (Chromotek, Germany). The samples were then analysed by Western blot probed with anti-FLAG antibodies to see if FLAG-Mp10 and FLAG-MpOS-D1 remained attached to the GFP-tagged proteins upon pull down. In my first experiment, I detected a band corresponding to the size of FLAG-Mp10 in the GFP-AMSH2 pull down, whereas such a band was not seen in the GFP-AMSH1 and GFP-AMSH3 pull downs (Figure 6.8). Moreover, no bands corresponding to the size of FLAG-MpOS-D1 were detected. This experiment suggests that GFP-AMSH2 and FLAG-Mp10 interacted *in planta* or became associated with each other during the pull down procedure. Unfortunately, upon repeating this experiment an additional 3 times, I did not detect bands of FLAG-Mp10. I also tried using anti-FLAG beads (Sigma-Aldrich, USA) to pull down FLAG-Mp10 and FLAG-MpOS-D1 and detect GFP-tagged AMSH proteins with GFP antibodies on Western blots, but these experiments were unsuccessful. It is therefore still unclear whether Mp10 and AMSH2 interact with each other *in planta*, as there is no evidence that the initial results found are reproducible.

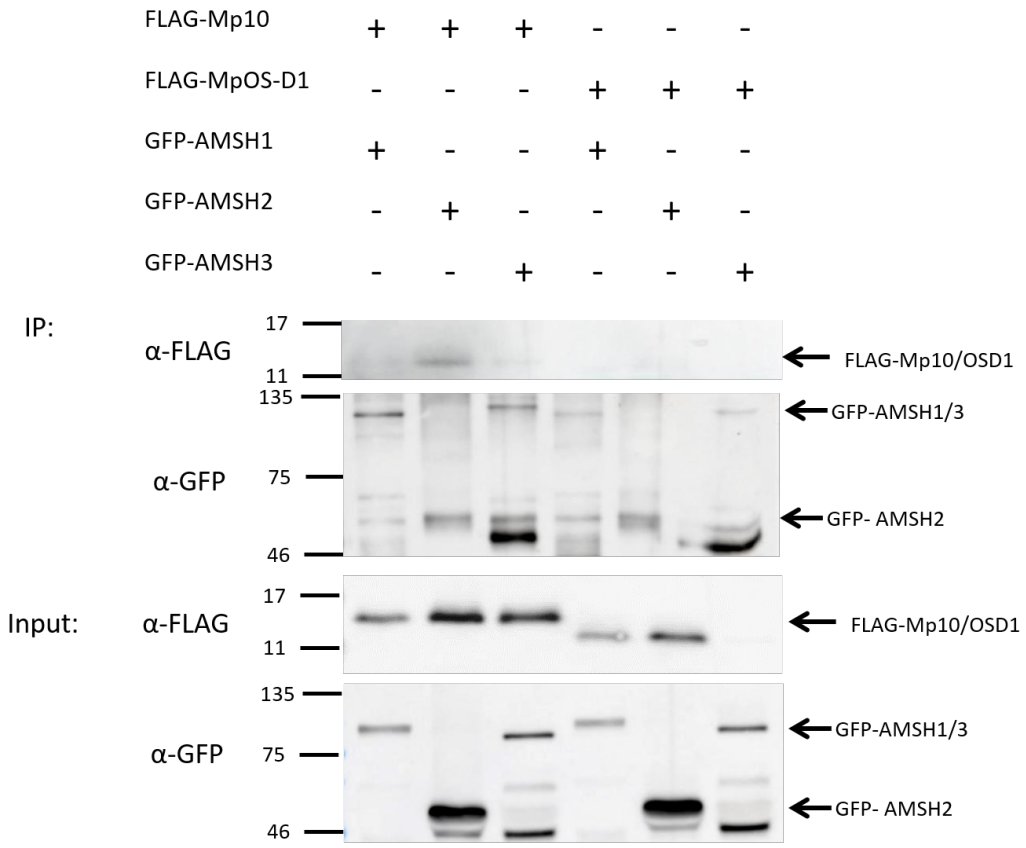


Figure 6.8: **AMSH2 and Mp10 may interact in planta**. Coimmunoprecipitation of proteins in *N. benthamiana* leaves transiently producing FLAG-tagged Mp10 and MpOS-D1, and GFP-tagged AMSH1, AMSH2.1 and AMSH3. 1 out of 4 experiments showed a band corresponding to FLAG-Mp10 when expressed with GFP-AMSH2.

6.2.5 AMSH2 has a different localisation to AMSH3 *in planta*

Whereas AMSH1 and AMSH3 have been investigated previously, little is known about the function of AMSH2. Both AMSH1 and AMSH3 are deubiquitinating enzymes (DUBs) that interact with ENDOSOMAL COMPLEX REQUIRED FOR TRANSPORT-III (ECRT-III) subunits in plant cells and are required for intracellular transport of vesicles late in the endosomal trafficking pathway and vacuole biogenesis (490; 492). In addition, AMSH3 is involved in the degradation of the chitin receptor CERK1 after ubiquitination, providing a link to the regulation of plant defenses, including the trafficking of immune receptors (493). AMSH3 localises in the plant cell cytosol and colocalises with late endosomal markers, such as ARA6 (492; 494). To better understand the role of AMSH2 in plant cells, I wished to investigate whether AtAMSH2 has the same cellular localisation as AtAMSH3. I cloned both AtAMSH2 and AtAMSH3 into the pB7WGF2 vector, which added an N-terminal GFP tag, and expressed them alongside RFP-Ara6, which labels late endosomes (495), in *N. benthamiana*.

I found that both AMSH2 and AMSH3 colocalised with Ara6, showing an endosomal distribution. However, unlike AMSH3 and Ara6, AMSH2 was also present in the nucleus (Figure 6.9). This suggests that both AMSH2 and AMSH3 are present in late endosomal vesicles, but that AMSH2 also targets the plant cell nucleus. AMSH2 is smaller than AMSH3 (25 kD versus 57 kD), and GFP-AMSH2 may locate to the nucleus because of its smaller size rather than this being biologically relevant. Moreover, with GFP being an N-terminal fusion, it is possible that the GFP portion of GFP-AMSH2 is cleaved off and that GFP migrates to the nucleus without the AMSH2 portion. Thus, further studies are necessary to better understand the localization of AMSH2 in relation to AMSH3 (and AMSH1), including the use of markers that label various cellular membranes and organelles.

6.2.6 Mp10 and AMSH2 alter the cellular localisation of the immune receptor FLS2

As Mp10 suppresses the calcium and ROS bursts induced by flg22, I investigated if Mp10 is involved in the trafficking of the PRR which detects flg22, FLS2 . I transiently expressed *A. tumefaciens* constructs for RFP-tagged Mp10 together with one for GFP-tagged FLS2 in *N. benthamiana* leaves. I included constructs for RFP-MpOS-D1 and RFP-GUS as controls.

As expected, AtFLS2-GFP in the presence of the control RFP-GUS labelled the pe-

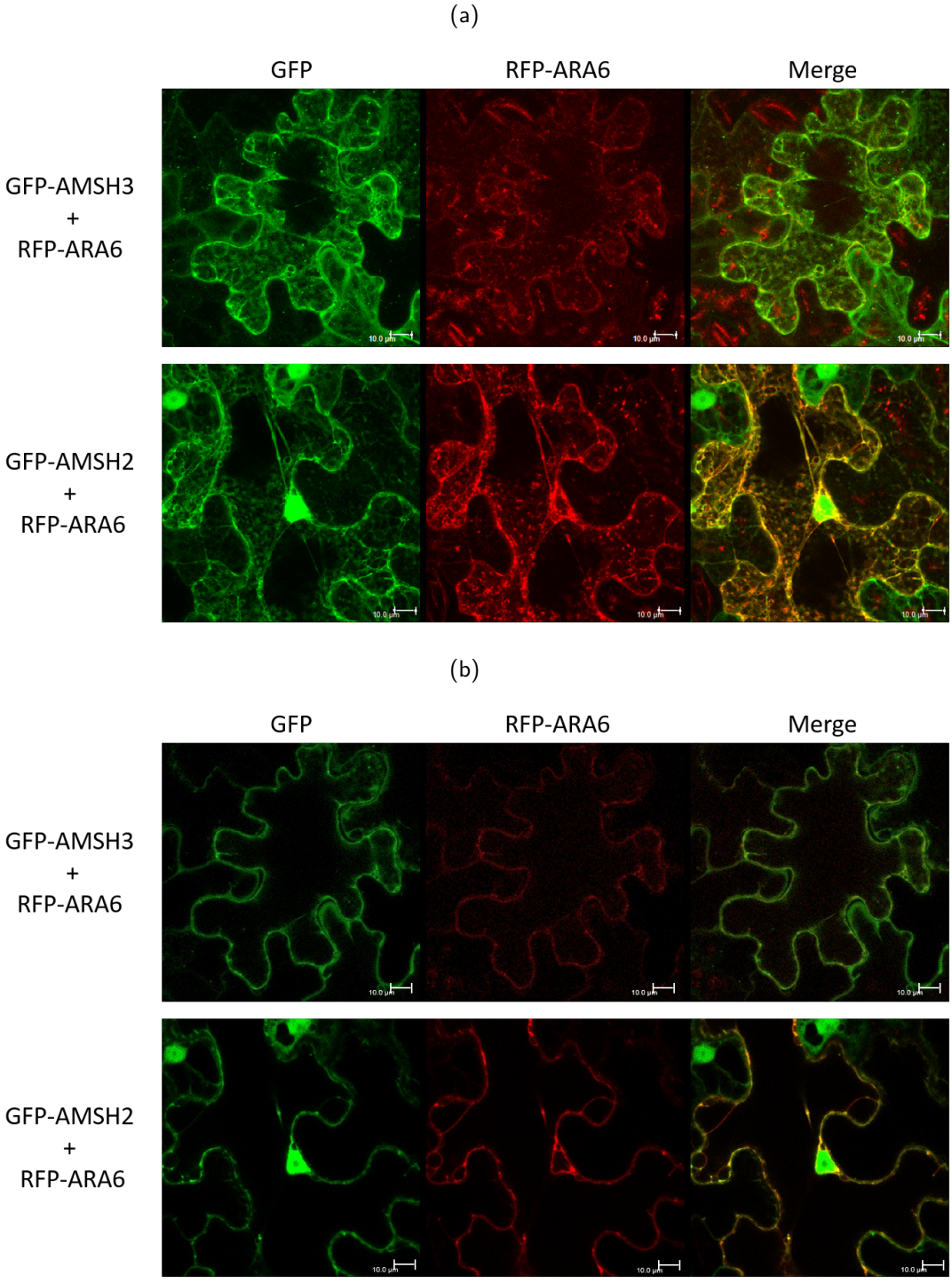


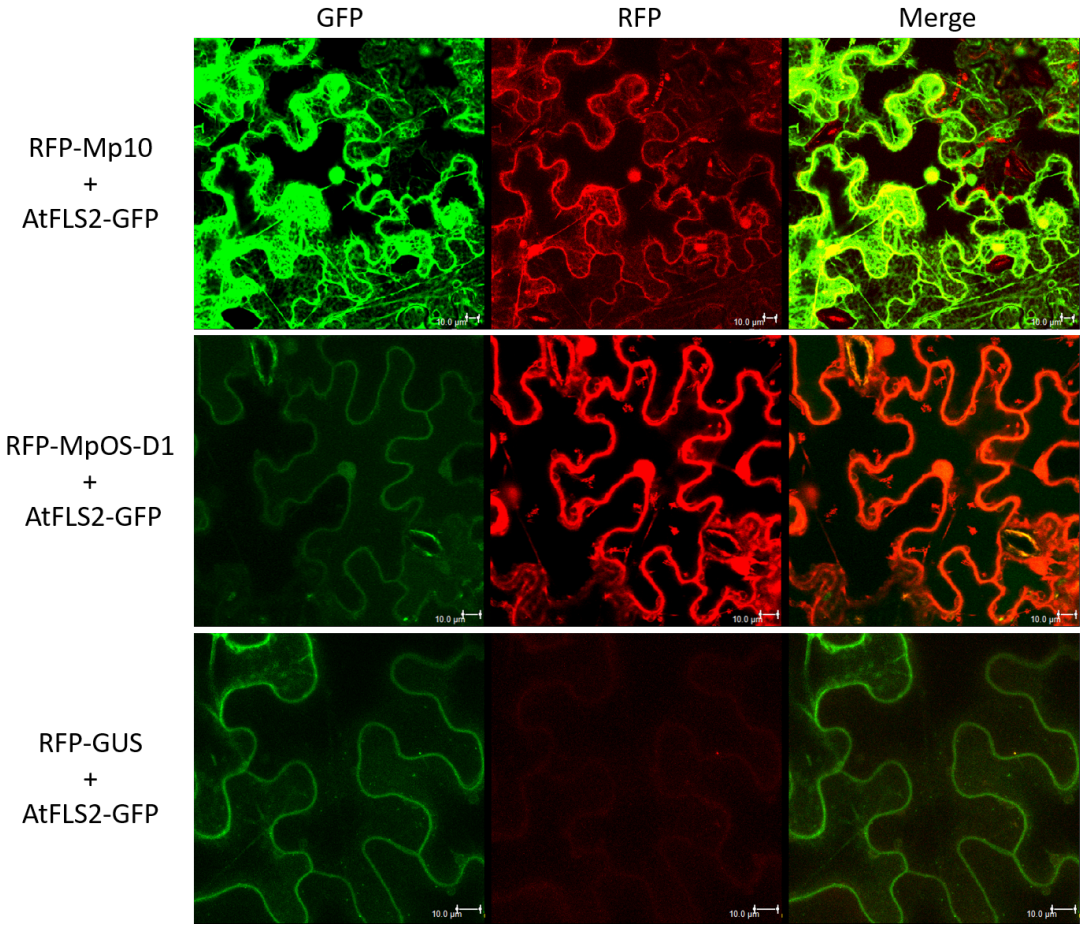
Figure 6.9: **AMSH2 has an overlapping but different localisation to AMSH3 *in planta***. RFP-tagged ARA6 was expressed transiently in *N. benthamiana* leaves alongside GFP-tagged AMSH2 or AMSH3. 6.9a z-projections and 6.9b optical sections of GFP and RFP signals in *N. benthamiana* leaf cells. All scale bars are $10\ \mu\mathrm{m}$.

riphery of the plant pavement cells consistent with FLS2 localization to the cell membrane (156), although the RFP signal of the RFP-GUS control was low (Figure 6.10a, lower panel). AtFLS2-GFP also localized to the periphery of the cells in the presence of RFP-MpOS-D1, for which high red fluorescence was detected (Figure 6.10a, middle panel). However, in the presence of RFP-Mp10 AtFLS2-GFP localization was not restricted to the cell periphery, but appeared to localize to the cell cytoplasm, nucleus, vesicle-like structures and filament-like strands throughout the cell (Figure 6.10a, upper panel). The altered localizations of AtFLS2-GFP in the presence of RFP-Mp10 were observed in roughly half of the cells in which green and red fluorescence was detected. Sam Mugford (RA in the Hogenhout lab) repeated some of these experiments and also found that RFP-Mp10 altered the cellular localization of tomato (*Solanum lycopersicum*) FLS2-GFP. SlFLS2-GFP did not locate to the nucleus, but nonetheless appears to locate to both the plasma membrane and tonoplast, including in cytoplasmic strands separating the vacuole (Figure 6.10b). Together, these results indicate that Mp10 affects the localisation of both Arabidopsis and tomato FLS2-GFP.

AMSH3 and 1 have been implicated in cellular trafficking, including that of cell immune receptors (490; 493) and AMSH2 may have a similar role. To test this, SlFLS2-GFP was produced in *N. benthamiana* leaves in which NbAMSH2 expression was knocked down by transiently expressing an RNAi construct to AMSH2. This revealed that knock-down of AMSH2 also disrupts FLS2 localization in the plant cell, as SlFLS2-GFP is localized in the cytoplasm and nucleus in leaves infiltrated with an RNAi construct for NbAMSH2, but not in the ones infiltrated with an RNAi construct for GUS (control) (Figure 6.11).

More confocal studies are required to better understand how heterologous expression of Mp10 and knock-down of AMSH2 may phenocopy each other. The data produced so far provides evidence that both Mp10 and AMSH2 alter FLS2 trafficking. FLS2 is required for mediating the calcium and ROS bursts to flg22 (Chapters 3 and 4), which is suppressed by Mp10 (Chapter 5) and by knock-down of AMSH2 (this chapter, Figure 6.7c). Together, these results suggest that Mp10 acts by suppressing AMSH2 activity.

(a)


GFP
RFP
Merge
RFP-Mp10
+
AtFLS2-GFP
RFP-MpOS-D1
+
AtFLS2-GFP
RFP-GUS
+
AtFLS2-GFP

(b)

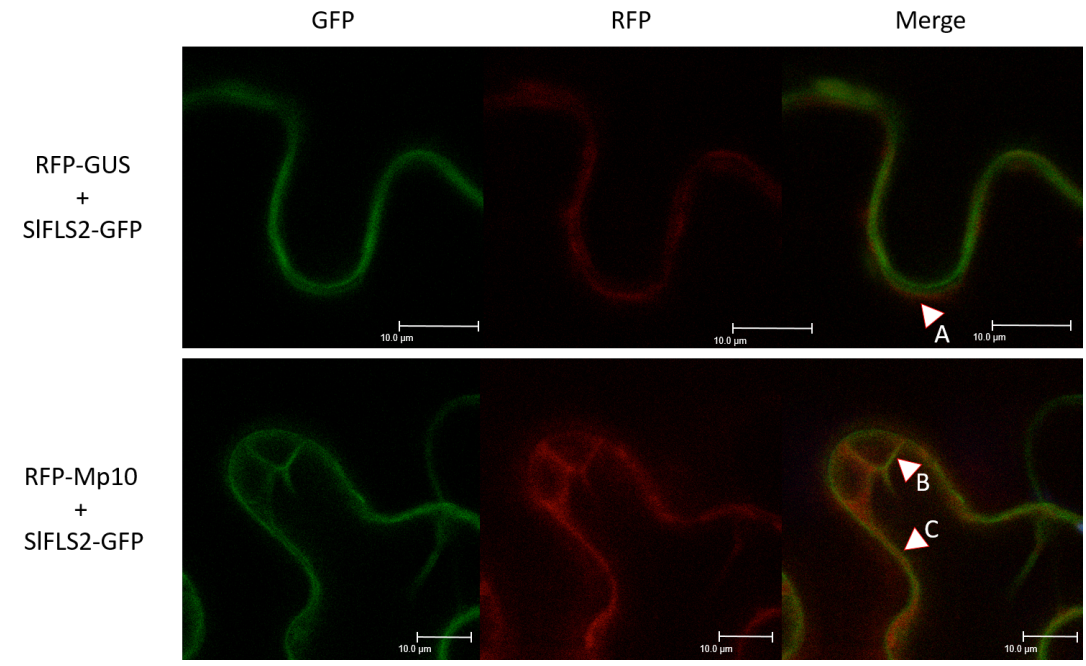

GFP
RFP
Merge
RFP-GUS
+
SlFLS2-GFP
RFP-Mp10
+
SlFLS2-GFP
A
B
C

Figure 6.10 *(previous page)*: **Mp10 alters cellular localization of the FLS2 immune receptor**. 6.10a AtFLS2-GFP localisation is altered upon coexpression with RFP-Mp10. GFP-tagged AtFLS2 was expressed transiently in *N. benthamiana* leaves alongside RFP- tagged Mp10, and MpOS-D1 and GUS controls. Images show z-projections of GFP and RFP signals in *N. benthamiana* leaf cells. When expressed with RFP-MpOS-D1 or RFP-GUS, AtFLS2-GFP signal can be seen on the periphery of the cell, consistent with a plasma membrane localization. When expressed with RFP-Mp10, AtFLS2-GFP signal can be seen in the cell cytoplasm, nucleus and filament-like strands throughout the cells. All scale bars are $10\ \mu\mathrm{m}$. 6.10b SlFLS2-GFP localisation is altered upon coexpression with RFP-Mp10. GFP-tagged SlFLS2 was expressed transiently in *N. benthamiana* leaves alongside RFP- tagged Mp10, and a GUS control. Images show z-projections of GFP and RFP signals in *N. benthamiana* leaf cells. When expressed with RFP-GUS, SlFLS2-GFP signal can be seen on the periphery of the cell, consistent with a plasma membrane location, but not on the inside edge of the cytosol (A). When expressed with RFP-Mp10, SlFLS2-GFP signal can be seen on the cell periphery but also in trans-vacuolar cytoplasmic strands (B) and on the inside edge of the cytosol, consistant with the tonoplast (C). All scale bars are $10\ \mu\mathrm{m}$. Experiment carried out by Sam Mugford.

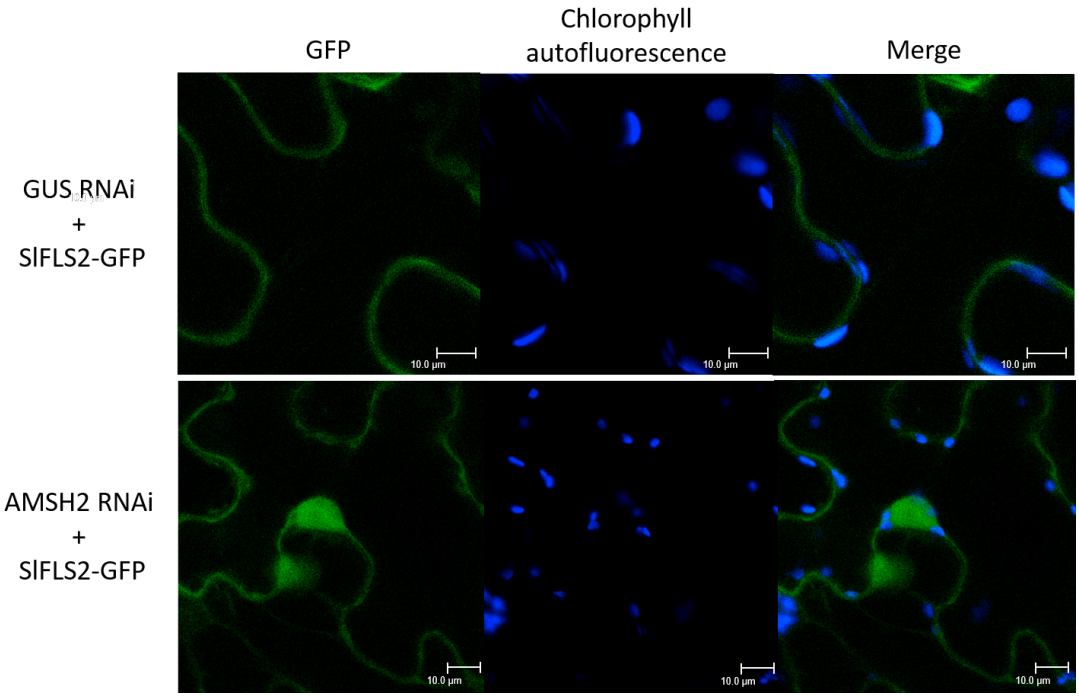


Figure 6.11: **Knock-down of AMSH2 in *N. benthamiana* alters cellular localization of FLS2**. GFP-tagged SlFLS2 was expressed transiently in *N. benthamiana* leaves alongside RNAi constructs for dsAMSH2 and a dsGUS control. Transient expression of an RNAi construct against NbAMSH2 in *N. benthamiana* leaves led to SlFLS2-GFP localisation in the nucleus as well as on the cell periphery. This was not seen in leaves infiltrated with an RNAi construct for GUS, where SlFLS2-GFP expression was only seen on the cell periphery. Images show z-projections of GFP and chlorophyll auto-fluorescence signals in *N. benthamiana* leaf cells. All scale bars are $10\ \mu\mathrm{m}$. Experiment carried out by Sam Mugford.

6.3 Discussion

The yeast two-hybrid screen of Mp10 against an insect-exposed Arabidopsis protein library identified AMSH2 as a possible Mp10 interactor. It is encouraging that out of over 500,000 independent clones, only three were found and confirmed to be interactors, all of which corresponded to AtAMSH2. We also used yeast two-hybrid to check the interaction between Mp10 and AMSH2 homologs AMSH1 and AMSH3, and only found interaction of Mp10 with AMSH2, though more investigations are needed to check that all proteins were expressed in yeast. The finding that heterologous expression of AMSH2 in *N. benthamiana* increases the ROS burst in response to a PAMP, whereas expression of AMSH1 and 3 does not, indicates a biological significance for Mp10 interaction with AMSH2, rather than with AMSH1 or 3. However, *amsh1* mutants display chlorosis under certain conditions (490), a phenotype that is also seen when Mp10 is expressed *in planta* (46). It is a possibility that Mp10 interacts with AMSH1 and 3 as well as AMSH2, and it is this that negatively effects the plant when Mp10 is present at high levels. More investigation is needed to identify how specific the interaction of Mp10 with AMSH2 is, specifically *in planta*. My initial coimmunoprecipitation experiment suggested an interaction solely with AMSH2, but I have not been able to replicate this result.

We also checked the interaction of Mp10 with all predicted splice variants of AMSH2; AMSH2.1, AMSH2.2 and AMSH2.3. Mp10 was found to interact with both AMSH2.1 and AMSH2.3 equally well. This is unsurprising as the two are extremely similar. Mp10 did not interact with AMSH2.2, which differs quite substantially from AMSH2.1 and AMSH2.3. This lack of interaction with the AMSH2.2 variant can be used to narrow down the region of AMSH2 involved in Mp10 interaction. The C-terminal of all AMSH2 splice variants is the same, so Mp10 must interact with the N-terminal of the protein; either in front of, or at the front portion of, the MPN domain (see Figure 6.4b). An alternative hypothesis is that the AMSH2.2 splice variant is not as stable as AMSH2.1 and 2.3 in yeast, leading to a lack of interaction. Further investigation using AMSH2 mutants in a yeast two-hybrid screen could check protein expression and narrow down the zone of interaction further. This may identify regions of AMSH2 that can be changed to stop Mp10 interaction without a loss of function of the protein.

Interestingly, we found that a double mutant of Mp10 that can no longer block the ROS and calcium bursts, Mp10 Y40F W120A, can also interact with AMSH2.1 and AMSH2.3. This indicates that binding to AMSH2 alone is not enough to confer effector action. Mp10

is a chemosensory protein (CSP), and CSPs are known to bind small hydrophobic ligands that induce a conformational change of the CSP (448). The mutations present in Mp10 Y40F W120Y are located both on the exterior and in the binding pocket of Mp10 (see Chapter 5). As the mutations do not alter AMSH2 binding, a process that would be predicted to occur on the exterior of Mp10, it is possible that loss of ligand binding in the internal pocket or prevention of conformational change is why Mp10 Y40A W120Y cannot block calcium and ROS bursts. The presence and binding of some other factor by Mp10 may therefore be required for its effector action. It would be useful to investigate whether Mp10 Y40F W120Y also disturbs FLS2 localisation, as wild-type Mp10 does. This would clarify whether Mp10-induced change in receptor localisation is responsible for the interference with ROS and calcium bursts.

Mp10 is able to block both the ROS and calcium bursts that form an early part of PAMP-triggered immune (PTI) responses, suggesting that its target is involved in early PTI. As AMSH2 levels in the plant have an effect on the ROS burst, this indicates that its action is upstream of ROS production, fitting with a role in early PTI. Not much is known about AMSH2 function; a T-DNA mutant with significantly reduced AMSH2 transcript levels has not yet been identified, so mutant analysis has not been possible (490). Lack of AMSH2 mutant discovery suggests that an absence of functional AMSH2 may be lethal; AMSH2 may therefore have an important role in the plant. The *N. benthamiana* homolog of AMSH2 also increased the ROS burst to flg22, though not significantly so, suggesting AMSH2 has the same function in different plant species. AMSH2 has been conserved in plants from ancestral angiosperms and is present in both monocots and eudicots (490). This indicates that AMSH2 may have the same function in immunity throughout angiosperms. Similarly to this, Mp10 is conserved and functional throughout aphid species (Chapter 5 and (316)). Mp10-AMSH2 interactions could therefore be a feature of many different aphid-plant interactions.

AMSH2 is likely to be acting as a deubiquitinating enzyme (DUB) in the plant, due to the presence of its MPN domain. This domain marks AMSH2 out as a metalloprotease type DUB, which is capable of cleaving K63-linked polyubiquitin chains (496). Human AMSH is capable of cleaving K63-linked polyubiquitin chains, but not the last ubiquitin molecule directly attached to protein receptors (497). In human cells, K63-linked polyubiquitination of the epidermal growth factor receptor (EGFR) is required for rapid endocytic sorting and receptor degradation (498). AMSH cleaves these K63-linked chains off the receptor, leaving monoubiquitin, which decreases the rate of receptor degradation and allows sustained

signaling activity of EGFR (498). Human AMSH has also been found to directly interact with the calcium sensing receptor (CaR) and modulate its trafficking and calcium signaling (499; 500). CaR is a G-protein-coupled receptor, demonstrating that AMSH has a role in regulation of different receptor types. AMSH2 in plants may have a similar role to human AMSH; removing polyubiquitin from receptors such as FLS2 in order to prolong immune signaling. I have found subunits of G-proteins to be involved in the defense response to aphids (Chapter 4), and plant G-proteins have also been implicated in calcium and ROS signaling (501). Therefore G-Protein Coupled Receptors (GPCRs) could also be possible targets of AMSH2, affecting PTI signaling. In agreement with a role for AMSH2 in PTI signaling, inhibition of AMSH2 activity by Mp10 or knock-down using RNAi, increases FLS2 localisation in the away from the plasma membrane and decreases the PTI ROS response. This is in contrast to addition of AMSH2, which increases the ROS response. Mp10 could therefore act by interfering with AMSH2 to increase the degradation rate of immune receptors, which in turn reduces the calcium and ROS bursts of early PTI.

Investigations are increasingly finding that, like animal receptors, plant immune receptors also undergo constitutive recycling between the plasma membrane and endosomal compartments as well as ligand-induced endocytosis leading to vacuolar degradation (502). FLS2 has been found to traffic through ARA6 labelled compartments after flg22 induction, where I have found that AMSH3 and AMSH2 proteins are also present (157; 494). As a DUB, AMSH2 should be opposing the action of proteins that attach ubiquitin onto proteins. In plant cells, it is E3 ubiquitin ligases that specify the target protein and add ubiquitin onto it (154). Interestingly, a group of E3 ubiquitin ligases, PUB22, 23 and 24, have been found to act as negative regulators of PTI. The pub22/23/24 triple mutant shows an enhanced ROS burst to flg22 and chitin, increased expression of defense-related genes and an increased resistance to pathogens (503). This suggests that these PUBs are involved in control of the early components of plant defense. AMSH2 may therefore be acting in opposition to the ubiquitinations carried out by PUB22/23/24 to ensure a balanced immune response.

Interestingly, PUB22 has been found to interact with subunit Exo70B2 of the exocyst complex and mediate its ubiquitination and degradation upon flg22 perception (504). Exo70B2 is involved in the regulation of PTI signaling in response to both flg22 and chitin via its role in vesicle trafficking, so has a role in both BAK1-dependent and -independent immune signaling (505; 504). This provides another potential function for AMSH2 in plant cells, with a role in the deubiquitination of exocyst components, as well as immune receptors

themselves. Either way, by targeting and preventing AMSH2 function, Mp10 would prevent the correct trafficking of receptors, leading to the inhibition of ROS and calcium bursts.

Though exact AMSH2 function in plant cells is unknown, the function of the AMSH2 homologs AMSH1 and AMSH3 has been identified. Both AMSH1 and AMSH 3 interact with components of ESCRT (Endosomal Sorting Complex Required for Transport) III; VSP24.1 and VSP2.1 (493; 490). This interaction is consistent with AMSH1 and 3 having a role in late endosomal/multivesicular body (MVB) trafficking, which is downstream of receptor endocytosis (506). AMSH1 and 3 may not have roles early in PTI, as the decision to recycle or degrade receptors occurs before receptors reach the late endosome (507). AMSH1 and 3 interact with ESCRT III components via the UPS8 domain at their N-termini (493; 490). AMSH2 lacks this N-terminal domain so is unlikely to interact with VPS proteins, indicating that it has a different role in the plant cell from AMSH1 and AMSH3. Lack of the N-terminal also makes AMSH2 a much smaller protein so it may move more freely in the plant cell cytoplasm than AMSH1 and 3, enabling AMSH2 to have different targets. This is in agreement with my confocal data showing that AMSH2 is distributed throughout the cell, unlike AMSH3.

AMSH3 and AMSH1 have both been implicated in plant-microbe interactions. When Katsiarimpa et al. expressed an enzymatically inactive form of AMSH3 in *N. benthamiana*, the effector AvrPtoB was no longer able to degrade the chitin receptor CERK1 (493). AvrPtoB works to remove CERK1 from the membrane by targeting it for degradation by ubiquitinating it (264; 453), so it seems that AMSH3 forms a part of this pathway below ubiquitination and is required for the endocytic degradation of CERK1. Decreased AMSH3 levels would therefore reduce pathogen success, which is the opposite of AMSH2, where reduction in levels decreases PTI responses and so would act to increase pathogen success. This further supports an AMSH2 function that is different to its homologs. AMSH1 mutants show altered pathogen susceptibility, with increased susceptibility to the necrotrophic fungus *Alternaria brassicicola* and increased resistance to the biotrophic fungus *Erysiphe cruciferarum*. However, this is suggested to be due to a lack of functional autophagy, leading to hyperactivation of the salicylic acid (SA) pathway, high PR gene transcript accumulation and increased levels of programmed cell death, rather than a direct action on components of plant immunity such as receptors (490). AMSH1 has also been found to be required for rhizobial infection and nodule organogenesis in *Lotus japonicas*, though its deubiquitination targets are unknown (508). Despite these links to plant-microbe interactions, neither AMSH1 nor AMSH3 have been identified as pathogen effector targets. AMSH2 as

a DUB therefore presents a novel target of pest/pathogen effectors.

The discovery of a DUB as a potential aphid effector target is interesting. In plant immunity, ubiquitination has roles in both PTI and ETI, including regulation of receptors involved in these systems (506). Plant pathogen effectors have been found that target components of the ubiquitination pathway both to promote and prevent ubiquitination (509; 510). Some bacteria can turn the plants own protein degradation system against them. For instance, the *P. syringae* effector HopM1 inhibits PTI in a 26S-proteasome dependent manner (511; 512). One of its targets is the Arabidopsis protein MIN7, which it targets for polyubiquitination, leading to MIN7 degradation by the 26S proteasome (270). AtMin7 is an ARF-GEF (adenosine diphosphate ribosylation factor, guanine nucleotide exchange factor) involved in vesicle trafficking, including that involved in callose deposition. HopM1 therefore suppresses cellular trafficking involved in immunity via activity of the plant ubiquitin system. AvrPtoB from *P. syringae* directly ubiquitinates the immune receptors CERK1 and FLS2, which targets them for degradation and reduces the ability of the cell to respond to PAMPs (265; 264). Conversely, pathogen effectors can also suppress the activity of the ubiquitin system to their own benefit, for instance the rice blast fungus, *Magnaporthe oryzae* produces the effector protein AvrPiz-t which interacts with the E3 ligase APIP6 to suppress its activity via degradation. APIP6 has a positive function in PTI, so this removal leads to increased susceptibility to the pathogen (513). Perturbation of the plant ubiquitin proteasome system via targeting a DUB like AMSH2 is therefore a valid route for an aphid effector to interfere with the plant immune response, including by altering cellular trafficking and increasing the rate of receptor degradation.

Chapter 7

The elucidation of aphid effectors that interact with plant NBS-LRR proteins and other aphid effectors

Contributors: Claire L. Drurey, Carlos Caceres, Sam Mugford, Richard Michelmore and Saskia A. Hogenhout

7.1 Introduction

So far, my investigations have focussed on the activation of PAMP-triggered immune pathways by aphids and the effector Mp10 present within GPA saliva that is able to subvert this. Though Mp10 can block the calcium and ROS bursts that form a part of the plant immune response, it also induces chlorosis in the plant (46). This chlorosis is dependent on SGT1, a ubiquitin-ligase associated protein that is required for certain types of NBS-LRR mediated disease resistances, probably via supporting R-protein complex formation and preventing their degradation (426). This evidence provides an indication that effector-triggered immunity may also play a role in the plant response to aphids.

Induction of effector-triggered immunity (ETI) occurs when plant resistance (R)-proteins detect a pathogen effector either directly or indirectly. All R-proteins contain a nucleotide-binding site (NBS) also known as an NB-ARC domain and most R-proteins are NBS leucine-rich repeat (NBS-LRR) proteins from either the Toll/interleukin-1 receptor (TIR)-domain containing or coiled coil (CC)-domain containing subfamilies. They have variable amino- and carboxy-terminal domains, which detect various effectors from diverse pathogens. The interaction between effector and R-gene is said to be gene-for-gene, with a specific effector encoded by a pathogen gene being detected by a specific R-protein encoded by a plant gene. ETI occurs downstream of PTI and is seen as an accelerated and amplified PTI response, leading to disease resistance and often involving a hypersensitive cell death response (HR) at the site of infection (273).

There is already evidence that R-proteins are involved in plant defense against insect pests. Several R-genes implicated in plant resistance to insect herbivores have been cloned including Bph14, Mi-1.2 and Vat. All are members of the CC-NBS-LRR subfamiliy. Bph14 confers resistance to brown planthopper in rice, involving the salicylic acid signaling pathway, callose deposition and trypsin inhibitor production and Vat confers resistance to one biotype of the melon-cotton aphid, *Aphis gossypii* (90; 514). Mi-1.2 confers resistance against phloem-feeding herbivores both above and below ground, including aphids, whiteflies, psyllids and root-knot nematodes (280; 81; 281; 282) and was recently also found to have an action against the zoophytophagous minute pirate bug, which does not feed from the phloem, but from the xylem, epidermal and mesophyll cells (515). This wide ranging recognition is unusual for an R-gene, and could hint at the presence of a shared effector that elicits defense in these separate herbivore species. Breeding programmes for insect resistance crops have elucidated quantitative trait loci (QTLs) involved in resistance

to specific aphid biotypes, particularly in wheat and barley. These loci are often found in areas rich for NBS-LRR proteins, for instance *Acyrthosiphon*-induced necrosis (AIN) in *Medicago truncatula*, which confers resistance to the bluegreen aphid, lays in a cluster of CC-NBS-LRR sequences (516).

Further support for the gene-for-gene model in plant-insect interactions comes from the study of the Hessian fly, a gall midge that is a pest on wheat. Wheat varieties carry resistance genes that are specific for certain hessian fly biotypes (517). Whereas the wheat R-genes have not yet been cloned and functionally characterized, the avirulence of Hessian fly biotypes maps to genomic regions encoding small predicted secreted proteins, which are expressed in Hessian fly salivary glands (518). It is therefore likely that the wheat resistance genes recognize the secreted proteins in Hessian fly saliva, conferring resistance to specific Hessian fly biotypes in certain wheat varieties, in a gene-for-gene model-like fashion. There is also evidence that specific amino acids in the salivary proteins are under strong selection in Hessian fly, so these proteins may be under selection to avoid recognition by the plant (518).

Components of ETI other than R-genes have also been implicated in aphid resistance. The chlorosis response to Mp10 in *N. benthamiana* requires SGT1, which acts a complex with HSP90 and RAR1 (Required for Mla12 Resistance 1) in order to chaperone and stabilise NBS-LRR proteins needed for ETI (519). Interestingly, both SGT1 and HSP90, but not RAR1, are needed for the resistance to potato aphids mediated by the R-gene Mi-1 (471). Phytoalexin deficient 4 (PAD4) has also been implicated in both aphid defense and ETI. PAD4 is a lipase-like protein that is required for ETI mediated by the TIR-NBS-LRR class of R-proteins, which includes SA-mediated HR (520). Interestingly, PAD4 but not SA is required for resistance to GPA in Arabidopsis (521; 522). PAD4 mediated resistance to aphids involves both antibiosis (a reduction in aphid fecundity) and antixenosis (deterrence of aphid settling and feeding) (521). The antibiosis and antixenosis effects can be split via mutation in the lipase domain of PAD4, suggesting that PAD4 affects aphids via involvement in more than one pathway (522). PAD4 is also involved in basal resistance, where it acts upstream of phytoalexin biosynthesis, including camalexin production (371). Camalexin production may contribute to the antibiosis response against GPA, as camalexin has negative effects on the aphid, reducing fecundity (238). A PAD4 role in both basal resistance and ETI may therefore be required for full resistance to aphids, though the lack of requirement for SA suggests that the pathways involved may be different to those elucidated in plant-pathogen interactions.

Though there are strong suggestions that R-genes in plants do detect effectors from insects, no R-gene-insect effector pair has been found to date. As we have identified a suite of candidate aphid effectors in the lab, including Mp10, which may be recognised by the plant in an R-gene mediated manner (46), it was decided to screen these candidate effectors for interactions with plant R-proteins. The Michelmore lab at the University of California, Davis has a plant NBS-LRR library, containing mainly Arabidopsis CC- and TIR-NBS-LRRs. We collaborated with them, sending the candidate aphid effectors in yeast two-hybrid vectors for high-throughput screening against the NBS-LRR library via yeast two-hybrid. The Michelmore lab results identified aphid effector candidates that could interact with NBS-LRR proteins, uncovering 23 potential R-proteins for aphid effectors, one of which was RPS4. We also identified a number of GPA effectors that may interact with each other.

7.2 Results

7.2.1 Yeast-two-hybrid screen of candidate aphid effectors against plant R-proteins

A functional genomics screen identified 43 candidate aphid effectors in GPA (46). I cloned these effectors, without their signal peptides, into Gateway-compatible pLAW10 and pLAW11 vectors for use in a yeast two hybrid screen (details can be found in Materials and Methods). I also included the genes encoding *Acyrthosiphon pisum* Ap1, Ap2 and ApC002 proteins, which are orthologs of the candidate effectors Mp1, Mp2 and MpC002 from GPA (44). The effector clones were sent to the Michelmore laboratory (University of California, Davis, USA). The Michelmore lab has a large library of sequences that encode partial NBS-LRR proteins from Arabidopsis, as well as crop species such as lettuce and tomato.

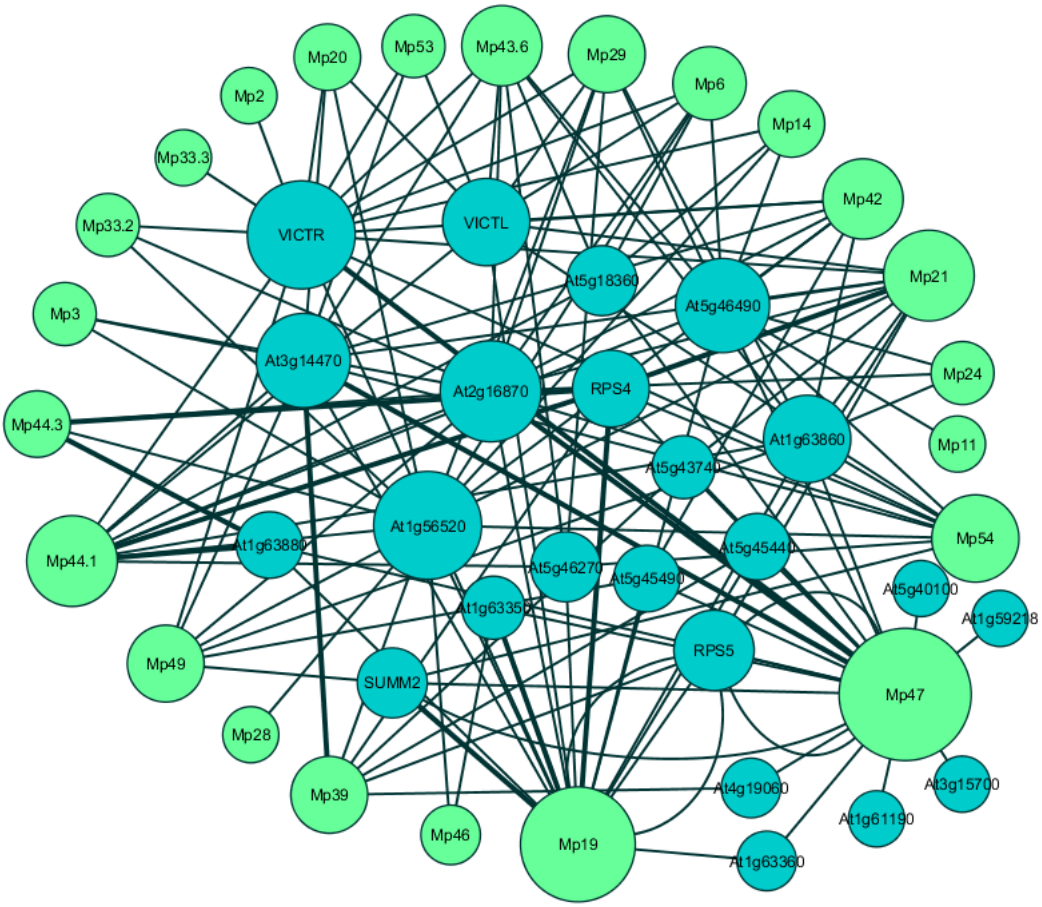
The effectors were tested for interactions with approximately 200 NBS-LRR clones containing sequences of TIR, CC, TIR and NBS, or LRR domains by yeast-two-hybrid assays. Interactions were tested four times, with the candidate effectors as prey (pLAW11) and the NB-LRR fragments as bait (pLAW10). Interactions were graded as weak or strong, depending on the time taken for the colony to grow after plating out. Figure 7.1a shows a schematic overview of all observed interactions with strong interactions as thick lines and weaker ones as thin lines, and the size of the node (representing a protein) correlated to the number of interactions. A table of all interactions identified can be found in Appendix E.

Of over 200 NBS-LRR protein fragments tested, 26 showed an interaction with the aphid proteins. The Arabidopsis NBS-LRR proteins VICTR, AT1G56520 and AT2G16870 show the most interactions with aphid effectors. Although multiple interactions may indicate 'stickiness' of these three NBS-LRR proteins, it is known that some R-proteins can recognise more than one pathogen effector. For example, the first R-gene cloned, Pto (resistance to *Pseudomonas syringae* pv. Tomato) can recognise both avrPto and avrPtoB from *Pseudomonas syringae* directly (523). Interestingly, some of genes of NBS-LRRs that interact with aphid effectors lie adjacently to each other in the Arabidopsis genome. For example, the genes of CC-NBS-LRRs AT1G63350 and AT1G63360, pulled out as interacting with Mp19 and Mp47, lie next to each other on chromosome 1. The TIR-NBS-LRRs AT1G63860 and AT1G63880 (both interact with Mp19, 44 and 47) are also near neighbours, with only one gene between them. VICTL and VICTR are known to be tandem genes, and both have been pulled out in the screen as interacting with a range of aphid

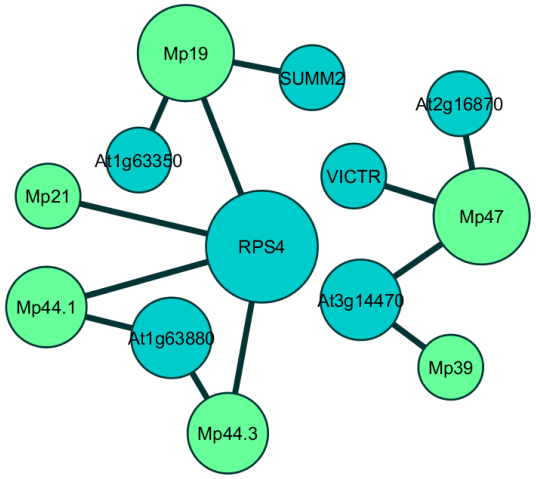
effectors. The TIR-NBS-LRR At5G46490 is also located very close to the VICTL and VICTR pair, and all three interact with Mp6, 19, 21, 29, 42, 43 and 44. NBS-LRRs are known to be located in clusters throughout the genome so perhaps identifying R-proteins located near each other chromosomally is to be expected, though this may also hint at the mechanism and evolution of R-genes, with pairs of proteins needed or tandem duplications producing variant R-proteins to adjust to aphid effector evolution (524; 525; 526). Finally, only 5 of the 23 NBS-LRR proteins identified as interacting with aphid effectors have been previously identified as conferring resistance to a pathogen, or being involved in pathogen resistance. These are RPS5 (AT1G12220, recognises *P. syringae* AvrPphB (527)), SUMM2 (AT1G12280, R-protein guarding MEKK1-MKK1/MKK2-MPK4 cascade (528)), AT1G61190 (recognises ATR39-1 from *Hyaloperonospora arabidopsidis* (529)), AT1G63880 (R-protein effective against *Leptosphaeria maculans* (530)) and RPS4 (AT5G45250, detects avrRps4 and PopP2 (531)). Given that NSB-LRR proteins often confer specific resistance to pathogens and pests (274) it is promising that the GPA effectors interact mostly with NBS-LRRs that have no known interactions with other effectors.

The clones of NBS-LRRs in the screen contained different parts of the NBS-LRRs. It was originally suggested that the LRR portion of NBS-LRR proteins acts as the effector binding domain, for instance the ATR1 effector is recognised by the LRR-domain of the TIR-NBS-LRR RPP1 (532), and mutating the LRR domain of CC-NBS-LRR Rx extends recognition of potato virus X strains (533). However it is increasingly found that effector interactions occur in other domains of R-proteins too, for instance the N R-gene from tobacco interacts with the p50 protein of the tobacco mosaic virus via its TIR domain (534). Only three LRR regions were pulled out as interactors of candidate aphid effectors. These are the LRR portions of RPS5, SUMM2 and the Arabidopsis CC-NBR-LRR gene AT1G63360, which interact with Mp19, 39, 47; Mp19, 47 and 54; and Mp19 and 47 respectively. All the other NBS-LRR portions pulled out as interacting with a candidate aphid effector contain the N-terminal TIR + NBS or TIR/CC domains. My results therefore support the idea that effector binding can occur at both the LRR domain of the R-protein, and the N-terminal region.

I compared the NBS-LRR proteins pulled out in the yeast two hybrid screen with the RNAseq data generated by the lab from aphid exposed Arabidopsis leaves (described in Chapter 6). Interestingly, of the 23 NBS-LRR proteins, which were identified as interacting with aphid effectors in the yeast two-hybrid screen, 7 were significantly transcriptionally changed in plants exposed to the pea aphid *A. pisum*, which does not colonize Arabidopsis



(a)



(b)

Figure 7.1: **Results of the yeast two-hybrid screen of aphid effector interactions with plant NBS-LRR proteins**. 7.1a Results of the yeast two-hybrid screen to detect aphid effector interactions with plant NBS-LRR proteins. I generated the yeast two-hybrid clones and the Michelmore laboratory (University of California, Davis, USA) conducted the yeast two-hybrid screen. 7.1b Simplified scheme showing only the strong aphid effector interactions with plant NBS-LRR proteins.
Aphid effectors are show in in green and plant R-proteins in blue. Thicker lines show strong interactions. Node sizes relate to the numbers of interactions found with that protein. Multiple connections between nodes shows interaction with more than one cloned segment of the NBS-LRR protein (e.g. both LRR and CC-NBS portions).

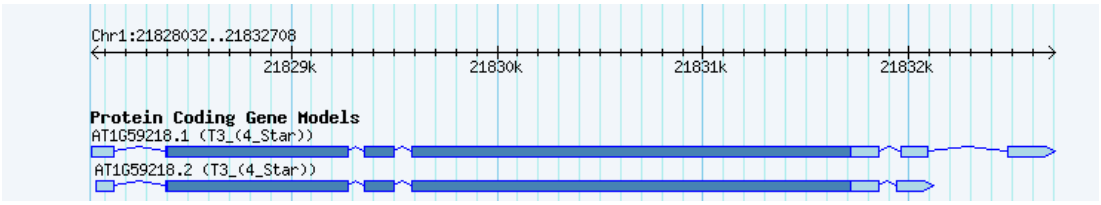
(see Table 7.1). Whereas in healthy plant tissue NBS-LRR genes are constitutively expressed at low levels, the expression of some NBS-LRR genes is upregulated in response to bacterial flagellin (535; 536). This overlap between the yeast-two-hybrid and RNA-seq data may therefore suggest that these NBS-LRR proteins play a role in detection of aphid effectors. Interestingly, none of these genes were significantly transcriptionally changed in response to GPA exposure. This is in agreement with the hypothesis that Arabidopsis is non-host to pea aphid, whereas this plant is colonized by GPA.

NBS-LRR Information		Yeast two-hybrid data		RNAseq data					
Gene Name	Type	Domain tested	Candidate aphid effectors interacted with	>1 splice variant detected	Gene model	M. persicae		A. pisum	
						log2 fold change	adjusted P value	log2 fold change	adjusted P value
SUMM2 (AT1G12280)	CC	CC	Mp47, Mp49	no	AT1G12280.1	0.193	1	1.319	0.001
		LRR	Mp19, 47, 54						
AT1G59218	CC	CC	Mp47	yes	AT1G59218.1	0.306	1	0.481	0.352
					AT1G59218.2	0.132	1	1.462	0.001
AT1G63360	CC	LRR	Mp19, Mp47	no	AT1G63360.1	0.216	1	1.065	0.002
AT1G63860	TIR	TIR	Mp19, 21, 24,29,42, 43.6, 44.1, 47, 49, 54	yes	AT1G63860.1	0.949	1	-0.352	1
					AT1G63860.2	0.191	1	-2.028	0.003
AT5G43740	CC	CC	Mp21, 39, 47	yes	AT5G43740.1	0.090	1	-0.707	1
					AT5G43740.2	0.189	1	1.69	0.003
AT5G46270	TIR	TIR	Mp19, 29, 43, 44.1, 54	no	AT5G46270.1	0.211	1	0.874	0.021
VICTL (AT5G46510)	TIR	TIR	Mp6, 19, 20, 21, 29, 42, 43, 44.1, 53, 54	no	AT5G46510.1	0.057	1	-1.404	0.011

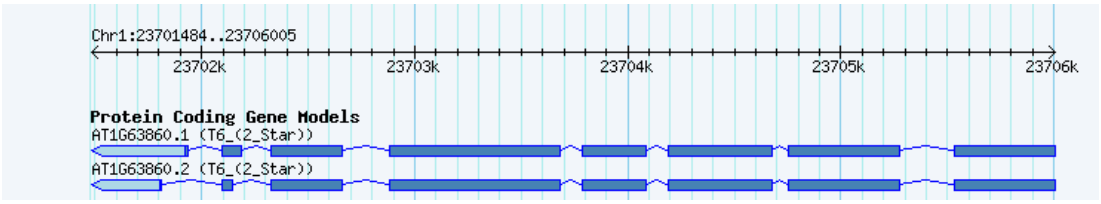
Table 7.1: **NBS-LRR proteins that interact with candidate effector proteins and are also differentially regulated in Arabidopsis plants exposed to aphids compared to non-aphid exposed plants**. Data listed are from the aphid effector - plant NBS-LRR protein yeast-two hybrid assays, and the RNAseq experiment (Chapter 6) of Arabidopsis plants exposed to GPA and the pea aphid (*A. pisum*). Not all differentially regulated R-genes from Arabidopsis are represented here, just those identified from the Y2H screen. Green indicates upregulation and red indicates downregulation of the gene in response to aphid exposure compared to non-aphid challenged controls.

For 3 of the 7 differentially regulated NBS-LRRs in pea aphid-exposed Arabidopsis, I found that two different splice variants of the gene were identified in the RNAseq data. Interestingly, only one of the two splice variants was found to be differentially regulated in each case. This may be due to misannotation of the gene, but the detection of both splice variants in our RNAseq data set suggests that both are transcribed, but only one is responsive to aphid exposure. For these three (AT1G59218, AT1G63860 and AT5G43740), the predicted full length-gene transcript was not differentially regulated by pea aphid exposure, whereas an alternative splice variant was (Figure 7.2). In the case of AT1G63860.2, this results in a shortened protein (862 amino acids compared to 988) lacking part of the carboxyl terminal downstream of the LRR domain as well as a truncation of the 3' untranslated region (UTR). The other two genes are not altered in protein sequence, but have a

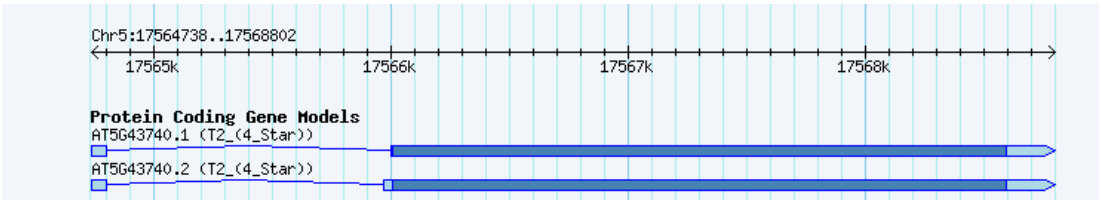
truncated 3' UTR (AT1G59218.2) or a longer 5' UTR (AT5G43740.2). These changes in the lengths of the UTRs could affect the stability or translation of the mRNA and so alter the amount of R-protein in the cell.



(a)



(b)



(c)

Figure 7.2: **Splice variants of R-proteins transcriptionally changed upon aphid exposure**. 7.2a Splice variant AT1G59218.2 which is significantly induced by *A. pisum* exposure has a shorter 3' UTR compared to AT1G59218.1, which is not induced. 7.2b Splice variant AT1G63860.2, which is reduced significantly upon *A. pisum* exposure has both a shorter carboxy terminal LRR of the deduced protein sequence and a shorter 5' UTR compared to AT1G63860.1, which is unchanged by *A. pisum* exposure. 7.2c Splice variant AT5G43740.2, which is significantly induced by *A. pisum* exposure has a longer 5' UTR compared to AT5G43740.1, which is not induced.
Gene models taken from The Arabidopsis Information Resource (TAIR) on www.arabidopsis.org

7.2.2 Further investigation of RPS4-GPA candidate effector interactions

Looking at the strong effector-NBS-LRR interactions of the yeast-two-hybrid screen only (Figure 7.1b), three candidate aphid effectors (Mp19, Mp21 and the two variants of Mp44, Mp44.1 and Mp44.3) interacted with RPS4. RPS4 is a well-known plant TIR-NBS-LRR required for resistance to strains of *P. syringae* expressing avrRps4 (537; 538). It requires the presence of a partner, RRS1, for recognition of effectors, which was not pulled out in this screen (531). To examine further the interaction of RPS4 with these three GPA effectors, I obtained *Agrobacterium tumefaciens* strains containing plasmids of both RPS4 and RRS1 from the lab of Jonathan Jones (The Sainsbury Laboratory, Norwich, UK) and conducted co-expression analyses in *Nicotiana tabacum* leaves, which show clear hypersensitive response (HR) responses in the presence of RPS4 and RRS1 and the effector avrRps4

(see Figure 7.3). A negative control of avrRps4 with the E187A mutation, which does not trigger HR was also included (539). I found that expression of the aphid effectors Mp19, Mp21, Mp44.1 and Mp44.3 with RPS4 and RRS1 did not lead to HR responses, whereas a HR was observed in the RPS4 and RRS1 plus avrRps4 control treatment (Figure 7.3a). Proteins corresponding to the sizes of tagged RPS4, RRS1, the GPA effectors and avrRps4 and the avrRps4 E187A mutant were detected on Western blots (see Figure 7.3b). Thus, the aphid effectors do not appear to trigger HR in the presence of RPS4 and RRS1.

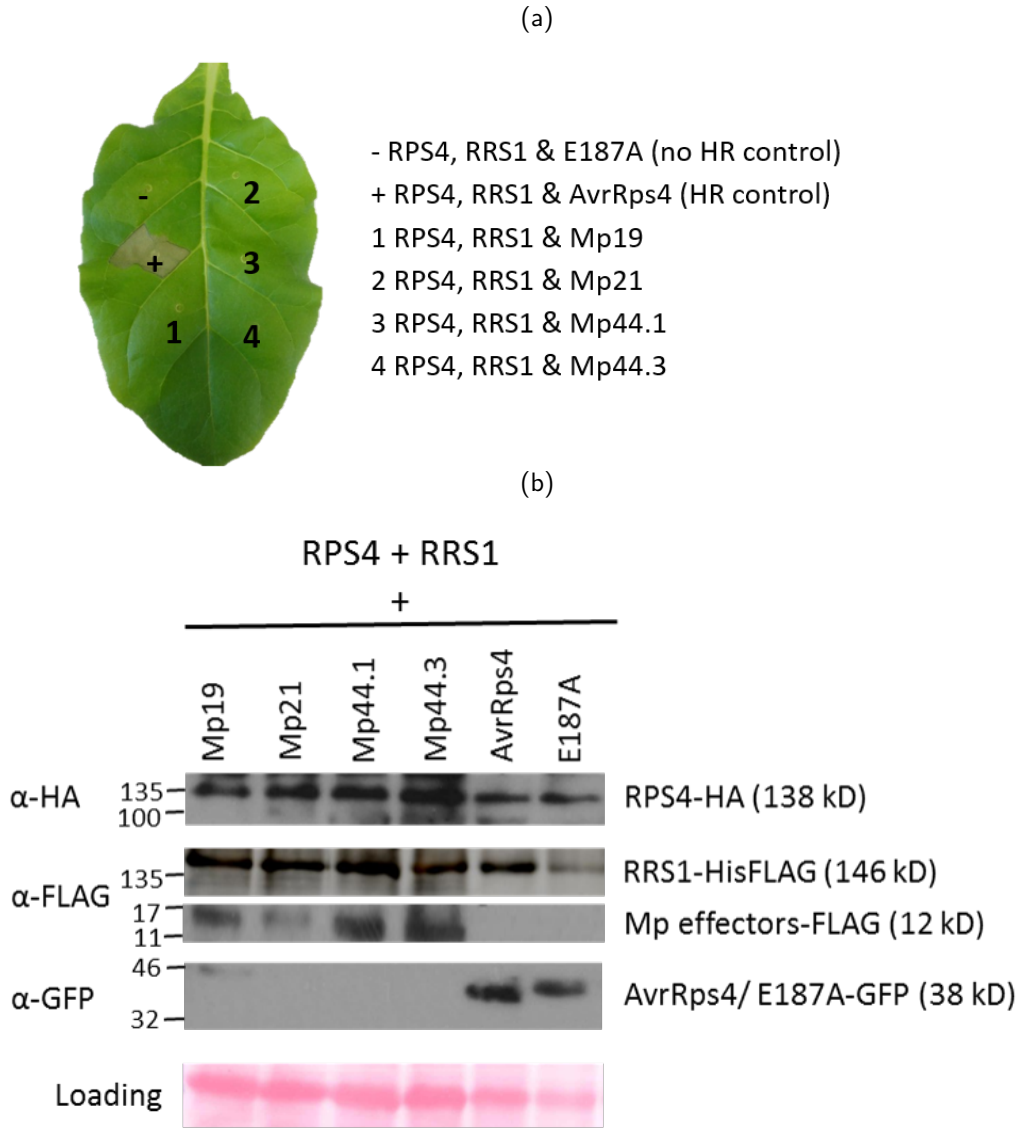


Figure 7.3: **Candidate GPA effectors found to interact with RPS4 in the yeast two-hybrid screen do not induce RPS4-mediated cell death responses**. 7.3a Mp19, Mp21, Mp44.1 and Mp44.3 do not activate a RPS4/RRS1 mediated hypersensitive response (HR). Agroinfiltration assays were performed in 3.5 to 4.5-week-old *N. tabacum* leaves, and image taken at 3 days after infiltration . This experiment was repeated 3 times, using 3 leaves per repeat, with the same results in each leaf. 7.3b Proteins corresponding to the sizes of HA-tagged RPS4, FLAG-tagged aphid effectors, FLAG-tagged RRS1 and GFP-tagged AvrRps4 and AvrRps4 E187A were detected in *N. tabacum* leaves. Tissue samples taken from a fourth infiltrated leaf at 2.5 dpi, before HR occurred.

Alternatively, aphid effectors may interact with NBS-LRRs in order to prevent their activation and the onset of ETI. I carried out an experiment to examine if Mp19, Mp21 and Mp44 could inhibit RPS4 activation by avrRps4. I included AvrRps4 E187A as a control in this assay because this mutant prevents RPS4-RRS1 activation by AvrRps4 (personal communication, Kee Sohn). The results from this assay were highly variable (see Figure 7.4). In half of the cases (2 experiments with 3 leaves each as biological controls), HR was observed in the presence of the aphid effectors at a similar level as the avrRps4 control, but in these cases HR also progressed in the presence of avrRps4 and avrRps4 E187A, which is supposed to inhibit HR. In the other half of the experiments (2 experiments with 3 leaves each as biological controls), the GPA effectors and avrRps4 E187A appeared to inhibit HR compared to the avrRps4 control. Whereas this second set of experiments suggests that the GPA effectors suppress RPS4-RRS1 activation, I cannot rule out the possibility that one of the components required for triggering the HR response is missing. This experiment therefore requires repeating and further assessment of protein presence in the infiltrated leaf areas by Western blot analyses.

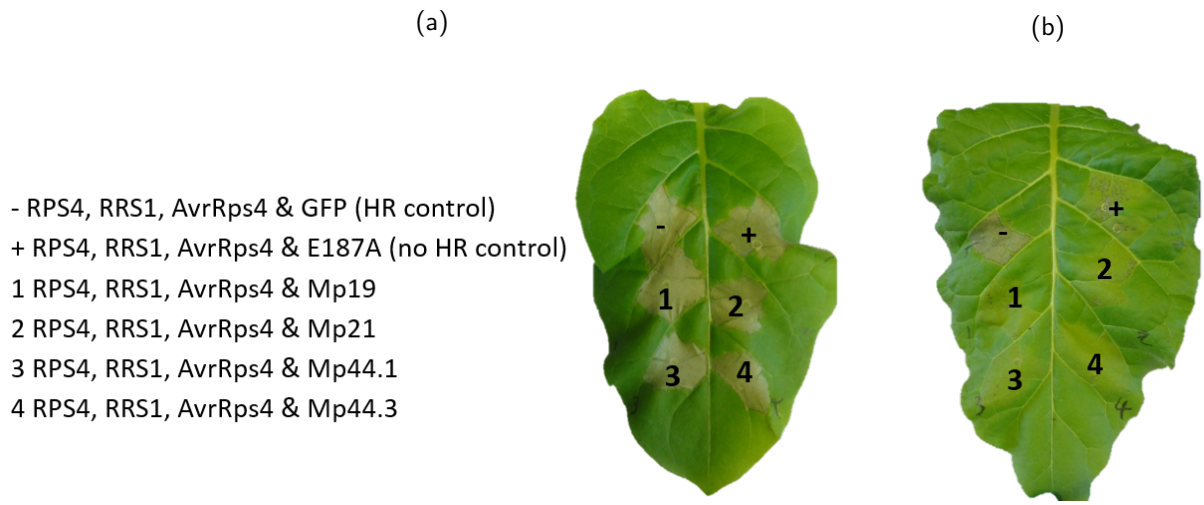


Figure 7.4: **Experiments to investigate inhibition of RPS4/RRS1-mediated cell death responses in *N. tabacum* showed variable results**. 7.4a Expression of Mp19, Mp21, Mp44.1 or Mp44.3 in *N. tabacum* did not prevent the AvrRps4-induced, RPS4/RRS1-mediated hypersensitive response in half of cases seen. 7.4b Expression of Mp19, Mp21, Mp44.1 or Mp44.3 in *N. tabacum* inhibited the AvrRps4-induced, RPS4/RRS1-mediated hypersensitive response in the other half of experiments.
Agroinfiltration assays were performed in 3.5 to 4.5-week-old *N. tabacum* leaves, and image taken at 3 days after infiltration . This experiment was repeated 4 times, using 3 leaves in each repeat, with two experiments showing each of the results shown above.

7.2.3 Aphid effector-effector interactions identified via yeast-two-hybrid

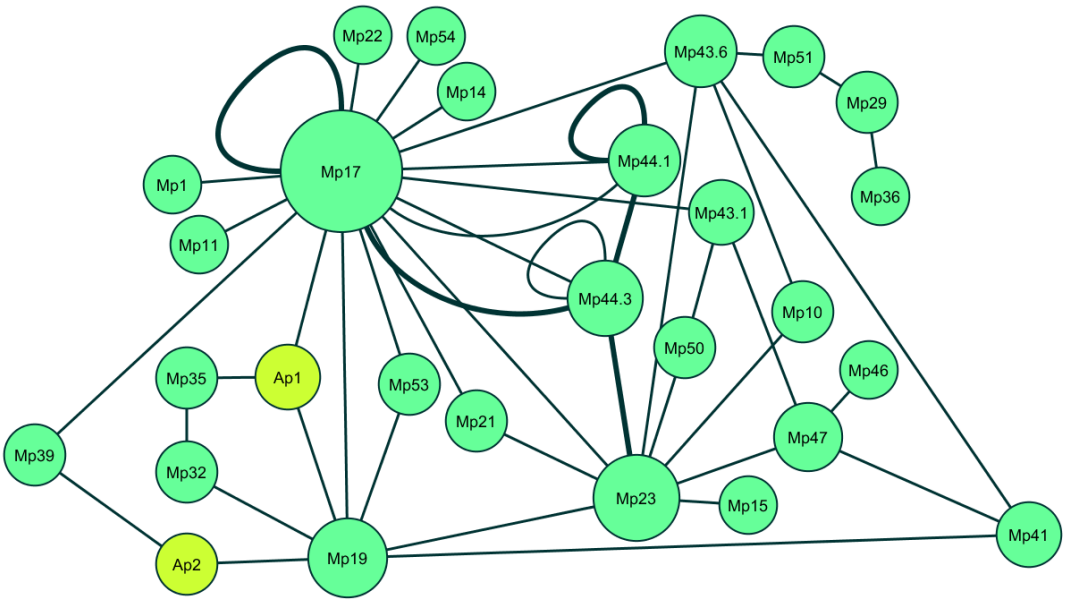
The Michelmore lab also conducted yeast two-hybrid analyses to assess aphid effector-effector interactions. Interestingly, several aphid effectors interacted with each other (see

Figure 7.5a, a table of all interactions identified can be found in Appendix E). From the figure, it can be seen that Mp17 and Mp23 show a large number of interactions with other candidate aphid effectors. This may be as they are "sticky" proteins, or are autoactive. However, Mp17 and Mp23 were not found to interact with any proteins in the yeast two-hybrid screen against plant NBS-LRR proteins, suggesting that they are not 'sticky', but show specific interactions with aphid proteins. The lack of interaction with plant NBS-LRRs also suggests that they are not delivered into the plant in order to be recognised. Both Mp17 and Mp23 are small proteins that contain a R&R (Rebers and Riddiford) domain belonging to the chitin binding 4 superfamily. The other candidate effectors do not contain this domain. The R&R consensus is present in the largest structural cuticular protein (CP) family in arthropods; the CPR family (540). CPR proteins are classified according to three different forms of the RR motif: RR-1, RR-2, and RR-3. Proteins with an RR-1 domain generally associated with soft or flexible cuticles, whereas those with RR-2 are preferentially found within hard cuticles (541). The RR-3 motif has only been found in a small number of sequences, and it is still unclear what their function is (542). Using CuticleDB, a relational database of Arthropod cuticular proteins, it was confirmed that Mp17 belongs to the RR-2 group and Mp23 to RR-1 group (543) (see Figure 7.6).

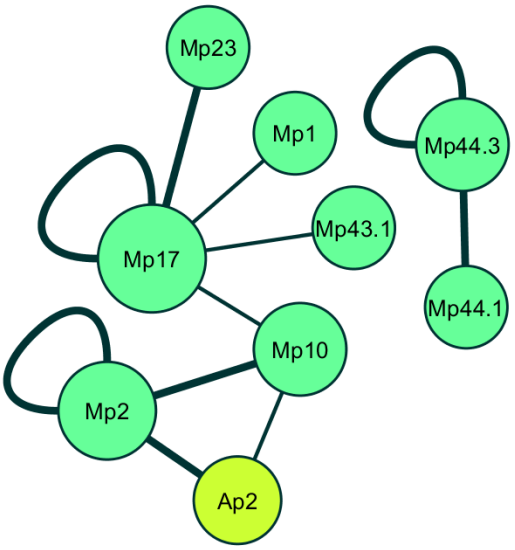
Mp17 shares 86% and 64% identity with CP3 and CP1 proteins from GPA (see Figure 7.6b) (544). With a chitin binding domain in the centre and numerous repeats and proline residues at the N- and C-termini, which are likely to form helicoidal structures, the structures of these proteins suggest that they anchor onto chitin domains (via the central chitin binding domain) and other proteins (via helicoidal structures). Therefore, there is a possibility that Mp17 may adhere to the inside of aphid stylets, and there, interact with aphid effectors to hold them within the stylet. The effectors may be released into the plant during aphid feeding.

7.2.4 Aphid effector-effector interactions verified in the lab

To confirm some of the effector-effector interactions, we carried out yeast-two hybrid assays in a different yeast two-hybrid system compared to the one used in the Michelmore lab. For this, the sequences corresponding to the mature protein (without signal peptides) of MpC002, Mp1, Mp2, Ap2, Mp10, Mp17, Mp19, Mp23, Mp43 and Mp44 were cloned into the pGAD (prey) and pLexA (bait) plasmids of the DUALhybrid yeast two-hybrid system (Dualsystems Biotech) for yeast two-hybrid analyses (Figure 7.7). Summaries of interactions can be found in Figure 7.5b. Mp23 and Mp43 showed autoactivation when in the

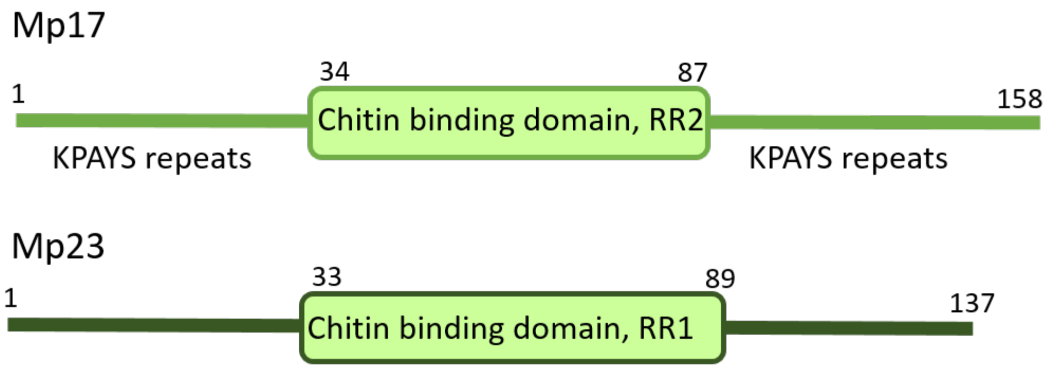


(a)



(b)

Figure 7.5: **Results of the yeast two-hybrid screen of aphid effector-effector interactions**.7.5a Results of the yeast two-hybrid screen to detect aphid effector-effector interactions. I generated the yeast two-hybrid clones and the Michelmore laboratory (University of California, Davis, USA) conducted the yeast two-hybrid screen. 7.5b Results of yeast two-hybrid interactions of some of the GPA effectors conducted in the Hogenhout laboratory. Not all interactions identified in the Michelmore screen could be repeated. Thicker lines show a strong interaction. Node size relates to the number of interactions found with that protein. Multiple connections between nodes shows interaction in both directions in Y2H.



(a)

```
                                                N-terminal repeats
MpCP2        TDVVLTGAPAAYVASSYTSHGPVPW-AYLQPYYQSAPLVAYTAYTAPAAKVIATAPAKIV
MpCP3        -----------YGRDGKQSSYPAPSAAYEQPKYPVSQYSAPSYEAKPQS---YSAPT---
Mp17         ---------------------------------------------Q---YSAPA---
MpCP1        ------------------SPVAAPI-YAAGPAYSAPAYSAAPAYAAPSS---YSAPS---
                                                                  :**:
                                                       RR-2 domain
MpCP2        TPPARTVPVAVAPAKLVAPAVPVAKVVQVVPAQFQPDPSYTFAYQVQDQITGDSKSQEET
MpCP3        -PSYNKQPSQ---SSSAYPSYPS---QKAHAEESYAPRPYKFEYSVNDEHTYGIKSQKEE
Mp17         ---YK--P------AYSAPAYSAP--KAYAPEPAYAPTPYNFEYSVNDPHTYDVHSQSEY
MpCP1        ---YK--P------AYKP--------AAYGHESYDAPAPYNFEYSVNDPHTYDVKSQSEY
                 .  *                                *.* *.*:*  *   :**.*
                          RR-2 domain
MpCP2        RQGD-VVKGRYSLIEPDGTRRTVDYTADPTNGFNAYVQKSDVQQAVF-VPSVSTDDVETI
MpCP3        SDGH-QVKGYYTLLEADGSRRTVEYTADE-NGFNADVKKEEAQGYNKAAPAYNKDS---Q
Mp17         SDGNGYVKGSYSLVEADGSIRTVEYTADDYNGFNAVVKNEGGYK----APSYSAPSYSAP
MpCP1        ADGNGYVKGSYSLVEPDGSTRTVEYTADDYNGFNAVVKKEGGYA----APAYSAPAYSAP
              :*.   *** *:*:* **: ***:****  ***** *::.         .*: .
                               C-terminal repeats
MpCP2        KVDTIEVEQARY-APSGSKPLKNT--LAVPE--------TKTKTGY-------------
MpCP3        AYKS---APAAYSAPSYNKQESS---Y----SAQPSYNAPAYNKQ--ESSYGAPAKQSQE
Mp17         AYK-PAYSAPAYSAPAYK-PAYSAPAYSAPAYSAPAYSAPAYKPAYKPAY----------
MpCP1        AYKAPAYAAPAYSAPAYKAPAYAAPAYSAPAYKSPSYSAPAYKAPYSAPSYSAPAYKAPS
               .        * **: .                          :

MpCP2        ----------------
MpCP3        YEAPSY----------
Mp17         ----------------
MpCP1        YSAPAYSAPAYAAPAY
```

(b)

Figure 7.6: **Candidate effectors Mp17 and Mp23 resemble cuticular proteins**. 7.6a Localization of domains in the linear Mp17 and Mp23 proteins. Both contain a chitin-binding domain at their centre. 7.6b Alignment of Mp17 with MpCP1, CP2 and CP3, minus signal peptides. Conserved residues are marked with an asterisk. Alignment carried out using Clustal Omega (445).

pLexA binding domain plasmid, but not in the pGAD plasmid. Mp17 did at first seem autoactive, but required more stringent conditions for true interactions to be revealed, for instance by growing the yeast colonies on growth media lacking adenine or on higher 3-amino-1,2,4-triazole (3AT) concentrations. Mp17 interacted with Mp1 and Mp10, both of which have some evidence of acting as aphid effectors (46; 44), and may interact with Mp23 and Mp43.1 as well. In addition Mp10 interacted with Mp2, and Mp2 interacted with Mp10 and itself and with the pea aphid (*A. pisum*) homolog of Mp2, Ap2. Mp44.3 interacted with Mp44.1 and itself. The other interactions identified in the Mitchelmore lab for MpC002, Mp1, Mp2, Ap2, Mp10, Mp17, Mp19, Mp23, Mp43 and Mp44 were not confirmed.

Taken together, the effector-effector screen revealed which GPA effectors may form hubs and interact with many other effectors. One candidate for the hub is Mp17. This protein has a RR-2 domain, which interacts with chitin, and two helicoidal structures, which may be responsible for Mp17 interactions with the other effectors. Mp44.1 and Mp44.3 are also interesting proteins for further research, as these interact with other effectors and with NBS-LRR proteins, including RPS4. For the latter, there is preliminary evidence that Mp44.1 and Mp44.3 may inhibit HR triggered by recognition of avrRps4 by the RPS4-RRS1 complex. A further avenue to explore is therefore whether Mp44.1 and Mp44.3 inhibit the triggering of ETI by other GPA effectors.

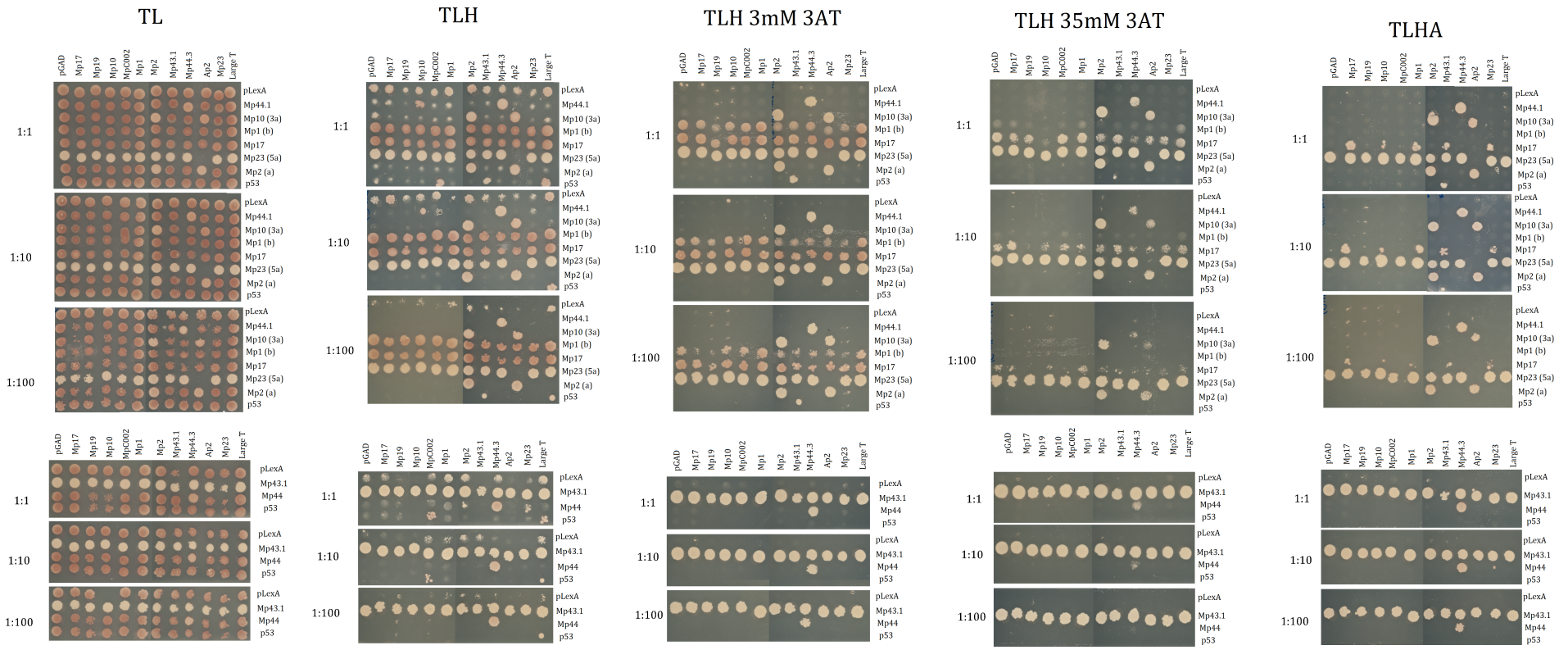


Figure 7.7: **Colony plates of the yeast-two-hybrid assay carried out in the Hogenhout laboratory to verify effector-effector interaction using the DualSystems kit (pGAD and pLExA)**. Yeast colonies were grown on selection media lacking tryptophan (T), leucine (L), hisitidine (H) and adenine (A), as indicated at the top of the plates, and in the presence of different concentrations of 3-amino-1,2,4-triazole (3AT). These experiments were repeated three times with the same results. Experiments were carried out by Carlos Caceres under supervision of myself and Sam Mugford.

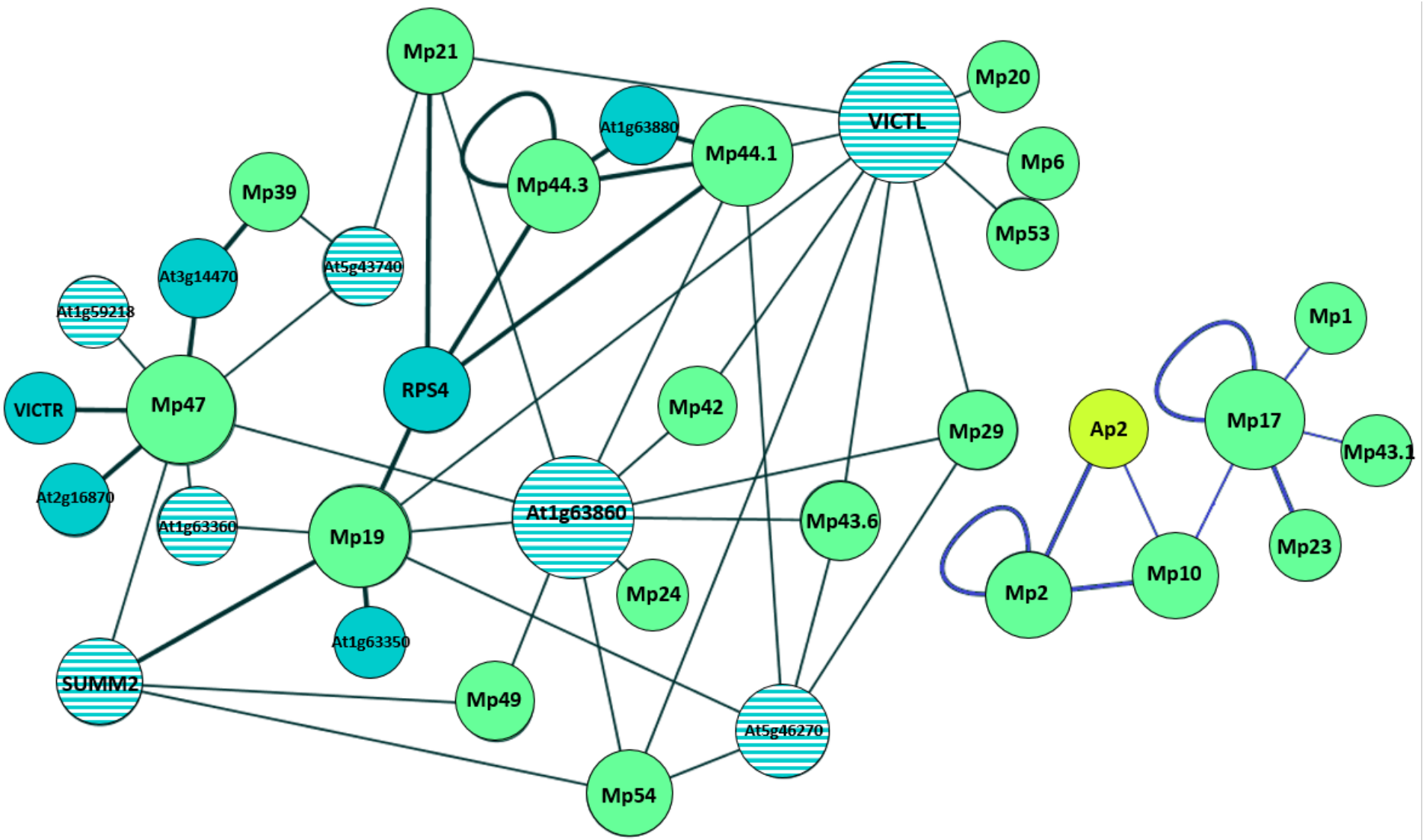


Figure 7.8: **Summary of effector-effector and effector-R-protein interactions found**. Effector-effector interactions verified in the lab are shown (blue lines). Interactions between candidate GPA effectors and NBS-LRRs that were found to be transcriptionally altered in the RNAseq dataset are shown (striped nodes), alongside the strongest effector-NBS-LRR interactions identified via the Michelmore yeast two hybrid screen. Thicker lines show a strong interaction. Node size relates to the number of interactions found with that protein.

7.3 Discussion

Sending our suite of candidate aphid effector proteins for a screen against plant R-proteins has opened doors for multiple avenues of further research. Some candidate aphid effectors were found to interact with several other effectors, but were not identified as interacting with plant NBS-LRR proteins. Likewise, GPA effectors that were identified as interacting with several NBS-LRR proteins were not pulled out as interacting with other effectors. This suggests that these interactions may be specific, and are not caused by general 'stickiness' of aphid effector proteins. Further investigations are needed to identify whether the interactions between candidate aphid effectors and both plant NBS-LRR proteins and other aphid effectors identified in the Y2H screen are biologically relevant and occur in the plant or aphid.

The strongest evidence we have thus far for ETI in plants in response to GPA is the induction of chlorosis and some cell death upon overexpression of the GPA effector Mp10 *in planta* which is dependent upon SGT1 (46). However, no NBS-LRRs were found to interact with Mp10 in the Michelmore lab yeast-two-hybrid screen, whereas this protein does interact with other aphid effectors. This also agrees with our own screens of Mp10 against our plant yeast-two-hybrid library, in which AMSH2 was pulled out as the sole interactor (Chapter 6). Perhaps the NBS-LRR protein was missing from the yeast two-hybrid library that was used to identify the AMSH2 target, or the conditions of this screen may have not been optimal to detect a NBS-LRR-effector interaction. It is also possible that other factors, including those in the plant host, are needed for an interaction to occur between an NBS-LRR and Mp10. An R-protein that perceives Mp10 may not interact with the effector directly, but sense Mp10 activity in the plant cell or Mp10 interaction with its target(s). Indirect detection, or "the Guard hypothesis", actually seems to be the more common method of effector detection by R-proteins (290).

The identification of several plant NBS-LRR proteins that interact with aphid effector proteins in yeast suggest that R-proteins may detect the presence of aphid effector proteins directly. Though R-proteins are found to detect the presence of effectors indirectly, there are examples of direct interactions of NSB-LRR proteins and effectors. For example, the TIR-NBS-LRR RRS1-R physically interacts with PopP2, an effector from *Ralstonia solanacearum* in order to confer resistance to the bacterium (288). Interestingly, this interaction requires the full-length RRS1-R protein, as individual domains of the NBS-LRR alone are not able to bind PopP2 (288). Binding of effectors to R-proteins has been found to occur at multiple sites. For instance the p50 protein of Tobacco mosaic virus is detected by the N R-gene

from tobacco via interaction at the TIR domain (534) and the rice blast fungus effector Avr-Pita is detected by the rice R-gene Pi-ta via interaction at the LRR-like domain (545). Multiple domains of NBS-LRR proteins were also found to interact with aphid effectors in the yeast two-hybrid screen. The LRR regions of three NBS-LRRs (RPS5, SUMM2 and AT1G63360) were pulled out as interactors of candidate aphid effectors. All the other identified interactions occurred with the N-terminal portions of NBS-LRRs, supporting the idea that effector interaction can occur at multiple sites on an R-protein.

NBS-LRRs are known to be located in clusters throughout the genome as the result of both segmental and tandem duplications, with high variation between NBS-LRRs, so as to detect many different effectors (524; 525; 526). It is increasingly found that NBS-LRR pairs are needed for disease resistance against a pathogen isolate or an effector, and many of these NBS-LRR pairs are linked by being located near each other in the genome, such as RRS1 and RPS4, and RRS1B and RPS4B which are required for recognition of AvrRps4 and PopP2, and AvrRps4 respectively (546; 547; 279). In this light, it is interesting that the Michelmore yeast-two-hybrid screen pulled out several pairs of NBS-LRR proteins located near each other in the Arabidopsis genome. VICTL and VICTR are known to be tandem genes (548), and both have been pulled out in the screen as interacting with a range of aphid effectors. The TIR-NBS-LRRs AT1G63860 and AT1G63880 are also near neighbours, with only one gene between them. AT1G63880 already has links to plant immunity; it has been identified as a gene that contributes to Col-0 Arabidopsis resistance against *Leptosphaeria maculans*, a hemibiotrophic fungal pathogen. AT1G63880 works together with another R-gene, AT1G64070, to give full resistance to the fungus, which involves callose deposition and camalexin induction (530). This is interesting, as the defense response to aphids in Arabidopsis also involves the induction of callose deposition and camalexin biosynthesis (Chapter 3) (340; 238). Though AT1G64070 was not found as an aphid effector interactor in this yeast two-hybrid screen, the TIR-NBS-LRR produced by another gene very near to AT1G63880, AT1G63860 was. Perhaps in response to aphids, it is these R-genes that work together, rather than the partner identified previously for fungal resistance. It would be interesting to investigate further whether these are acting in tandem to recognise aphid effectors. Perhaps both proteins would be needed to be expressed to see any recapitulation of the yeast-two-hybrid results or ETI responses *in planta*.

Comparing the results of the yeast-two-hybrid with the RNAseq dataset recently generated by our lab, it can be seen that seven of the NBS-LRRs identified as candidate aphid effector interactors are transcriptionally altered by pea aphid (*A. pisum*) exposure. NBS-

LRR proteins are expressed at low levels in all tissues within a plant, consistent with the need for a rapid response once a pathogen is detected. However, some NBS-LRRs are upregulated in response to bacterial flagellin, suggesting that exposure to pathogen elicitors can lead to a heightened sensitivity to attack (535; 536). The overlap between the yeast-two-hybrid and RNAseq data suggests that these NBS-LRRs have a role in aphid-plant interactions. It would be interesting to investigate these further, perhaps by looking at aphid fecundity on the NBS-LRR mutants or carrying out assays to investigate the HR response if they and the effectors they interact with are expressed together *in planta*. The seven NBS-LRR genes were transcriptionally changed in response to *A. pisum*, which cannot colonise Arabidopsis, but not in response to GPA, which can. An approach for further study may therefore be to test *A. pisum* homologs of the GPA effectors in NBS-LRR-effector interaction assays, as these R-proteins may be part of the mechanism behind Arabidopsis non-host resistance to *A. pisum*.

Of the seven NBS-LRRs found in the yeast two-hybrid screen that are transcriptionally changed by aphid exposure, three of the NBS-LRRs were present in the RNAseq dataset as two different splice variants. Of these splice variants, only one was significantly responsive to pea aphid exposure. These were the CC-NBS-LRRs AT1G59218.2 and AT5G43740.2, and the TIR-NBS-LRR AT1G63860.2. Induction of alternative splice variants of NBS-LRR proteins upon pathogen recognition has been observed in several plants, including tobacco, Arabidopsis and barley (549; 550; 551). The induction of alternative splice variants of the N and RPS4 R-genes in tobacco and Arabidopsis respectively are dynamically induced upon pathogen detection (549; 550). The truncated proteins which the alternative splice variants encode, can in turn, be seen to positively regulate disease resistance, by as-yet unknown mechanisms. More investigation is needed to confirm the existence and presence of splice variants of NBS-LRRs induced by aphid exposure in Arabidopsis, and whether this has a biologically relevant function, such as affecting the level of NBS-LRR protein in the cell.

I chose to carry out further investigations on the interaction between RPS4 and the candidate aphid effectors Mp19, Mp21 and Mp44. If one or more of these candidate aphid effectors pulled out in the screen really does interact with RPS4 directly, it would be the first effector shown to do so. The *P. syringae* effector AvrRps4, which was the first effector identified to be detected by RPS4, does not interact directly with the R-protein. RPS4 activation is believed to occur as a result of disruption of EDS1-RPS4 interactions at endomembranes by AvrRps4 (552). However, direct interaction of even EDS1 and AvrRps4 has not been seen, leading to the conclusion that these proteins also interact indirectly

(539). I showed that the expression of the aphid effectors alongside RPS4 and its partner RRS1, which has been found to be required for RPS4 mediated resistance (531), did not give the HR phenotype in *N. tabacum*. RPS4 is not an integral membrane protein, but it does associate with endomembranes in its non-activated state, with a small proportion localised to the nuclei (277). RPS4 accumulation in the nucleus is needed for triggering immunity after activation by its cognate effector AvrRps4 (277). Mp19, 21 and 44 are all small proteins (11, 13 and 12 kD respectively) with no strong localisation predictions, so should be present throughout the cytoplasm and within the nucleus. The vector I expressed them in, pGWB11, adds a C-terminal FLAG tag, but this should not be large enough to interfere with binding, any more than the C-terminal activation domain in pLAW11.

There may be slight variations of RPS4 between plant ecotypes that affect whether binding of an effector activates ETI. It is known that there are differences in RPS4 sequence between different Arabidopsis accessions, and these relate to differing susceptibilities to *P. syringae* expressing AvrRps4 (538). The RPS4 and RRS1 I used in the HR assays was from the Arabidopsis ecotype Ws-2. There are already known differences between the recognition of effectors by these proteins as the RRS1-R allele found in Ws-2 can confer recognition of the *Ralstonia solanacearum* effector PopP2 as well as AvrRps4, but the RRS1-S allele found in Col-0 cannot, though it still recognises AvrRps4. This difference in recognition is linked to the polymorphisms found between the different accessions (553; 531; 287). Though RRS1-S cannot recognise PopP2, it was still found to interact with it in yeast-two-hybrid screens (288), suggesting that interaction alone is not sufficient for activation of ETI. This hints that the interaction seen in the Michelmore screen between RPS4 and the aphid effectors may activate ETI in only some accessions with specific variants of RPS4. Screening RPS4 variants from other ecotypes or this alternative RRS1/RPS4 pair would therefore be an interesting next step.

In pairs of R-genes and effectors that interact directly, there would be strong balancing selection present, locking them into coevolutionary conflict where effector changes to escape recognition are matched by new host recognition capacities (554). This can be seen in the extreme levels of amino acid polymorphism present within both the Arabidopsis R-gene RPP13 and the avirulence gene that it recognises from downy mildew, ATR13 (555; 556). Different polymorphisms of aphid effectors are also found, for example Mp44.1 and Mp44.3, which were both identified as RPS4 interactors. This may hint that selection is acting on GPA effectors to prevent recognition by R-genes. The interactions between different alleles of aphid effectors found in a population and R-proteins present in plant populations could

therefore make an interesting area for further study.

It is possible that the binding of aphid effectors to RPS4 does not trigger cell death, but instead prevents it. RPS4 may detect the presence of another effector through guarding an effector target, and Mp19, 21 or 44 may in turn prevent this from happening. I looked at inhibition of RPS4 signaling in my assays and this gave me confusing results. If binding of the aphid proteins to RPS4 prevents its translocation into the nucleus or blocks activation of the protein somehow, stopping R-protein signaling, then I would expect that, in the presence of AvrRps4 and the aphid effector there would be no HR. This was seen in half of the experiments carried out, but not all of them. This assay is difficult to carry out, as it depends on multiple proteins (RPS4, RRS1 and AvrRps4) being expressed at the same time in the correct proportions to trigger HR. Western blot assays can be used in further repeats to provide more confidence in assay results. However it may be better to try other assays to detect RPS4-aphid effector interactions in plants, for instance co-immunoprecipitations in *N. benthamiana* or Arabidopsis protoplasts. More investigations are needed to see if the interaction between candidate aphid effectors and RPS4 truly occurs within plants, and whether this affects the TIR-NBS-LRRs ability to initiate ETI.

Like RPS4, several other NBS-LRRs that were identified as aphid effector interactors have also been identified previously as being involved in the immune responses to plant pathogens. The CC-NBS-LRRs RPS5 and SUMM2 are two such proteins. RPS5 was identified as interacting with Mp19, 21, 39 and 47. Like RPS4, it was characterised as giving resistance against *P. syringae* strains, in this case those expressing the effector protein AvrPphB (557). AvrPphB targets and cleaves a family of kinases involved in regulating PAMP receptor signaling at the plasma membrane. This includes the kinase PBS (AvrPphB susceptible protein 1), which associates with RPS5 at the R-proteins CC domain (527; 558). After AvrPphB proteolytic action on PBS1, PBS1 binds to the LRR domain of RPS5 which activates the R-protein (559). Both CC and LRR domains of RPS5 are therefore needed for recognition of effector action. In the screen, both CC and NBS-LRR portions of RPS5 were pulled out as interacting with aphid effectors suggesting that both domains of RPS5 are also involved in detection of aphid effectors. SUMM2, which was found to interact with Mp19, 47, 49 and 54, guards protein kinases MEKK1-MKK1/MKK2-MPK4 of the PAMP-triggered MAP kinase cascade. SUMM2 is thought to detect changes in MPK4 activity that occur as a result of pathogen effector action, such as that of HopAI1 from *P. syringae*, which inhibits kinase acitivities (528). The identification of two different R-proteins that are known to guard protein kinases involved in immunity suggests that this

aspect of plant signaling is targeted by the aphid. Protein kinases have important roles in plant immunity, involved in both direct detection of elicitors as transmembrane receptor kinases, and in mitogen-activated protein kinase cascades (MAPK) (560). As such they are an ideal class of proteins for effectors to target. It would be interesting to look at the levels of phosphorylation by protein kinases, such as those in the MAPK cascade, in the presence of different effectors to see if they do have an effect on this area of cellular signaling.

Not only were several candidate aphid effector-R-protein interactions discovered, but a number of aphid effector-effector interactions were identified as well, some of which could be repeated in our lab using a different yeast-two-hybrid system. We found that Mp17 seems to be acting centrally in these interactions, being the candidate effector that interacts with the most other proteins, namely Mp1, Mp10, Mp19 and Mp43. There is also a link with Mp2 via Mp10. It is interesting that three of the proteins we have characterised in the lab as likely to have an effector action, Mp1, 2 and 10, should all have a link with Mp17 (46; 44). Mp17 was not found to interact with any NBS-LRR proteins in the yeast two-hybrid screen. Overexpression of Mp17 in *N. benthamiana* did not produce a phenotype, nor does it interfere with the flg22-induced ROS burst (46); the presence of an RR2 chitin-binding domain suggests that it would remain attached to aphid exoskeleton, rather than ending up in the plant. Together, this hints that Mp17 may have an important role in effector regulation or delivery, rather than acting in the plant. If this is the case, silencing of Mp17 in GPA should have a larger effect than silencing any one of the effectors alone. The creation of stable dsMp17 Arabidopsis is currently underway for use in investigations such as this. In the future, it would also be interesting to introduce Mp17 with a mutated chitin-binding domain into the interaction, or in a silenced system to see if this could interfere with effector delivery. Two residues have been found in hard-cuticle type R&R consensus sequences that are essential for chitin binding, so these could be used (561).

Relatively recently, a new anatomical structure has been described within the aphid stylet, termed the acrostyle (14). The acrostyle has been found to harbour a high concentration of cuticular proteins with the RR-2 motif (15). As Mp17 also has this motif, and expressed in salivary glands, I suggest that Mp17 may be binding within the acrostyle. Experiments using Mp17 antibodies on dissected stylets would be able to confirm this. The only functional property confirmed so far for the acrostyle is the capacity to trap and release protein P2 of cauliflower mosaic virus (CaMV) within the common duct (15; 14). This capacity for binding and release may be used by the aphid itself for collection of its own effector proteins and release within the correct saliva type when feeding from plant cells. This would

ensure a release at concentrations needed to be effective, and would also ensure grouping of effectors if any are needed to overcome the ETI caused by recognition of another effector.

Mp43 is also an interesting protein as it contains an EF hand domain, which is known to bind calcium. As calcium is an important signaling ion in plant immunity, perhaps calcium binding by this aphid protein can inhibit the calcium signaling in plant immunity. Calcium binding proteins have already been found in aphid saliva, though these were larger than Mp43 (29; 32). These proteins were thought to prevent the calcium-triggered occlusion of sieve elements by forisomes, though this has been disputed (562). A calcium-binding protein has also been identified in the saliva of the green rice leafhopper, *Nephotettix cincticeps* (483). This protein, NcSP84, also contains EF-hands and could be detected in the phloem sap of rice exposed to the leafhoppers, showing delivery via the saliva. The expected function of NcSP84 is also to bind calcium that moves into sieve elements in response to puncturing by the stylet, so suppressing occlusion. Two other proteins with a predicted EF-hand motif were also found in the salivary glands of the rice brown planthopper, *Nilaparvata lugens* (563). Finding potential calcium binding proteins in the saliva of these different phloem feeding insects suggests that it is a universal component in the phloem feeders arsenal against plant defense responses. Identification of Mp43 in a potential aphid effector complex suggests that calcium sensing could regulate effector release, with effectors delivered only when needed after a plant immune response has been induced.

The interaction of aphid effectors with each other introduces the possibility that multiple effectors may work together to prevent detection by NBS-LRRs. One effector may 'protect' or 'prevent' another effector from triggering ETI. Effector interplay is not currently well studied, but examples of effector-effector interaction, effectors functioning as a "team" together or effectors antagonising each other are becoming increasingly known (564). One example of this is the interaction of VirF and VirD5 effectors from *Agrobacterium*. VirF is a bacterial encoded F-box protein that interacts with the plant host SCF (Skp1-Cul1-F-box protein) ubiquitin E3 ligase complex in order to uncoat the T-DNA upon reaching the host nucleus (565). However, F-box proteins are themselves short-lived and unstable. VirD5 binds and stabilises VirF, preventing its degradation, so allowing it to fulfil its function in the nucleus (566). As more interactions between effectors from pathogens such as this come to light, it looks like effector-effector interactions may play a greater role in host colonisation than previously suggested by studying effectors in isolation. Whether an aphid effector could prevent ETI triggered by another effector by interacting directly with the R-protein is unknown. Few pathogen effector proteins target R-genes directly. Some effectors

are able to prevent ETI, such as *P. syringae* HopD1 and HopZ1a, but these effectors target components downstream of R-proteins, rather than the R-proteins themselves (567; 568). The PopP2 effector from *Ralstonia solanacearum* interacts with the TIR-NBS-LRR protein RRS1-R to stabilise it. RRS1-R is believed to act as a negative regulator of plant immunity, so stabilising it is advantageous to the pathogen. However, RRS1-R can also detect the enzymatic activity of PopP2, leading to the activation of ETI, suggesting that coevolution between the two organisms has turned RRS1-R from a target into a sensor (569).

The identification of 23 different NBS-LRR proteins that interact with one or more of the 46 potential aphid effectors screened is encouraging, and provides a base for further investigations. Although a few R-proteins have been identified that are required for resistance to insect herbivores, the effectors that they detect either directly or indirectly have not yet been found. This work may therefore lead to a novel finding. The multiple aphid effector-effector interactions identified suggest exciting possibilities for aphid effector delivery, or effectors that work together to subvert plant immunity, which could be researched in more detail.

Chapter 8

Discussion

8.1 Summary of research

This study began with a simple aphid-plant interaction model (Chapter 1), which involved recognition of unknown GPA elicitors by unknown plant pattern recognition receptors (PRRs), leading to immune signaling and defense responses involving components that were also largely unknown. Effectors in the saliva of aphids were thought to suppress defense responses, modulate other plant pathways and be detected by plant R-proteins leading to ETI. This interaction is analogous to the "zig-zag model" of plant immunity (273). In the research of the last four years, the plant-aphid interaction model has become more defined with the identification of specific components involved in all stages of the interaction (see Figure 8.1).

My research revealed that proteinaceous elicitors of GPA induce Arabidopsis defense responses independently of wounding by the insect (Chapter 3). Further investigations using protease inhibitors suggest that it is not proteases present within GPA extract whose action is perceived (Chapter 3). The *Buchnera aphidicola* chaperonin GroEL has been identified as an aphid elicitor (363). The results in this thesis partly confirm that GroEL may be an elicitor, but also show that more than one elicitor is detected by Arabidopsis, leading to the activation of more than one defense pathway.

The LRR-RLK BAK1 is required for the ROS burst and induced resistance response to GPA elicitors (Chapter 3). PRRs that directly bind elicitors and are known to interact with BAK1, including FLS2, EFR, PEPR1 and PEPR2, are not required for defense responses against GPA (340). RNAseq data generated from insect-exposed Arabidopsis resulted in the identification of 31 RLKs and RLPs that are transcriptionally changed upon exposure to aphids. I found that Arabidopsis mutants for three of these receptors showed altered responses to GPA extract but not flg22 in ROS burst assays, suggesting that these receptors may play a role in the perception of GPA elicitors (Chapter 4). Together these data provide evidence that aphids have at least two elicitors that are perceived by plant PRRs leading to a PTI-like defence response. This is supported by the fact that both the 3-10 kD and the greater than 10 kD fractions of aphid extract induce plant defense responses (340).

I identified further immune signaling pathway components induced in Arabidopsis upon perception of aphid elicitors. Both the Arabidopsis NADPH oxidase, RBOHD, and the G-protein β subunit, AGB1, are required for the generation of a full ROS response to aphid elicitors (Chapters 3 and 4). GPA has a higher fecundity on the *agb1* mutant, but not on

the *bak1-5* mutant, suggesting the AGB1 pathway mediates successful defense responses against GPA. The induction of the defence gene *PAD3*, which encodes a protein involved in the biosynthesis of camalexin, which is toxic to GPA (Kettles et al., 2013; Chapter 3), is reduced in the *agb1* mutant, but not *bak1-5* plants (Chapter 4). However, callose deposition in response to aphid elicitors is reduced in the *bak1-5* plants. Together, these data suggest that aphids have at least two different elicitors, which activate two distinct plant defense response pathways.

I made progress with the identification of aphid effectors that suppress plant defence responses. The aphid effector candidate Mp10 suppresses plant ROS and calcium bursts triggered by flg22 and GPA elicitors. Knock down of Mp10 via plant-mediated RNAi reduces fecundity of GPA on Col-0 Arabidopsis, but not on the *bak1-5* mutant (Chapter 5), indicating that Mp10 is a genuine effector that contributes to aphid fitness and is likely to act in the BAK1 pathway of plant immunity against aphids. Mp10 mutant analyses identified two residues important for its ROS and calcium burst suppression function. Modelling of the Mp10 structure using known CSP structures revealed that both residues are present within the central binding pocket, which in CSPs is known to bind small hydrophobic molecules (Chapter 5). Thus, the two residues may be important for binding of a ligand in the central pocket of Mp10. However, these residues are also predicted to be exposed towards the exterior of Mp10 (Chapter 5), so they could also be responsible for direct interaction with other proteins, including a plant target.

A plant target for Mp10, AMSH2, was identified via yeast two hybrid screens. Whereas experiments to confirm interactions of Mp10 and AMSH2 *in planta* are ongoing, it is clear that AMSH2 has a role in the PTI ROS response to flg22 and the trafficking of FLS2 to the plasma membrane (Chapter 6). Given that Mp10 also suppresses the flg22-induced ROS burst, these results provide evidence that the Mp10 interaction with AMSH2 is biologically relevant. My research has thus shed light on molecular interactions between plant and aphid proteins, and uncovered a function of a conserved plant protein in immune receptor regulation.

Mp10 also induces chlorosis when expressed in *Nicotiana benthamiana* that is dependent on SGT1 and so may be part of an ETI response (46). I found that Mp10 mutants that no longer block the ROS and calcium bursts to flg22 also cause chlorosis (Chapter 5). This indicates that induction of chlorosis is not dependent upon Mp10 effector action. Salicylic acid, which is required for some ETI responses, is not needed for the chlorosis triggered by

Mp10 (Chapter 5).

To find out if GPA effectors, including Mp10, interact directly with plant R-proteins, a yeast two-hybrid screen of 43 GPA effectors against a library of plant NBS-LRR protein domains was carried out. Several GPA effectors interacted with NBS-LRR domains, whereas no interactions were detected for Mp10 (Chapter 7). Future work should reveal whether or not the interactions discovered between GPA effectors and plant NBS-LRRs are biologically relevant, and if they trigger or suppress NBS-LRR action.

The GPA effectors were also screened against each other in yeast two-hybrid assays which unexpectedly revealed a number of effector-effector interactions. Mp10 interacts with Mp2 (Chapter 7), which is another effector studied in the Hogenhout lab (Pitino et al., 2013). Some candidate aphid effectors interacted with multiple other candidate effectors leading to the platform hypothesis, which proposes that some effectors may act in the tip of aphid stylets to hold other effectors in place and allow quick release into the plant upon plant cell puncture. Alternatively, the effectors may traffic together to their site of action inside the plant.

Taken together, I have unravelled components involved in the PTI, ETS and ETI responses that occur during the insect-plant interaction, and that determine whether or not an aphid can colonize a plant. Components identified in this thesis will inform further research and aid in the development of new approaches to obtain aphid resistant crops.

8.2 Aphids and plant immunity

8.2.1 Aphid elicitors and their perception in Arabidopsis

Aphid elicitors

Insects and insect saliva were known to induce plant defense responses (115). We chose to work with whole aphid extract to identify and characterise elicitors present in GPA. By using whole GPA extract, it was found that elicitors present within the aphid itself could induce specific plant defense responses reminiscent of PTI, including induction of a ROS burst, callose deposition, induction of defense genes and an induced resistance response. This plant response to GPA elicitors is specific, as though proteinase K also induced a ROS burst, it did not generate an induced resistance response to GPA. The use of aphid extract

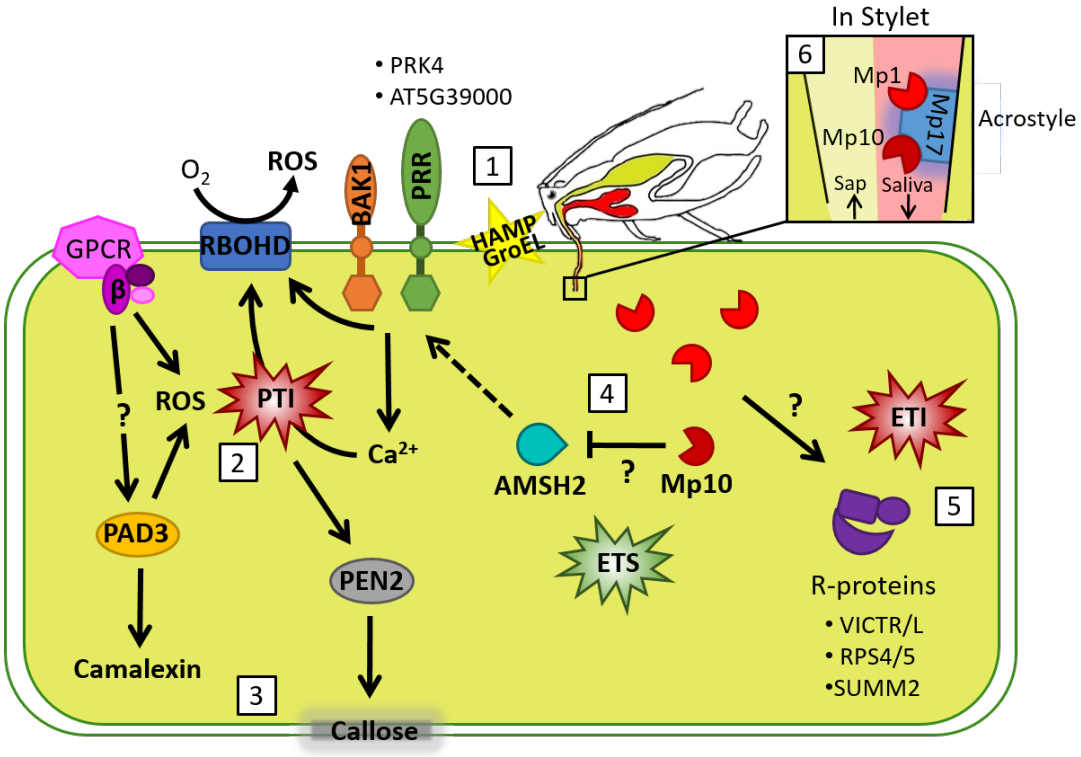


Figure 8.1: **Model of plant-aphid interactions: what we know now**
1. Multiple GPA elicitors, possibly including GroEL (363), induce immune responses (Chapter 3). BAK1 is required for PTI-like defense responses to aphids (Chapter 3). My investigations have also identified two plant PRRs, PRK4 and AT5G39000, which may detect aphid elicitors in a BAK1-dependent or independent fashion (Chapter 4).
2. A ROS burst is induced downstream of BAK1, which requires RBOHD. Calcium is also required for full RBOHD activation (348; 181). Callose deposition via the action of PEN2 is induced downstream of BAK1 and RBOHD. The G-protein subunit AGB1 is involved in another immune signaling pathway after aphid perception, and *PAD3* induction occurs because of this pathway, independently of BAK1. *PAD3* induction contributes to the ROS burst ((340); Chapter 3; Chapter 4).
3. Callose deposition is induced by GPA extract ((340); Chapter 3) and may inhibit aphid feeding due to reinforcement of cell walls, making probing harder. Callose can also block phloem sieve tubes, preventing the flow of phloem sap to the aphid feeding site (201). Effective resistance against aphids involves camalexin, produced by the action of PAD3. Camalexin is toxic to aphids, so provides an effective means of defense against the insects (238).
4. Aphid effector Mp10 works in the BAK1 immune pathway to block the calcium and ROS bursts that form a part of PTI, thereby inducing effector-triggered susceptibility (Chapter 5). Mp10 may interact with AMSH2, a plant DUB, which has a role in PRR-mediated ROS production and cellular trafficking of PRRs (Chapter 6).
5. Mp10 induces SGT1-dependent chlorosis suggestive of ETI (46) that is independent of SA accumulation (Chapter 5). GPA effectors interact with several plant NBS-LRR proteins in yeast two-hybrid experiments, and these interactions may induce or inhibit ETI (Chapter 7).
6. Candidate aphid effectors interact with each other (Chapter 7). Mp17 contains a chitin-binding domain suggestive of a location in the acrostyle. It may form a platform for targeted effector delivery.

also enabled the discovery of three PRRs which are involved in the ROS response to GPA elicitors but not flg22: CRK7, PRK4 and AT5G39000. I also found that elicitors in GPA extract trigger different immune pathways to flg22 in Arabidopsis, as *PAD3* induction is dependent upon BAK1 in response to flg22 but not GPA extract, where it is instead downstream of AGB1. Together, these findings demonstrate that the use of GPA extract was a good starting point to elucidate plant pathways involved in defense responses to GPA. The next step is to characterise the components of GPA extract that are the eliciting factors.

Further investigations showed that the elicitors were likely to be proteinaceous, as boiling and digestion by proteinase K both removed the eliciting activity of GPA extract. David Prince narrowed down the identity of GPA elicitors to 3-10 kD and greater than ($>$) 10 kD proteins found in both GPA extract and GPA saliva (340; 316). This agrees with previous work showing that the 3-10 kD fraction of GPA saliva causes induced resistance (47). Though both the 3-10 kD and $>$ 10 kD fractions generate an induced resistance response to GPA, only the 3-10 kD fraction was found to induce a ROS burst (340), showing that the ROS response is not always required for the induced resistance response to GPA. Nevertheless, the induced resistance response is absent in both *bak1-5* and *pad3*, indicating that responses downstream of both BAK1 and PAD3 contribute to plant defense against GPA. Identification of components of the different fractions of GPA extract and testing whether they also induce defense responses could be used to further identify the eliciting factors. Proteinaceous elicitors could be investigated by mass spectrometry of the GPA extract fractions. However, GPA extract will be a complex mixture as it is derived from whole aphids. An easier method may be to confirm the identified PRRs that detect aphid elicitors and then identify what component of GPA extract binds to them.

The finding that boiling and proteinase K treatment of GPA extract leads to a loss of both the ROS burst and induced resistance responses suggests that the elicitor/s is a protein or peptide. The lack of eliciting activities after boiling suggests that a three dimensional structure of a protein, such as an enzyme, contributes to eliciting activity. As the ROS burst response to aphids takes place over a longer period of time than that of the bacterial elicitor flg22, I wondered whether Arabidopsis was perceiving elicitors (DAMPs) that were produced by the action of an enzyme in aphid saliva, of which there are many (31; 34; 35; 32). Enzymatic activity could also cleave aphid proteins to produce elicitors 3-10 kD in size. In order to investigate this, I chose to investigate proteases, which could produce smaller peptides from aphid proteins. A protease itself could have elicitor action, as a serine protease elicitor from *Pseudomonas aeruginosa* capable of inducing plant immune

responses in Arabidopsis has been identified (193). I found that the use of several protease inhibitors did not impact the GPA-extract induced ROS burst, suggesting that the elicitor is not a protease (Chapter 3). Other enzymes found in aphid saliva may be responsible for non-protein DAMP production.

Work carried out by Chaudhary et al. found that GroEL, a molecular chaperone from the aphids obligate symbiont *Buchnera aphidicola*, was capable of inducing plant defense responses, including defense gene induction, callose deposition and a ROS burst (363). It therefore seems that one of the elicitors from aphids is actually bacterial in origin. GroEL is 60 kD in size, so would correlate with the greater than 10 kD elicitor found in our own studies. However, we found that this fraction did not induce a ROS burst in Arabidopsis (340). Peptides produced from the cleavage of GroEL by enzymes present in aphid saliva could lead to the production of GroEL-derived elicitors of 3-10 kD in size, but I did not find that protease inhibitor treatment affected ROS. The boiling of GroEL was found to increase, rather than decrease its eliciting activities which is at odds with our results, and those of others who found a loss of eliciting activity upon boiling (363; 47; 340). Perhaps the timing of boiling after aphid elicitor collection could explain the differences between the results, as enzymes may have time to produce 3-10 kD elicitors before being denatured if boiling is delayed. This is something that could be investigated in the future.

The presence of multiple fractions that induce defense responses in plants suggests that there is more than one elicitor present in aphids. This indicates that Arabidopsis is capable of perceiving different components of aphids, possibly using different receptors. The presence of multiple elicitors on one biotic threat is common, for instance pathogenic bacteria can contain flagellin, EF-Tu and peptidoglycan, all of which are detected by Arabidopsis using different receptors (139; 141; 342).

Pattern recognition receptors which detect aphids

It was found that the ROS burst and callose deposition in response to GPA extract is dependent upon BAK1 action, as these responses are lost in the *bak1-5* mutant, which is deficient only in innate immune signaling (147). This indicates that BAK1 is upstream of both these immune responses in its capacity as an immune coreceptor. The ROS burst and callose deposition triggered by GroEL are also greatly reduced in *bak1-5*, which suggests that it is GroEL that is perceived in a BAK1-dependent manner (363). BAK1 is involved in the perception of many pathogens, including bacteria, fungi, oomycetes and

viruses (392; 393; 394), as well as being involved in the JA response to chewing herbivores in *Nicotiana attenuata* (110). The finding that BAK1 is also involved in the response to aphids suggests that it has a conserved function in the detection of biotic agents. As BAK1 plays a major role in brassinosteroid hormone perception (145; 412), BAK1 is in an ideal position to control the balance between plant growth and immunity. A role of BAK1 in the crosstalk between plant growth and immune pathways has been shown (570).

In the light of BAK1 involvement in many different responses, there must be something that provides specificity to the plant interaction with aphids. Based on what is known about BAK1 action, it is likely that BAK1 acts as a co-receptor in aphid elicitor perception (148). PRRs that specifically bind aphid elicitors and then interact with BAK1 will therefore provide the specificity needed in the plant-aphid interaction. My investigation of RLKs and RLPs that are differentially regulated by insect exposure identified two RLKs that may be acting as PRRs for aphid elicitors. T-DNA mutants of PRK4 and AT5G39000 both showed a reduced ROS response to GPA extract compared to wild-type Col-0 plants (Chapter 4). One or both of these RLKs may interact with BAK1 in aphid perception, and an action for the future is to check the interaction of these RLKs with BAK1 in aphid challenged plant tissues.

The type of RLK found can also hint at the type of elicitor that is detected. PRK4 contains extracellular LRR domains, like the PRRs FLS2 and EFR (114). This suggests that the elicitor it binds to is a protein (409), which could make it the GroEL PRR. The response of PRK4 mutants to GroEL should therefore be tested. On the other hand, AT5G39000 is an RLK from the *Catharanthus roseus* RLK1-like kinase (CrRLK1L) family which contain extracellular malectin-like domains that are predicted to bind to carbohydrates (571). CrRLK1L proteins have been implicated in diverse roles including cell elongation, polarised growth and fertilisation as well as biotic and abiotic stress responses (571). Carbohydrate binding of these receptors in order to sense cell wall status has been conjectured (572), so AT5G39000 could be involved in sensing perturbations to the cell wall that components of aphids and aphid saliva, such as pectinases, cellulases and glucosidases (36; 34) may cause. Further testing of AT5G39000 mutant response to carbohydrates found in aphids or the predicted products of salivary enzymes would be needed to test these hypotheses.

Though the carbohydrate binding of CrRLK1Ls has not been proven, the CrRLK1L FERONIA (FER) binds to proteins, including the peptide hormone rapid alkalinisation factor (RALF) and glycosylphosphatidylinositol-anchored proteins (GPI-APs) (573; 574).

Interaction of FER with ligands leads to NADPH-dependent ROS production and calcium signaling, which regulate polarised root hair growth, cell wall integrity and pollen tube rupture (574; 575; 576). Through interacting with multiple cellular components, FER is able to mediate distinct signals under different cellular and developmental conditions and environmental challenges. There is therefore the possibility that AT5G39000 could also bind to proteins, causing a ROS burst, and have multiple roles within plant cells.

Two T-DNA mutants of CRK7 showed an increased ROS response to GPA extract, suggesting that the function of CRK7 in wild-type plants is to suppress the ROS response to aphids (Chapter 4). The extracellular portion of CRK7 contains Gnk2/DUF26 domains of unknown function, so the type of substrate it might bind is unknown. CRK7 is involved in mediating the responses to extracellular ROS (406). It may be that this RLK is involved in the balance between an effective immune response and one which might negatively effect the plant. Molecules produced in ROS generation are capable of damaging the plant, so ROS levels need to be tightly controlled in order to avoid detrimental side-effects (577). I found that the ROS burst to flg22 remained unchanged in the *crk7* mutants, which may indicate that CRK7 functions specifically in the plant response to aphids.

The dynamics of the ROS response to flg22 and aphid elicitors are different, with the ROS burst to GPA extract taking place over a longer timeframe. CRK7 could be involved in the regulation of ROS bursts which take place over longer periods of time. It would be interesting to test the *crk7* mutants with other elicitors that also show longer ROS burst signatures, such as the oomycete INF1 (144). Whether this increased ROS is biologically relevant to the plant-aphid interaction is unknown. Assays of other defense responses and aphid success are required to identify whether the increased ROS in *crk7* plants is translated into increased resistance.

8.2.2 Intracellular signaling induced by aphid perception

The ROS burst that occurs as a result of GPA elicitor perception requires the NADPH oxidase RBOHD (Chapter 3). RBOHD is also needed for the ROS response to pathogen elicitors (178). In flg22 perception, RBOHD is activated downstream of BAK1 by the action of BIK1, which is phosphorylated and released from BAK1 upon elicitor perception (151). BIK1 then directly interacts with and phosphorylates RBOHD, leading to ROS production (180; 171). *bik1* mutants therefore lose flg22-induced resistance to *P. syringae*, allowing increased bacterial growth (151). The need for both BAK1 and RBOHD in the ROS re-

sponse to aphid elicitors suggests that the same pathway of transphosphorylation events mediated by BIK1 in pathogen interactions is also active in aphid perception, providing the link between elicitor perception and activation of signaling (180; 171). Unexpectedly, GPA shows reduced feeding and fecundity on *bik1* mutant plants, with increased aphid-induced hydrogen peroxide accumulation and symptoms of HR (152). This suggests that BIK1 is a negative, rather than positive regulator of immunity against aphids. BAK1-mediated RBOHD activation after aphid elicitor perception may therefore use another, BIK1-independent pathway.

BIK1 activates RBOHD via phosphorylation in response to flg22 detection, but this is only part of RBOHD regulation after elicitor perception. Calcium also affects RBOHD function, both by directly binding to the NADPH oxidase at EF-hand motifs present in the cytosolic region of the protein, and via phosphorylation by calcium-dependent protein kinases (181; 182; 183). Both BIK1- and calcium-mediated activation of RBOHD are therefore required for a full ROS burst response (348). Work undertaken in the lab has since found that calcium bursts are induced in Arabidopsis in response to both GPA extract and GPA feeding (Thomas Vincent, unpublished). This shows that both ROS and calcium signaling are both involved in PTI responses to aphids.

I found that the G-protein β subunit, AGB1 was also required for full elicitation of the ROS burst in response to GPA elicitors (Chapter 4). However the ROS response in *agb1-2* mutant plants was not as low as that seen in *bak1-5* plants, suggesting that AGB1 does not have a central role in the activation of ROS signaling. It may play a role in regulation of ROS production by RBOHD, or be responsible for ROS from another source entirely. Although the primary ROS burst following pathogen recognition occurs in the apoplast by the action of oxidases such as RBOHD, ROS produced in other cellular compartments, such as the chloroplasts, is also implicated in defense (174). AGB1 is needed for the RBOHD/F-mediated ROS burst induced by the bacterial PAMPs flg22 and elf18 and is suggested to work in the same pathway as RBOHD in response to the biotroph *Pseudomonas syringae*, though not in resistance against the necrotrophic fungus *Plectosphaerella cucumerina* (194; 195). Further investigation is required to see if AGB1 affects RBOHD-mediated ROS in response to aphids.

In Arabidopsis, AGB1 works within a G-protein coupled receptor (GPCR) complex made up of a 7-transmembrane pass-regulator of G-protein signaling (7TM-RGS) protein called AtRGS1, and α-, β- and γ-subunits that act together in signaling (404). The Arabidopsis

genome encodes one α subunit, (GPA1) one β subunit, (AGB1) and two γ subunits (AGG1 and AGG2) (420). It would be interesting to identify whether these components of the GPCR complex as well as AGB1 are required for defense responses to insects, as this would confirm that AGB1 is working as part of a complex in immune signaling. As GPCR complexes contain a receptor portion, the G-protein may not only be involved in intracellular signaling, but could act as a receptor of aphid elicitors directly. The α-, β- and γ-subunits of the heterotrimeric G-protein complex are required for perception of a protease elicitor from *Pseudomonas aeruginosa* in Arabidopsis (193). These G-protein components were found to function upstream of a MAPK cascade in immunity against the bacteria, rather than downstream of or parallel to the MAPK cascade as was found in flg22 perception (194). This led to development of a model for a novel immune pathway, in which the G-protein subunits interact with a sensor that directly perceives the presence of the protease elicitor and activates downstream defense signaling (193). Though my protease inhibitor assays (Chapter 3) suggest that the eliciting activity of GPA extract does not involve a protease, other activities of elicitors from aphids may be perceived by G-protein components. Investigations of MAPK activation in response to GPA extract in *agb1-2* and other G-protein mutants will shed further light on whether G-proteins are acting as receptors or signal transducers in the response to aphids.

I found that *PAD3* expression was not induced as highly in response to GPA extract in an *agb1* mutant compared to wild-type Col-0 Arabidopsis, whereas *PAD3* expression was equally induced in *bak1-5* and Col-0 plants (Chapter 4). This suggests that *PAD3* induction, and so camalexin production occurs downstream of AGB1, but not downstream of BAK1. It is not known how AGB1 regulates *PAD3* induction and whether this occurs directly or involves intermediates. In the response to pathogens, *PAD3* is induced by both the WRKY transcription factor WRKY33 and the NAC family transcription factor ANAC042 (376; 377). These transcription factors may therefore be induced via G-protein signaling. More investigation is needed to dissect the AGB1-PAD3 pathway further and to find out whether WRKY33 and ANAC042 are involved in PAD3 expression induction downstream of AGB1. Nevertheless, this work demonstrates that two signaling pathways are involved in aphid perception, one involving BAK1 and another involving AGB1. Interestingly, I found that GPA fecundity was increased on *agb1* mutant plants, but not on *bak1-5* mutants. This suggests that defense responses downstream of AGB1 are effective against GPA and act to limit their numbers on Arabidopsis. The induction of PAD3 may play a role in this effective resistance.

8.2.3 Defense responses induced by aphids

Exposure to GPA extract leads to induction of *PAD3*. PAD3 is the cytochrome P450 responsible for the final steps in camalexin synthesis from tryptophan via dihydrocamalexic acid (235; 236; 237). *PAD3* is induced at aphid feeding sites around points of stylet entry, and aphids show a higher fecundity on *pad3* mutant plants (238). Camalexin is responsible for this effect on GPA, as it is taken up by aphid feeding and reduces the fecundity of aphids that feed on it (238). I found that *PAD3* is induced downstream of AGB1 and GPA shows higher fecundity on *agb1* mutant Arabidopsis. The increased performance of GPA on the *agb1* mutant is therefore likely to be due to the lack of induction of camalexin biosynthesis in these plants. Pea aphids, which do not use *Brassica* species, including Arabidopsis, as hosts, survive better on *agb1* plants compared to wild-type Col-0 Arabidopsis, suggesting it is likely that PAD3 and camalexin production also play a role in non-host resistance of plants to aphids, though more experiments, including investigation of pea aphid success on *pad3* plants, are required to confirm this.

I also found that PAD3 has a feedback effect on ROS production in response to GPA elicitors but not to flg22 (Chapter 4). In the *pad3* mutant, the ROS burst in response to GPA extract is reduced to similar levels as that seen in the *bak1-5* mutant, suggesting a role just as central as the co-receptor. In PTI, induction of camalexin biosynthesis has been identified as downstream of the ROS burst, rather than regulating it (421; 422). Though the same immune components, such as BAK1, RBOHD and PAD3, are found in the response to insects and pathogens, their regulation may therefore be different. It is not known whether PAD3 impacts ROS by altering its extracellular generation, such as by affecting RBOHD function, or whether intracellular ROS is involved. It is also unknown whether PAD3 itself directly affects ROS, or whether the camalexin that it synthesises has a feedback role. Carrying out ROS burst assays in the presence of differing amounts of camalexin would further test this. Camalexin itself has been found to increase intracellular ROS in human cancer cells, so it could have a similar affect in the plant (375).

Unlike PAD3 and camalexin, the induction of callose deposition is downstream of BAK1, as callose deposition in response to GPA extract is lost in a *bak1-5* mutant (Chapter 3). Callose deposition is also dependent upon PEN2, a myrosinase involved in indole glucosinolate metabolism (227; 205). Callose deposition in the cell walls of palisade cells of the leaf may affect aphids as they probe, as their stylet moves intracellularly. Callose may also affect feeding from the phloem; callose deposition on sieve plates in the phloem is linked to

resistance against the phloem feeder *Nilaparvata lugens* in rice (201). Interestingly, AGB1 contributes to PEN2-mediated resistance to *Magnaporthe oryzae* in Arabidopsis (403), so G-protein signaling could also be involved in callose induction in response to aphids, as well as BAK1. Checking callose deposition in the *agb1* mutant would shed light on the interaction between the AGB1 dependent aphid immune pathway and callose deposition.

I found that CYP81F2 was induced by aphid elicitors (Chapter 3). CYP81F2 encodes a P450 monooxygenase involved in indole glucosinolate biosynthesis, located above PEN2 in the biosynthesis pathway (227) (see Figure 5 in Chapter 3). *pen2* mutant plants do not show altered resistance to GPA (47). However, aphid fecundity does increase slightly on the *cyp81f2* Arabidopsis mutant (341; 238). This suggests that indole glucosinolates have a greater role than callose in aphid defense, though the effect of both PEN2 and CYP81F2 is minor compared to PAD3. An explanation for this may be that GPA is very effective at suppressing PTI responses regulated via BAK1, which includes PEN2-mediated callose deposition. I have generated some evidence for this via work on the aphid effector Mp10, which suppresses PTI in the BAK1 signaling pathway.

8.2.4 Aphid effectors and their delivery into cells

Though exposure to aphid extract induces an array of plant signaling and defense responses, aphids like GPA are still capable of colonising plants. The action of effectors delivered into the plant via insect saliva is thought to be behind this overcoming of plant defense (24). A suite of candidate effectors from GPA has been identified in the lab (46). I have contributed evidence that one of these proteins, Mp10, functions as an effector inside the plant host. First, I showed that knock-down of Mp10 in GPA has a negative effect on aphid fecundity on Col-0 but not *bak1-5* plants, indicating that Mp10 acts by suppressing PTI downstream of BAK1 (Chapter 5). Secondly, preliminary data has revealed that Mp10 antibodies label plant cell cytoplasm near aphid feeding sites (based on the presence of stylet tracks), providing evidence that Mp10 is delivered into the plant by aphid feeding, strengthening the hypothesis that Mp10 function is in the host plant (Chapter 5). Thirdly, when expressed *in planta* Mp10 suppresses the ROS and calcium bursts to flg22 and GPA extract, which require BAK1. Finally, Mp10 is able to modulate the cellular location of the PRR FLS2, perhaps through interactions with the plant protein AMSH2.

A function of Mp10 inside plant cells may be unexpected as Mp10 has homology to insect chemosensory proteins (CSPs) which are known to have roles in sensory organs and

the development of insects (429). However, CSPs are small stable molecules that could adapt to multiple roles, including in a plant host (433). Macrophage migration inhibitory factors (MIFs) are another group of aphid proteins that are thought to have known roles inside aphids, in this case in their own immune system, but also have effector function in plants (578; 259). Transgenic lines that produce Mp10 under an inducible promoter have been generated and are currently being tested. Given that Mp10 expression induces chlorosis upon expression *in planta*, this could compromise the PTI suppression activities of Mp10, making it difficult to investigate ETS.

A number of GPA effectors are known to be required for aphid success on plants; silencing Mp1, Mp2 and MpC002 reduces aphid fecundity, and expression of the aphid proteins in Arabidopsis increases GPA fecundity (43; 44). The fact that expression in Arabidopsis improves GPA fecundity indicates that Mp1, Mp2 and MpC002 have activity inside the plant host. In contrast, the *A. pisum* homologs of these effectors (Ap1, Ap2 and ApC002) do not improve GPA fecundity when expressed in Arabidopsis, indicating the the *A. pisum* effectors are not active in Arabidopsis (44). It remains to be tested if the *A. pisum* effectors are active in legumes, which are the hosts of the pea aphid. However, RNA-mediated knock-down of ApC002 in *A. pisum* reduces feeding ability and drastically increases mortality of the aphid on *Vicia faba* and medicago (both legumes), indicating that ApC002 does have a role in the legume host (41; 42).

GPA effectors may interact with each other. Yeast two-hybrid analyses of effector-effector interactions identified an unexpected number of interactions (Chapter 7). It is possible that the effectors interact with each other so that they can be delivered together into plant cells to target the same pathways, including perhaps the prevention of ETI. Pathogen effectors that interact with each other, such as VirF and VirD5 from *Agrobacterium tumefaciens* are known, though not many are well characterised (564; 566). Effector-effector interactions may play a larger role in plant pathogen/pest success than previously thought.

Many effectors, including Mp1, Mp2 and Mp10 interact with Mp17. This may indicate that Mp17 is simply a 'sticky' protein, and liable to show interactions in a yeast two-hybrid screen. However, candidate effectors from phytoplasma were also included in the yeast two-hybrid screen, none of which interacted with Mp17. Mp17 was also not found to interact with any NBS-LRRs in the screen against plant R-proteins. This indicates that Mp17 interaction with multiple proteins is specific for aphid effectors. Mp17 may therefore act as a central hub. Mp17 is a cuticular protein with an RR-2 chitin binding domain (Chapter 7)

and proteins containing RR-2 motifs are present at a high concentration in the acrostyle, an anatomical structure at the tip of the stylet (14). Mp17 does not modulate the flg22-induced ROS burst, or produce a phenotype upon overexpression *in planta*, suggesting that it has a function in the aphid rather than the plant (46). A hypothesis resulting from these observations is that Mp17 binds to chitin within the acrostyle via its RR-2 motif, and provides a platform for effector collection and eventual delivery into the plant.

Mp17 could also have a regulatory role. Unlike organisms such as bacteria and fungi that use effectors to manipulate plant defense, GPA is a complex multicellular insect. Effectors from the aphid may therefore need to have a layer of regulation to ensure correct delivery into the plant rather than to different aphid tissues. Although the food canal and salivary canal are separate along most of the length of the stylets, they do join before the end, forming a common duct, where two way trafficking of saliva and phloem sap occurs (13). The common duct is where the acrostyle is located (14). Proteins such as Mp17 may be required to ensure that effectors such as Mp10 are released or activated only when needed. This could be important as effectors that perturb plant cell systems may also be able to affect insect cells. Similarly, some pathogen effectors such as proteases are synthesised and delivered to plant cells in inactive forms, and only activated once inside the plant cell. For example, *P. syringae* effector AvrRpt2 is a cysteine protease that requires cleavage at a specific site by a plant factor for activation of protease activity (579).

Holding effectors at the tip of the stylets may also allow the aphid to sense plant immune status. Several aphid salivary proteins that are identified as candidate effectors contain calcium-binding domains, including Mp43, which also interacts with Mp17 (Chapter 7) (29; 32). As the calcium burst plays a central role in plant innate immune signaling (165) (Chapter 3; unpublished data Thomas Vincent), some aphid proteins may be acting as sensors of this, leading to aphid effector release only when required. This may prevent the negative effects caused by effector delivery at the wrong time and location. For example, some effectors may only be released into the plant upon the suppression of ETI by other effectors. More investigation into these aphid-effector interactions is needed, including testing if these interactions actually occur within aphids and plants, and if these interactions are biologically relevant.

8.2.5 Mp10 and its plant target AMSH2

A yeast two-hybrid screen of Mp10 against an Arabidopsis protein library was used to identify potential Mp10 interactors within the plant, and pulled out a single candidate: AMSH2 (Chapter 6). AMH2 is predicted to be a deubiquitinating enzyme (DUB), and has not been implicated in the plant immune response before, though the AMSH2 homologs AMSH1 and AMSH3 affect plant-microbe interactions via their function in the control of intracellular trafficking (490; 492; 508). Mp10 interaction with AMSH2 suggests that AMSH2 is also involved in BAK1-mediated defense responses. I found that AMSH2 affects the flg22-induced ROS burst in *N. benthamiana*. Heterologous expression of AMSH2 increased the ROS burst, suggesting that AMSH2 has a positive effect on PTI responses, whereas transient silencing of AMSH2 reduced the ROS burst, confirming an action as a positive regulator (Chapter 6). Thus, like Mp10, AMSH2 also regulates the elicitor-induced ROS burst. As both Mp10 expression and silencing of AMSH2 result in a reduction of the ROS burst to flg22, these results suggest that Mp10 may inhibit AMSH2 action. Whereas the exact role of AMSH2 in a plant cell is not known, AMSH2 has a conserved MPN domain, suggesting that this protein has deubiquitinating (DUB) activity (496). Possible AMSH2 activity as a DUB combined with the evidence that Mp10 acts upstream of the calcium and ROS bursts in a BAK1-dependent manner suggests that AMSH2 has a role in the regulation of immune receptors by ubiquitination. Perturbation of immune receptor localisation by expression of Mp10 or silencing of AMSH2 supports this hypothesis (Chapter 6).

Control of immune receptor location and signaling by ubiquitination has been investigated in the response to pathogens, using the flg22-FLS2 elicitor-PRR pair. These studies have found that, after flg22 perception and PRR complex activation, BAK1 phosphorylates the plant U-box (PUB) ubiquitin ligases PUB12 and PUB13. This leads to PUB12/13 association with FLS2, which they then ubiquitinate (155). Ubiquitination of FLS2 leads to its degradation, probably via endocytosis and trafficking to the vacuole (157; 502). AMSH2 may therefore be acting at the cell membrane in opposition to ubiquitination by PUB12/13, preventing FLS2 endocytosis and enabling immune signaling to occur for longer (Hypothesis 1 in Figure 8.2 below). In contrast, AMSH2 silencing or AMSH2-activity inhibition by Mp10 may result in increased FLS2 ubiquitination, thereby increasing FLS2 endocytosis and reducing the amount of PRR signaling at the cell membrane, reducing the cells ability to sense elicitors and to induce a ROS response.

It is also possible AMSH2 action occurs further downstream of receptor internalisa-

tion, in a more similar location to its homolog AMSH3, which localises to late endosomes (492; 494). There is a possibility that flg22-induced internalised FLS2 is present as an active receptor complex with the ability to continue signaling from endosomes, though there is no direct evidence for this (502). AMSH2 action and removal of ubiquitin at this point may therefore allow prolonged signaling to occur from the receptors endosomal location, or facilitate recycling back to the cell membrane (Hypothesis 2 in Figure 8.2 below). In contrast, AMSH2 silencing or AMSH2-activity inhibition by Mp10 would prevent the recycling of FLS2 to the cell membrane or increase the rate of degradation after internalisation.

The change to FLS2 and BAK1 localisation in *N. benthamiana* upon coexpression with Mp10 was seen without the need for elicitor challenge, suggesting that Mp10 interaction with AMSH2 does not interfere with elicitor-induced receptor endocytosis. In the absence of pathogen elicitors both BAK1 and FLS2 are located at both the plasma membrane and in endosomal compartments, suggestive of constitutive endocytic recycling (580; 157; 502). The receptors also need to be trafficked to the plasma membrane after synthesis. Both recycling and receptor delivery affect the number of receptors that are available in the plant cell plasma membrane for immune signaling, and ubiquitination plays a role in the regulation of both recycling and receptor delivery (506). Thus, FLS2 ubiquitination levels also affect its localization to various cellular components in the absence of any pathogen. Other E3 ubiquitin ligases have been implicated in the control of plant immune responses, providing other ubiquitinating enzymes that AMSH2 action may oppose.The E3 ubiquitin ligases PUB22, 23 and 24 have been identified as negative regulators of PTI (503). PUB22 targets a subunit of the exocyst complex, which mediates vesicle tethering during exocytosis (504). AMSH2 could therefore regulate deubiquitination of FLS2 directly or act by opposing the action of PUB22; both would affect the delivery of FLS2 to the cell membrane (Hypothesis 3 in Figure 8.2 below).

No matter at what stage AMSH2 acts, knock down of AMSH2 or Mp10-mediated inactivation of AMSH2 would prevent receptor localisation at the cell membrane. This would reduce the number of receptors present on the surface of the cell that in turn would reduce the detection of elicitors, resulting in the inhibition of ROS and calcium bursts and the defense responses that occur downstream of them. AMSH2 functional characterization is investigated in relation to FLS2, which does not play a role in the perception of aphid elicitors (340). However, Mp10 does suppress the ROS and calcium burst to both flg22 (which acts via FLS2) and GPA elicitors (which does not). It is therefore possible that AMSH2 is involved in the trafficking of multiple receptors in addition to FLS2 that require BAK1 for

signaling. Further work is required to confirm Mp10-AMSH2 interaction *in planta* and how AMSH2 may affect defense responses to GPA.

As a CSP, Mp10 is a small globular protein consisting of 6 alpha helices that form an internal pocket which is capable of binding small hydrophobic molecules (429; 430; 431). Binding of a ligand to the pocket has been found to induce a conformational change in CSPs (448). In olfaction and gustation, binding of a chemical in the central pocket leads to transport by the CSP to odorant or gustatory receptors in the cell membrane of sensory neurones (433; 581). The tyrosine (Tyr) and tryptophan (Trp) residues at position 40 and 120 respectively, which are essential for the Mp10 calcium and ROS burst suppression activity, are located within the internal hydrophobic pocket of Mp10 (Chapter 5). These residues may therefore regulate the docking of small molecules inside the pocket essential for Mp10 effector action, possibly involving a conformational change. It may therefore be binding to a small molecule or a conformational change afterwards that is required for Mp10 ROS and calcium suppression activity. However, these residues may also be present on the surface of Mp10, and so regulate binding there.

The Mp10 Y40F W120Y mutant, which does not block ROS or calcium bursts in response to flg22, is still able to bind AMSH2 (Chapter 6), indicating that the Mp10 interaction with AMSH2 is not important for Mp10-mediated suppression of the ROS and calcium burst. Mp10 may need to undergo conformational change after AMSH2 binding in order to suppress calcium and ROS bursts, which mutation of Tyr(40) and Trp(120) prevents. It is also possible that docking of a small hydrophobic molecule inside the pocket, dependent upon Tyr(40) and Trp(120) is required in addition to AMSH2 binding for Mp10 ROS and calcium burst suppression activity to occur. There may also be third component involved, which is recruited via the Tyr(40) and Trp(120) residues of Mp10 . Nonetheless, the Tyr(40) and Trp(120) residues of Mp10 are unlikely to be involved in Mp10 interactions with AMSH2, even though these residues are exposed to the surface of the globular Mp10 structure. Further investigations into whether Mp10 mutants are also able to affect receptor localisation would clarify whether this is responsible for the ROS and calcium burst suppression by Mp10. The deubiquitination activity of AMSH2 could also be investigated in the presence of Mp10 and mutants to see how this is affected by effector binding.

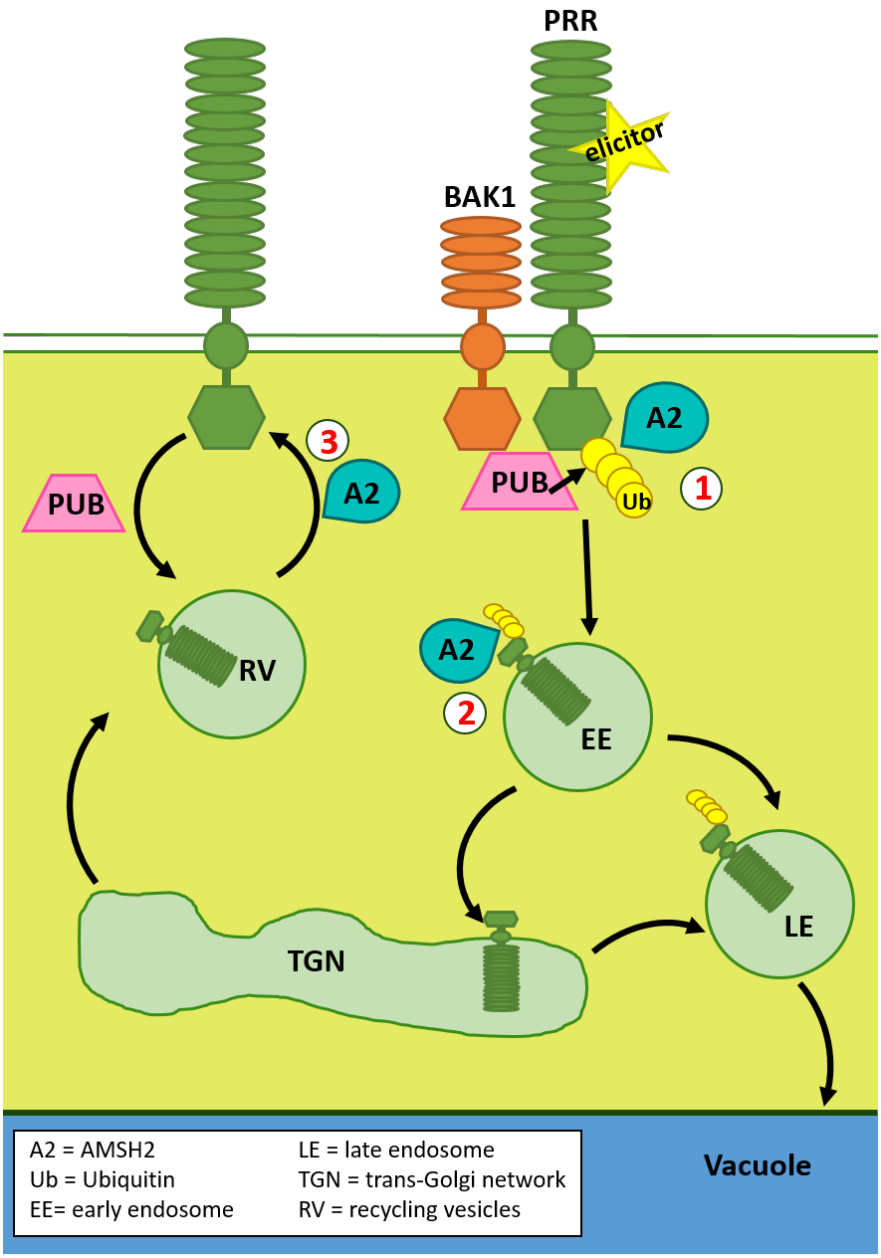


Figure 8.2:
AMSH2 could be acting on several different pathways in the control of receptor localisation. In all three cases, knock-down of AMSH2 or Mp10-mediated inhibition of AMSH2 activity would lead to more ubiquitinated PRRs and a greater number of PRRs present intracellularly, rather than on the plasma membrane.
1. AMSH2 may directly oppose the ubiquitination of receptors carried out by PUB12/13 after elicitor perception has occurred. Ubiquitination leads to endocytosis and degradation, whereas AMSH2-mediated deubiquitination activity may prevent endocytosis of the receptor and so prolong immune signaling at the plasma membrane.
2. AMSH2 may deubiquitinate receptors that have been endocytosed, redirecting the receptors from degradation to one of recycling or slowing down the process of degradation and allowing endosomal immune signaling to continue for longer.
3. AMSH2 may regulate the steady state recycling of receptors. The DUB may directly remove ubiquitin from receptors or components involved in intracellular trafficking that have been ubiquitinated by the action of PUBs (potentially PUB22/23/24), thereby increasing the numbers of receptors at the plasma membrane.

8.2.6 Aphid effector-triggered immunity

Mp10 induces chlorosis when expressed in *N. benthamiana*, which is dependent upon SGT1 (46). SGT1 is a ubiquitin-ligase associated plant protein that is required for plant cell death responses, including those involved in effector-triggered immunity (426). SGT1 involvement therefore suggests that Mp10 is recognised by the plant using an intracellular immune receptor, leading to ETI. Expression of Mp10 in *N. benthamiana* also led to a reduction in the fecundity of GPA reared on the plants, suggesting that ETI triggered by Mp10 has an effect on aphids (46).

In ETI, SGT1 forms a complex with HSP90 in order to chaperone NBS-LRR proteins and ensure their correct conformation. RAR1 (required for Mla12 resistance) regulates the HSP90-SGT1 complex, resulting in the stabilisation of NBS-LRR proteins (519). Both SGT1 and HSP90, but not RAR1, are required for Mi-1 mediated resistance to potato aphids (471). SA is also involved in ETI, including being required for Mi-1 mediated resistance (285), but my data shows that SA does not contribute to Mp10-mediated chlorosis in *N. benthamiana*. SGT1 is also involved in the chlorosis response induced by the JA-analogue coronatine, which is produced by *Pseudomonas syringae*, suggesting SGT1 involvement in a JA-mediated signal transduction pathway, independent of SA-dependent ETI. (582). The involvement of SGT1 in Mp10-induced chlorosis therefore requires further investigation. The requirement for HSP90, RAR1 and components of the JA signal transduction pathway could be tested to check whether Mp10 chlorosis is due to ETI triggered by NBS-LRR recognition or is independent of R-proteins and occurs via JA pathways.

Despite evidence for inducing ETI, Mp10 was not found to interact with any NBS-LRR proteins in the yeast two-hybrid screen (Chapter 7). This may be because the R-protein responsible for its recognition was not in the NBS-LRR library used. We also screened Mp10 against an insect-exposed Arabidopsis protein library, which was likely to contain some NBS-LRRs, and this did not identify any R-proteins as interactors either (Chapter 6). The lack of an interactor could be due to lack of direct interaction between Mp10 and the R-protein that detects it. Many R-proteins function by detecting perturbation of systems in the plant cell by guarding immune components, or decoys that look like immune components (290; 302). The R-protein that detects Mp10 may therefore interact with and guard AMSH2, or a similar protein, instead of directly binding Mp10.

Though Mp10 Tyr(40) and Trp(120) mutants are not able to suppress the flg22-induced

calcium and ROS bursts, these Mp10 mutants still induce the chlorosis response when expressed either systemically or locally via agroinfiltration in *N. benthamiana* (Chapter 5). This suggests that interference with PTI is not required for Mp10 recognition. Interestingly, the Mp10 mutant Y40F W120Y still interacts with AMSH2 (Chapter 6), so it may be Mp10-AMSH2 interaction that is detected by an R-protein guard, rather than inhibition of AMSH2 action. Another GPA CSP, MpOS-D1 was also found to cause chlorosis in *N. benthamiana*, and this does not appear to interact with AMSH2 (Chapter 5 and 6). Some common aspect of GPA CSPs may therefore be recognised by plants. This could be tested by using the other identified CSPs from GPA (Chapter 5) in similar plant and aphid assays.

I found that the chlorosis response to Mp10 still occurred in *N. benthamiana* expressing the NahG transgene, which prevents accumulation of SA (451) (Chapter 5). This suggests that the response triggered by Mp10 is SA-independent. This is unusual for ETI responses, which are generally seen as SA-dependent (583). However, SA is not needed for all ETI responses, for instance SGT1 and SA are both required for resistance to *Phytophthora infestans* in *N. benthamiana*, but SA is not needed for the hypersensitive response, which is a part of ETI (451). Therefore, though SA is not needed for the chlorosis response to Mp10, this does not mean the phytohormone is not required at all in defense against aphids. Both SGT1 and SA are required for the ETI mediated by the R-gene Mi-1 against potato aphids in tomato (471; 285). On the other hand, SA signaling is not needed for defense against GPA in Arabidopsis (372). The defense responses triggered by Mp10 may therefore involve different pathways than those commonly found in ETI. SA requirement may also differ depending on the plant and aphid species investigated.

The yeast two-hybrid screen of candidate aphid effectors against NBS-LRR proteins found a number of different interactions, suggesting that aphid proteins may well be detected *in planta*. Interestingly, the candidate effector Mp42 was found to interact with several R-proteins in the yeast two-hybrid screen. In a previous screen of candidate effectors carried out in the lab, Mp42 was found to reduce aphid fecundity when expressed in *N. benthamiana*, suggesting that it triggers ETI (46). One of the R-proteins identified as an Mp42 interactor may therefore be responsible for perception of Mp42 and the reduction in aphid fecundity seen (Chapter 7).

I carried out follow-up investigations on the R-protein RPS4 and the GPA effectors it was identified to interact with; Mp19, Mp21 and Mp44. I found no evidence for the induction of ETI when these proteins were expressed in *N. tabacum* together, nor did I find

dependable evidence for the suppression of ETI in the same system (Chapter 7). This may be due to a lack of ETI components downstream of GPA effector recognition by RPS4 in *N. tabacum*, rather than in Arabidopsis, which RPS4 was cloned from. However HR induced by AvrRps4 recognition by RPS4 still occurs in *N. tabacum*, suggesting this is not the case, though I cannot rule out the absence of components specific to aphid defense pathways. The variation of RPS4 between different Arabidopsis ecotypes may also explain why I saw no ETI phenotype, as differences in recognition of effectors between RPS4 variants has been found (553; 531; 287). Before investigations continue, the confirmation of aphid effector-RPS4 interaction *in planta* should be carried out.

Interestingly, several NBS-LRRs identified as aphid effector interactors are encoded next to each other in NBS-LRR clusters within the Arabidopsis genome (Chapter 7). NBS-LRRs are increasingly found to work in pairs in resistance responses to pathogen effectors, and many of these are located near each other in the Arabidopsis genome, for instance RRS1 and RPS4, and RRS1B and RPS4B (546; 547; 279). NBS-LRRs may also work in pairs to detect and initiate immune responses to GPA effectors. I also found evidence for the involvement of alternative splice variants of NBS-LRR proteins in responses to aphids (Chapter 7). Alternative splicing of NBS-LRRs has been identified as a means of regulation of immune responses in *N. tabacum* and Arabidopsis (549; 550), so the alternative splice variants I identified may be playing a similar role here. It would be interesting to confirm whether the R-gene splice variants are differentially induced upon aphid exposure, and how this effects protein levels in the plant. Complex regulation of R-genes is required as activation of ETI can have serious consequences for the plant, due to the induction of cell death. The utilisation of splice variants and requirement for a partner may be how the plant maintains this control.

8.2.7 Applications and implications of this study

Aphids, particularly GPA, have been found to develop resistance to existing pest control methods (72; 73; 514; 1). Understanding defense mechanisms already in place within the plant host can allow the development of strategies which enhance plant resistance responses, or the introduction of identified defense components to plants which do not naturally have them. Several possibilities for the enhancement of aphid resistance in crop plants can be identified from my research.

The AGB1 dependent regulation of PAD3 and camalexin production has been identified as an effective means of plant defense against aphids. Camalexin is only found in Ara-

bidopsis and a few related Brassica species (584). However, camalexin is synthesised from precursors involved in auxin signaling, which are likely to be present in many plant species (241). It may therefore be possible to introduce the genes responsible for camalexin synthesis, including PAD3, into other species where they would utilise the common metabolic intermediates. Metabolic engineering of the camalexin biosynthesis pathway has already been done transiently in *N. benthamiana*. The introduction of four camalexin biosynthesis genes, including PAD3, was sufficient confer production of camalexin, providing proof of this concept (585). Stable transformation has not been reported, so it is unknown whether introduction of the camalexin biosynthetic machinery has fitness payoffs. The channelling of resources into camalexin production at the expense of auxin signaling could cause undesirable side effects. The presence of extra metabolites could also alter plant properties such as taste, which would be undesirable in a crop plant.

Camalexin may be an effective defense against generalist insect species such as GPA, but Brassica specialists may already have a high tolerance to this phytoalexin. However, brassica specialists would be unlikely to move onto non-brassica plants expressing camalexin, due to the presence of other defense responses that prevent their colonisation. The cabbage aphid (*Brevicoryne brassicae*) shows increased fecundity on *pad3* plants (175), suggesting that despite adaptation to this plant family, camalexin is still an effective means of population control. Camalexin was not directly measured here, so the response on *B. brassicae* may be due to the loss of feedback on immune signaling that I have found PAD3 provides in response to aphid elicitors. This feedback response may also increase immune responses in other plant species, aiding plant defense responses.

Plant resistance genes against aphids such as Mi-1 and Vat are already utilised in crop protection (514). Symptoms produced upon Mp10 expression in *N. benthamiana* suggest that R-proteins may also have a role in the detection of this aphid effector. Although the yeast two-hybrid screen carried out to identify interactions between candidate aphid effectors and NBS-LRRs did not identify any R-proteins from Arabidopsis that interacted with Mp10, several potential R-proteins that may recognise other effectors were identified. These R-proteins can be screened for the ability to confer aphid resistance. The durability of plant resistance genes in agriculture is variable, with some such as Vat being used successfully extensively in areas without resistant biotypes, and others such as Nr being overcome within a short timeframe (87; 88). The stacking of R-genes into one genotype may provide more durable forms of resistance, especially a combination of those from different gene clusters which represent different interactions between R-genes and their cognate effectors, as it

will be less likely for aphid pests to alter multiple effectors at the same time (586; 587). If interaction of the identified R-proteins and aphid effectors is confirmed, several could be deployed together in this way. However, resistance is often found in specific aphid biotypes, rather than being developed or evolved by one clone, so the introduction of aphid biotypes from elsewhere is a large factor in the breakdown of aphid resistance (514; 588). Once an elicitor which triggers ETI is identified, it will be possible to survey its variation across different biotypes, which could then inform R-gene stacking, so that R-genes are utilised that will work against a broad range of biotypes.

The mutation of effector targets may also be a viable way of introducing resistance into susceptible lines. Mp10 interacts with AMSH2, which may be how the effector can interfere with BAK1-dependent plant immunity. The use of AMSH1, AMSH3 and all three AMSH2 splice variants in yeast two-hybrid screens has narrowed down the region of AMSH2 involved in Mp10 interaction to the N-terminal region of the DUB. Further investigation using AMSH2 chimeras or mutants could narrow down this zone of interaction further, and possibly identify regions of AMSH2 that can be altered to stop Mp10 interaction without the loss of DUB function. Engineering of this altered form of AMSH2 into crops would prevent Mp10 binding and allow the PTI response to occur upon perception of aphid elicitors. Homologs of Mp10 from other aphid species also seem to be active in blocking immune responses (316), suggesting this effector is used by aphids other than GPA. This method of crop protection could therefore give resistance to multiple species of aphid.

My work has shown that the same plant defense pathways are utilised in response to both insects and pathogens. Introducing a PRR that can activate these pathways in response to aphid elicitors may therefore be successful, as it will utilise immune signaling pathways and downstream defense responses that are already present within the plant. I identified two RLKs that are required for the ROS burst to GPA extract. If the action of these receptors in the perception of aphids is confirmed, transferring them between plant species may be able to increase plant resistance to aphids. Proof of concept has already been seen in the transfer of the Brassica specific PRR EFR from Arabidopsis into the solanaceous plants *N. benthamiana* and *Solanum lycopersicum* (tomato), which led to increased resistance to a range of different phytopathogenic bacteria (589). PRRs detect conserved components of plant pests, which makes it likely that these elicitors are unable to change to avoid recognition. It is also more difficult to generate new effectors that target a PRR than it is to mutate or delete existing effectors to avoid recognition by an R-protein. The use of several PRRs, or a combination of both PRRs and R-proteins may therefore provide

broad-spectrum resistance against multiple species of aphid.

The evidence suggests that one or both of the PRRs I identified may be interacting with BAK1 in order for immune signaling to occur. As I found that Mp10 is active against the BAK1-mediated immune response to aphids, it may be best to focus on components of non-BAK1-mediated pathways to discover sources of aphid resistance, as it seems that GPA is already able to overcome BAK1-mediated resistance. As AGB1 was found to be upstream of PAD3 induction, and so the production of camalexin which is an effective defense against aphids, it may be best to focus on elucidation of this immune pathway and identify the receptors involved, which may be GPCRs.

I also identified an RLK, CRK7, which appears to have a negative effect on the immune signaling to aphids. If CRK7 is responsible for damping down the immune response normally, perhaps unleashing plants from this regulation will allow a greater defense response. CRK7 mutants should therefore be checked for an increased resistance to aphids. However, removal of immune response control systems could have fitness consequences for the plant. Defense responses are generally curtailed to reserve resources that are required for growth. Many mutants that show constitutive or increased defense responses also have growth defects (113), it is therefore important to consider and investigate the balance between growth and immunity before removing regulators of plant immunity.

The discovery that elicitors from aphids can lead to plant defense responses, including induced resistance, suggests that aphid elicitors may be utilised as a crop treatment to prime plant defense. Pre-treating plants with the elicitor harpin from plant pathogenic bacteria, has been found to induce resistance against aphids (590). Several compounds are already utilised as elicitors in integrated pest management programmes to enhance disease resistance of crop plants, with varying degrees of success. One example is Elexa, developed by Glycogenesys Inc (Boston, USA), which contains 4% chitosan, a derivative of chitin, as its active ingredient and is able to protect a range of crops against important pathogens (591). Application of an elicitor that induces resistance to aphids would enable producers to boost crop immune response over periods when aphids are likely to become a problem, such as when large levels of alates are detected.

However, elicitors may not be entirely successful. The level of induced resistance seen in response to GPA extract was not large, with reduction in fecundity varying between 20-40%. This may be due to the presence of effectors such as Mp10 which are effective in

suppressing plant defense responses. It has also been found that the elicitors are heat sensitive, and so likely to denature and become inactive under field conditions. The discovery of GroEL as a specific aphid elicitor may lead to use of this protein as a crop elicitor as this seems to be heat stable (363). DAMPs produced during aphid feeding could also be potential targets for field application development. There is also the repeated issue here of the growth-immunity interaction, as the priming of plant defense often counteracts plant growth and so crop yield. The application of PAMPs to seedlings leads to growth inhibition (114). Treatment of crop plants by elicitors may therefore not be beneficial to agriculture.

The identification of aphid genes which are required for insect success on plants, such as Mp10 and C002 also opens the door for control of pest insects by RNAi mediated gene silencing. RNAi has been used as a tool in the lab to investigate the impact of certain genes on aphid fitness (43). I found that silencing of Mp10 by 40-60% led to a reduction of GPA fecundity of about 20%. Similar results have been seen when silencing other aphid effectors (44). A larger reduction in fecundity can be achieved over more aphid generations; a 40-60% reduction was obtained after rearing GPA on dsMp2 and dsMpC002 plants for 4 weeks (45). High levels of silencing could therefore be possible in a field setting, where multiple generations of one species live on one plant. A reduction of aphid reproduction by 40-60% in these conditions would dramatically decrease aphid population growth, leading to a reduction in agricultural losses due to aphids. This method of pest control would only affect insects that feed on the plant, so would avoid negative effects on beneficial insects such as pollinators and aphid predators. Targeting of genes such as those encoding effectors also means that the effect is specific for aphids. Other animals without the gene that eat the plant are unaffected. This method therefore holds promise for control of aphid pests.

Overall, my research has found that components of plant immunity, from PTI to ETS and ETI, play a role in insect-plant interactions. It appears that there is one toolkit for biotic interaction in plants, including ROS, calcium, BAK1 and G-proteins, with some components such as PRRs and R-proteins used to provide specificity and enabling the tailoring of the plant defense response to the attacker. Research on the molecular interaction between a model plant and aphid has identified multiple avenues for applications in agriculture. A combination of approaches will likely be the most successful method of aphid control; combining methods such as RNAi of aphid effector genes, and the introduction of PRRs and R-genes will multiply the effects that they can have.

Appendices

Appendix A

The leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 and the cytochrome P450 PHYTOALEXIN DEFICIENT3 contribute to innate immunity to aphids in Arabidopsis. Prince et al., 2014

The Leucine-Rich Repeat Receptor-Like Kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 and the Cytochrome P450 PHYTOALEXIN DEFICIENT3 Contribute to Innate Immunity to Aphids in Arabidopsis[1][C][W][OPEN]

David C. Prince[2], Claire Drurey, Cyril Zipfel, and Saskia A. Hogenhout*

Department of Cell and Developmental Biology, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, United Kingdom (D.C.P., C.D., S.A.H.); and The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, United Kingdom (C.Z.)

The importance of pathogen-associated molecular pattern-triggered immunity (PTI) against microbial pathogens has been recently demonstrated. However, it is currently unclear if this layer of immunity mediated by surface-localized pattern recognition receptors (PRRs) also plays a role in basal resistance to insects, such as aphids. Here, we show that PTI is an important component of plant innate immunity to insects. Extract of the green peach aphid (GPA; *Myzus persicae*) triggers responses characteristic of PTI in Arabidopsis (*Arabidopsis thaliana*). Two separate eliciting GPA-derived fractions trigger induced resistance to GPA that is dependent on the leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 (BAK1)/SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE3, which is a key regulator of several leucine-rich repeat-containing PRRs. BAK1 is required for GPA elicitor-mediated induction of reactive oxygen species and callose deposition. Arabidopsis *bak1* mutant plants are also compromised in immunity to the pea aphid (*Acyrthosiphon pisum*), for which Arabidopsis is normally a nonhost. Aphid-derived elicitors induce expression of PHYTOALEXIN DEFICIENT3 (PAD3), a key cytochrome P450 involved in the biosynthesis of camalexin, which is a major Arabidopsis phytoalexin that is toxic to GPA. PAD3 is also required for induced resistance to GPA, independently of BAK1 and reactive oxygen species production. Our results reveal that plant innate immunity to insects may involve early perception of elicitors by cell surface-localized PRRs, leading to subsequent downstream immune signaling.

Close to a million insect species have so far been described, and nearly one-half of them feed on plants (Wu and Baldwin, 2010). Within these plant-feeding insects, most feed on a few related plant species, with only 10% feeding upon multiple plant families (Schoonhoven et al., 2005). Plant defense to insects include several layers (Bos and Hogenhout, 2011; Hogenhout and Bos, 2011). Physical barriers, volatile cues, and composition of secondary metabolites of plants are important components that determine insect host choice (Howe and Jander, 2008; Bruce and Pickett, 2011). In addition, plants induce a variety of plant defense responses upon perception of herbivore oral secretions (OS), saliva, and eggs (De Vos and Jander, 2009; Bruessow et al., 2010; Ma et al., 2010; Wu and Baldwin, 2010). These responses may provide full protection against the majority of insect herbivores, and insects that are able to colonize specific plant species likely produce effectors in their saliva or during egg laying that suppress these induced defense responses (Bos and Hogenhout, 2011; Hogenhout and Bos, 2011; Pitino and Hogenhout, 2013).

Aphids are sap-feeding insects of the order Hemiptera and are among the most destructive pests in agriculture, particularly in temperate regions (Blackman and Eastop, 2000). More than 4,000 aphid species in 10 families are known (Dixon, 1998). Most aphid species are specialists and use one or a few closely related plant species within one family as host for feeding and reproduction. Examples are pea aphid (*Acyrthosiphon pisum*), cabbage aphid (*Brevicoryne brassicae*), and English grain aphid (*Sitobion avenae*) that colonize plant species within the legumes (family Fabaceae), brassicas

[1] This work was supported by the Biotechnology and Biological Sciences Research Council (grant nos. BB/J004553/1 to S.A.H. and BB/G024936/1 to C.Z.), the John Innes Foundation (to S.A.H.), the Gatsby Charitable Foundation (to C.Z.), and Biotechnology and Biological Sciences Research Council studentships (to D.C.P. and C.D.).

[2] Present address: The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, United Kingdom.

* Address correspondence to saskia.hogenhout@jic.ac.uk.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Saskia A. Hogenhout (saskia.hogenhout@jic.ac.uk).

[C] Some figures in this article are displayed in color online but in black and white in the print edition.

[W] The online version of this article contains Web-only data.

[OPEN] Articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.114.235598

(Brassicaceae), and grasses (Gramineae), respectively. The green peach aphid (GPA; *Myzus persicae*) is one of few aphid species with a broad host range and can colonize hundreds of plants species in over 40 plant families, including brassicas (Blackman and Eastop, 2000). Aphids possess mouthparts composed of stylets that navigate to the plant vascular system, predominantly the phloem, for long-term feeding. However, before establishing a long-term feeding site, these insects display a host selection behavior by probing the upper leaf cell layers with their stylets, a behavior seen on host and nonhost plants of the aphid (Nam and Hardie, 2012). When the plant is judged unsuitable, the aphid takes off to find an alternative plant host. It is not yet clear what happens in the initial stages of insect interactions with plants.

Plants sense microbial organisms (including bacteria, fungi, and oomycetes) through perception of conserved molecules, named microbe-associated molecular patterns and pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) to induce the first stage of plant immunity, termed PAMP-triggered immunity (PTI). PTI is effective against the majority of plant pathogens. Bacterial and fungal PAMPs characterized so far include bacterial flagellin (or its derived peptide flg22), bacterial elongation factor (EF)-Tu (or its derived peptide elf18), bacterial lipopolysaccharides and bacterial cold shock protein, chitin oligosaccharides, and the oomycete elicitin INF1 (Boller and Felix, 2009)

Plant PRRs are either receptor-like kinases (RLKs) or receptor-like proteins. Most leucine-rich repeat (LRR)-type PRRs associate with and rely for their function on the small regulatory LRR-RLK BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 (BAK1)/SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (SERK3; Monaghan and Zipfel, 2012). For example, in Arabidopsis (*Arabidopsis thaliana*), flg22 and elf18 bind to the LRR-RLKs FLAGELLIN SENSITIVE2 (FLS2) and EF-TU RECEPTOR (EFR), respectively, leading to a quasi-instant association with BAK1 (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006; Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010; Roux et al., 2011; Sun et al., 2013). BAK1 is required for optimal downstream immune signaling events, such as mitogen-activated protein kinase (MAPK) activation, reactive oxygen species (ROS) bursts, callose depositions, induction of immune genes, and induced resistance. Similarly, BAK1 is a positive regulator of innate immune responses triggered by the Arabidopsis LRR-RLKs PLANT ELICITOR PEPTIDE1 RECEPTOR1 (PEPR1) and PEPR2 that bind the Arabidopsis-derived damage-associated molecular pattern *A. thaliana* Peptide1 (AtPep1; Krol et al., 2010; Postel et al., 2010; Roux et al., 2011) and by the tomato (*Solanum lycopersicum*) LRR receptor-like protein Ve1 that recognizes Ave1 derived from *Verticillium* spp. (Fradin et al., 2009; de Jonge et al., 2012). Consistent with the role of BAK1 downstream of numerous PRRs, BAK1 is required for full immunity to a number of bacterial, fungal, oomycete, and viral pathogens (Heese et al., 2007; Kemmerling et al., 2007; Fradin et al., 2009; Chaparro-Garcia et al., 2011; Roux et al., 2011; Kørner et al., 2013).

Notably, it has been recently shown that the ortholog of BAK1 in *Nicotiana attenuata* regulates the induction of jasmonic acid (JA) accumulation upon herbivory (Yang et al., 2011a). However, immunity to insects was not affected when *BAK1* was silenced, and the observed effect on JA accumulation may be due to an indirect effect on brassinosteroid (BR) responses, for which BAK1 is also an important positive regulator (Li et al., 2002; Nam and Li, 2002). Therefore, it is currently unclear if BAK1 is involved in the early recognition of insect-derived elicitors leading to immunity.

We discovered that the key regulatory LRR-RLK BAK1 participates in plant defense to an insect herbivore. We found that extracts of GPA trigger plant defense responses in Arabidopsis that are characteristic of PTI. Arabidopsis *bak1* mutant plants are compromised in defense to GPA, which colonizes Arabidopsis, and to pea aphid, for which Arabidopsis is a nonhost. BAK1 is required for ROS bursts, callose deposition, and induced resistance in Arabidopsis upon perception of aphid-derived elicitors. One of the defense genes induced by GPA-derived extracts encodes PHYTOALEXIN DEFICIENT3 (PAD3), a cytochrome P450 that catalyzes the conversion of dihydrocamalexic acid to camalexin, which is a major Arabidopsis phytoalexin that is toxic to GPA (Kettles et al., 2013). *PAD3* expression is required for Arabidopsis-induced resistance to GPA, independently of BAK1 and ROS. Our results provide evidence that innate immunity to insect herbivores may rely on the early perception of elicitors by cell surface-localized PRR.

RESULTS

We first investigated if GPA-derived elicitors trigger cellular responses characteristic of PTI responses, including the induction of PTI marker genes, ROS bursts, and callose depositions (Boller and Felix, 2009). Aphids secrete saliva into the plant while probing and feeding; however, the plant is not only exposed to aphid saliva, but also aphid mouthparts and honeydew. In addition, aphid saliva collected from feeding membranes differs in composition depending on the medium into which it is secreted (Cherqui and Tjallingii, 2000; Cooper et al., 2010). Studies of aphid saliva have identified proteins that were not detected in the salivary gland (Carolan et al., 2011), did not possess secretion signals (Harmel et al., 2008), or originated from bacterial endosymbionts (Filichkin et al., 1997). Therefore, the composition of aphid saliva is complex and unlikely to be entirely represented by collecting secretions from feeding membranes. Aphid honeydew contains proteins from the aphid plus its endosymbiotic bacteria and gut flora, including known PAMPs (Sabri et al., 2013). In light of this, we opted to expose the plant to whole aphid extracts rather than aphid saliva only.

Treatment of Arabidopsis leaves with a GPA-derived extract up-regulates transcript levels of genes encoding FLG22-INDUCED RECEPTOR-LIKE KINASE1 (FRK1), CYTOCHROME P450, FAMILY 81, SUBFAMILY F, POLYPEPTIDE2 (CYP81F2), and PAD3/CYP71B15 (Fig. 1A), which are markers for early immune signaling, indolic glucosinolate production, and camalexin biosynthesis, respectively (Zhou et al., 1999; Asai et al., 2002; Bednarek et al., 2009). These genes have been previously shown to be induced by both protein and carbohydrate elicitors (Gust et al., 2007; Denoux et al., 2008). The levels of gene inductions to GPA-derived extract and flg22 were similar, except for *pad3*, which was more up-regulated in GPA-derived extract than in flg22-treated leaves (Fig. 1A). Callose deposition is a commonly observed plant response to elicitors, the timing of which depends on the elicitor used (Luna et al., 2011). We assayed callose deposition 24 h after elicitor treatment and observed increased numbers of callose deposits in Arabidopsis leaves treated with GPA-derived extract compared with a buffer control, although not quite as high as in flg22-treated leaves (Fig. 1B). Similarly, an ROS burst was observed in Arabidopsis leaves treated with GPA-derived extract (Fig. 1D). This ROS burst was however delayed compared with that of the flg22 treatment; the ROS burst to flg22 occurred within 10 to 20 min (Fig. 1C), while that to GPA-derived extract occurred after 1 h. At this time, the flg22-induced ROS levels were returning to base level (Fig. 1D). Nonetheless, these data show that GPA-derived extract contains one or several elicitors that trigger PTI-like plant responses.

We next investigated whether PTI-like responses triggered by GPA-derived extract required components involved in PTI. Flg22-triggered ROS burst is

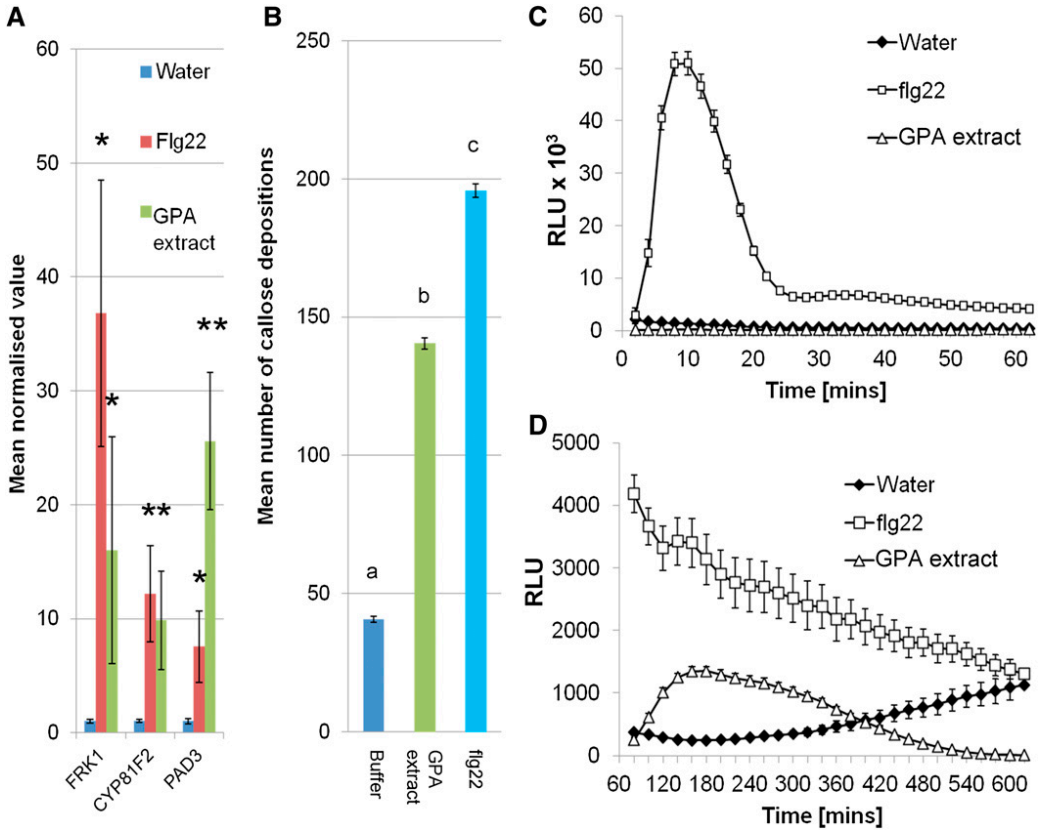


Figure 1. Plant defense elicitations to GPA-derived extract are characteristic of PTI. A, GPA-derived extract elicits the expression of PTI marker genes. Bars show the means ± SE of target gene expression levels of four independent experiments (n = three per experiment). Asterisks indicate significant differences in GPA fraction compared to water (Student's t probabilities calculated within GLM), with $^*P < 0.05$ compared to water control for each gene and $^{**}P < 0.05$ between flg22 and GPA-derived extract treatment. B, GPA-derived extract elicits callose deposition. Data shown are mean callose deposits produced per 1.34 mm^2 of leaf upon each treatment with means ± SE of three independent experiments (n = 12 leaf discs per experiment). Different letters indicate significant differences between the treatments (Student's t probabilities calculated within GLM) at $P < 0.05$ (n = 36, $F_{2,103}$ = 2039.93). C and D, Col-0 leaf discs were elicited with water, 12.5 nM flg22 (in water), and GPA-derived extract (in water), and ROS bursts in these leaf discs were measured using luminol-based assays at 0 to 60 min (C) and 60 to 600 min (D) after elicitation. Graphs show means ± SE of n = 32 leaf discs per replicate. Data of one representative experiment are shown. The experiment was repeated three times with similar results. RLU, Relative light units. [See online article for color version of this figure.]

dependent on the NADPH-oxidase *A. thaliana* respiratory burst oxidase homolog D (AtRbohD; Nühse et al., 2007; Zhang et al., 2007). We previously found that an aphid candidate effector *M. persicae* candidate effector10 suppresses the flg22-mediated ROS burst (Bos et al., 2010), a response that also requires BAK1 (Chinchilla et al., 2007; Heese et al., 2007). Because BAK1 is an essential regulator of many PTI responses characterized so far (Monaghan and Zipfel, 2012), we also investigated if BAK1 was required for the PTI-like responses to GPA-derived extract. The GPA-derived extract-triggered ROS burst was reduced in the semi-dominant *bak1-5* mutant and was completely absent in *AtrbohD* (Fig. 2A). Flg22-triggered callose deposition requires biosynthesis of 4-methoxylated indole glucosinolates, mediated by CYP81F2 (Clay et al., 2009), and is diminished in mutants of *PENETRATION2 (PEN2)*, which encodes a myrosinase involved in glucosinolate metabolism (Lipka et al., 2005; Bednarek et al., 2009; Clay et al., 2009; Luna et al., 2011). As GPA-derived extract induces *CYP81F2* expression (Fig. 1A), we investigated whether PEN2 and BAK1 were required for GPA-triggered callose depositions. The number of callose deposits was significantly reduced in *bak1-5* and *pen2-1* mutants compared with ecotype Columbia (Col-0) after treatment with GPA-derived extract (Fig. 2B). Together, these data provide evidence that PTI-like responses to GPA-derived extract require components involved in PTI responses.

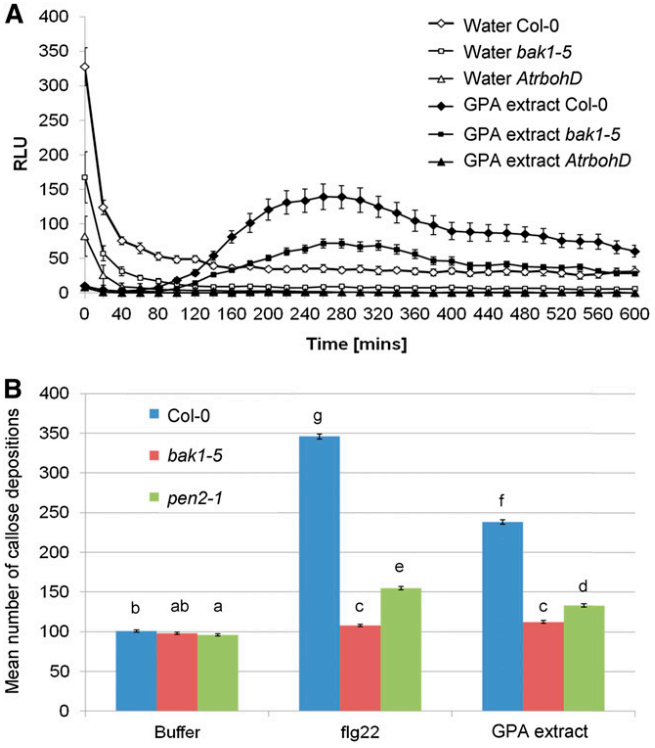


Figure 2. Plant defense elicitations to GPA-derived extract require components of PTI. A, GPA-derived extract elicits an ROS burst in wild-type Col-0 that is reduced in *bak1-5* and absent in the *AtrbohD* mutant. ROS bursts were measured over a 600-min period. Graph shows means ± SE of $n = 16$ leaf discs per replicate. White symbols represent water-treated leaf discs, and black symbols represent GPA-derived extract-treated leaf discs. Data of one representative experiment are shown. The experiment was repeated three times with similar results. B, GPA-derived extract-elicited callose deposition is significantly reduced in *bak1-5* and *pen2-1*. Data shown are mean callose deposits produced per 1.34 mm^2 of leaf upon each treatment with means ± SE of three independent experiments ($n = 12$ leaf discs per replicate). Different letters indicate significant differences between the treatments (Student's *t* probabilities calculated within GLM) at $P < 0.05$ ($n = 36$, $F_{10,323} = 1388.15$). [See online article for color version of this figure.]

As very little is known about plant cell surface perception of insect-derived elicitors, we further investigated the role of BAK1 in immunity to aphids. In addition to its role in PTI signaling, BAK1 is also involved in BR responses (Li et al., 2002; Nam and Li, 2002), light signaling (Whippo and Hangarter, 2005), and cell death control (He et al., 2007; Kemmerling et al., 2007). Null *bak1* mutants are compromised in all of these areas. The ethyl methane sulfonate mutant *bak1-5* has a substitution in the cytoplasmic kinase domain that leads to compromised innate immune signaling but is not impaired in BR or cell death control (Schwessinger et al., 2011), allowing its use to investigate the relevance of BAK1 in resistance to pathogens with different lifestyles (Roux et al., 2011). We investigated GPA performance on *bak1-5*, the null mutant *bak1-4* (He et al., 2007), and a null mutant of BAK1-LIKE1 (BKK1)/SERK4, *bkk1-1*, which is the closest paralog of BAK1 and similarly controls PTI, BR, and cell death responses (He et al., 2007; Roux et al., 2011). GPA reproduction on wild-type Col-0 and *bak1-5* plants were more similar than the reproduction rates of this aphid on *bak1-4* and *bkk1-1* plants (Supplemental Fig. S1). This suggests that the pleiotropic phenotypes, such as deregulated cell death, of the null mutants affect aphid performance (He et al., 2007; Kemmerling et al., 2007). These results are consistent with the response of the obligate biotrophic oomycetes *Hyaloperonospora arabidopsidis*, which showed decreased reproduction on *bak1-4* plants but no increase in reproduction on *bak1-5* plants for three *H. arabidopsidis* isolates (Roux et al., 2011). Therefore, we continued our investigation with the Arabidopsis *bak1-5* mutant alone.

Treatment with exogenous PAMPs enhances plant resistance to pathogens, and this is also known as induced resistance (Zipfel et al., 2004; Balmer et al., 2013). De Vos and Jander (2009) previously observed that GPA saliva proteins between 3 and 10 kD in molecular mass elicit induced resistance to GPA in Arabidopsis (De Vos and Jander, 2009). To investigate if BAK1 is involved in this response, wild type Col-0 plants were treated with GPA-derived extract, and GPA reproduction on these leaves was then assessed over a period of 10 d. Induced resistance was triggered by whole GPA-derived extract (Fig. 3A), the GPA--derived 3- to 10-kD fraction (Fig. 3B), and the 3- to 10-kD GPA saliva fraction (Supplemental Fig. S2). Induced resistance was reduced in the *bak1-5* mutant (Fig. 3,

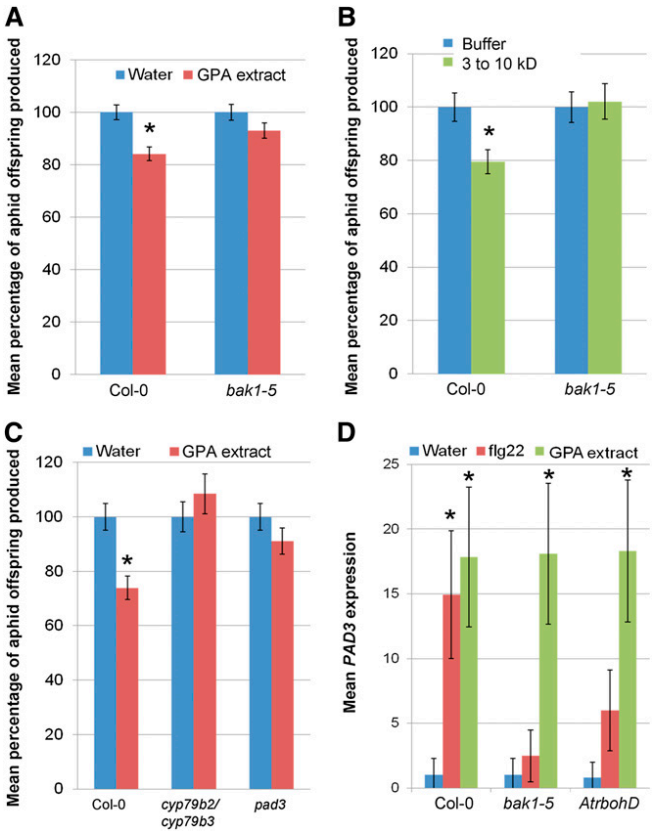


Figure 3. Plant defense responses elicited by GPA-derived extract are dependent on BAK1 and PAD3. A and B, Induced-resistance to GPA-derived extract (A) and GPA 3- to 10-kD fraction (B) is dependent on BAK1. Bars show the means ± SE of total nymphs produced per plant of six (A) and three (B) independent experiments. The nymph counts were normalized with the water or buffer controls set at 100%. Asterisks indicate significant differences to GPA fraction compared with water/buffer (Student's *t* probabilities calculated within GLM) with $*P < 0.001$ (Col-0 wild type, $n = 60$, $F_{1,19} = 17.88$) and $P = 0.063$ (A; *bak1-5* mutant, $n \geq 57$, $F_{1,115} = 3.45$) and $*P = 0.005$ (Col-0 wild type, $n \geq 28$, $F_{1,56} = 8.065$) and $P = 0.835$ (B; *bak1-5* mutant, $n \geq 25$, $F_{1,53} = 0.043$). C, Induced-resistance to GPA-derived extract is dependent on PAD3. Bars show the means ± SE of total nymphs produced per plant of three independent experiments. Nymph counts were normalized with the water control set at 100%. $*P < 0.001$ (Col-0, $n \geq 23$, $F_{1,46} = 15.5$), $P = 0.384$ (*cyp79b2/cyp79b3* mutants, $n \geq 16$, $F_{1,36} = 0.76$), and $P = 0.188$ (*pad3* mutant, $n \geq 19$, $F_{1,41} = 1.73$). D, GPA-derived extract-triggered *PAD3* expression is not dependent on BAK1 or AtRbohD. Bars show the means ± SE of target gene expression levels of three independent experiments (n = three per experiment). Expression levels were normalized with the water control of Col-0 set at 1. Asterisks indicate significant differences compared with water control (Student's *t* probabilities calculated within GLM) with $*P < 0.05$. [See online article for color version of this figure.]

A and B; Supplemental Fig. S2). These demonstrate that aphid elicitors present in whole GPA-derived extract and saliva are recognized in a BAK1-dependent manner, leading to immunity to GPA.

Next, we investigated if PAD3 is involved in Arabidopsis-induced resistance to GPA. The cytochrome P450 PAD3 catalyzes the conversion of dihydrocamalexic acid to camalexin, the major Arabidopsis phytoalexin, and acts downstream of CYP79B2 and CYP79B3 enzymes in the glucosinolate biosynthetic pathway (Zhao et al., 2002; Schuhegger et al., 2006). We previously demonstrated that camalexin is toxic to GPA (Kettles et al., 2013). Moreover, *PAD3* expression is induced upon perception of aphid elicitors (Fig. 1A), GPA saliva (De Vos and Jander, 2009), and GPA feeding (De Vos et al., 2005; Kettles et al., 2013).

We found that Arabidopsis *pad3* and *cyp79b2/cyp79b3* mutants do not show induced resistance to GPA upon treatment of plants with GPA-derived extract (Fig. 3C). To determine whether the PAD3-dependent induced resistance requires BAK1 and apoplastic ROS production, we measured *PAD3* induction in *bak1-5* and *AtrbohD* plants in response to GPA-derived extract. *PAD3* expression was reduced in *bak1-5* and *AtrbohD* in response to flg22 but not GPA-derived extract (Fig. 3D), suggesting that PAD3-dependent induced resistance to GPA-derived extract is independent of BAK1 and apoplastic ROS production. Therefore, Arabidopsis-induced resistance to GPA is dependent on BAK1 and PAD3 through separate signaling pathways.

We sought to characterize further the biochemical properties of the GPA-derived elicitors. The ROS burst and induced-resistance responses disappeared when GPA-derived extract was boiled (Fig. 4, A and B). The proteinase K-treated GPA-derived extract did not generate an induced-resistance response to GPA (Fig. 4B), even though proteinase K itself induced an ROS burst in Arabidopsis Col-0 that started at about 1 h after treatment and disappeared upon boiling of proteinase K (Supplemental Fig. S3, A and B). The 3- to 10-kD fraction induced an ROS burst, while fractions that are smaller than 3 kD and larger than 10 kD did not (Fig. 4C). Induced resistance to GPA was, however, observed for both the 3- to 10-kD and larger-than-10-kD fractions but not for the smaller-than-3-kD fraction (Fig. 4D). Altogether, these results indicate the presence of at least two eliciting fractions in GPA-derived extract, which are likely to contain heat-sensitive proteins or peptides.

Arabidopsis *bak1-5* mutant plants produce significantly less ROS in response to the GPA-derived 3- to 10-kD extract (Fig. 5A). BAK1 is a coreceptor that associates with several LRR-RLK-type PRRs, such as FLS2, EFR, PEPR1, and PEPR2 (Chinchilla et al., 2007; Heese et al., 2007; Postel et al., 2010; Roux et al., 2011), which perceive bacterial flagellin, bacterial EF-Tu, and the damage-associated molecular patterns AtPeps, respectively (Gómez-Gómez and Boller, 2000; Yamaguchi et al., 2006; Zipfel et al., 2006; Yamaguchi et al., 2010). However, Arabidopsis mutant lines in these PRRs did not show reduced ROS bursts to the 3- to 10-kD extract (Fig. 5, B and C). While the lysine-motif-RLK CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) does not require BAK1 for signaling, this receptor is involved in the perception of chitin

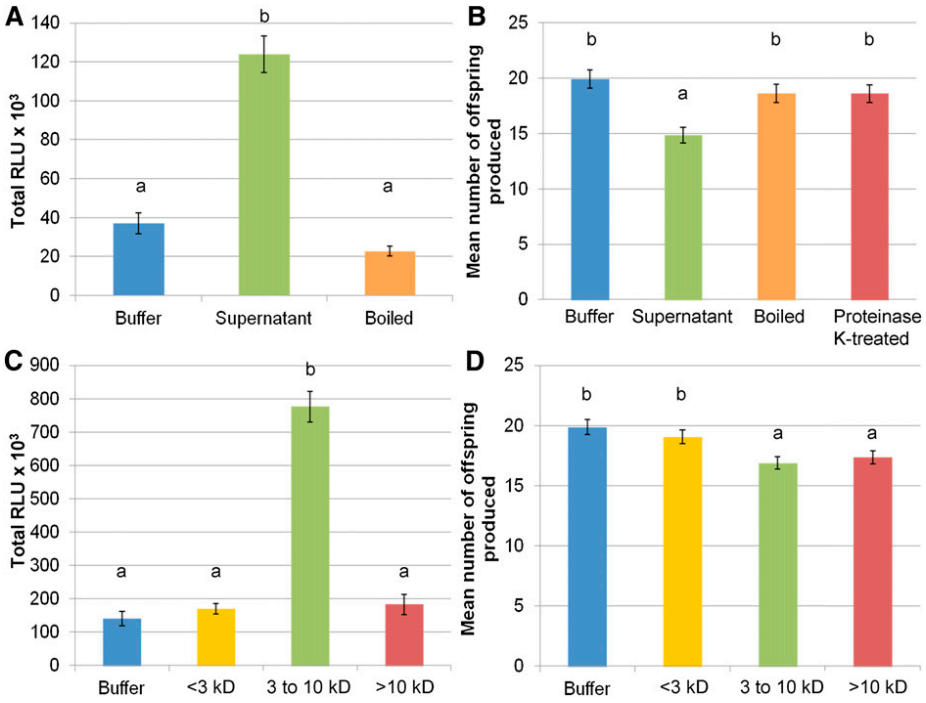


Figure 4. GPA-derived extract-eliciting activities disappear upon boiling and proteinase K treatments. A, Boiled GPA-derived extract does not elicit an ROS burst. ROS bursts were measured over a 600-min period. Bars show means ± SE of $n = 16$ leaf discs per replicate. Data of one representative experiment are shown. The experiment was repeated three times with similar results. Bars marked with different letters indicate significant differences at $P < 0.05$ using ANOVA. B, Boiled and proteinase K-treated GPA-derived extract do not elicit induced resistance. Bars show the means ± SE of total nymphs produced per plant of three independent experiments. Bars marked with different letters indicate significant differences at $P < 0.05$ (Student's t probabilities calculated within GLM; $n = 30$, $F_{3,119} = 7.688$). C, The 3- to 10 kD fraction of GPA-derived extract elicits ROS bursts. ROS bursts were measured over an 800-min period. Bars show means ± SE of $n = 16$ leaf discs per replicate. Data of one representative experiment are shown. The experiment was repeated three times with similar results. Letters indicates significant differences at $P < 0.05$ using ANOVA. D, Three- to ten-kilodalton and larger-than-10-kD GPA-derived extracts elicit induced resistance. Bars show the means ± SE of total nymphs produced per plant of six independent experiments. Letters indicate significant differences at $P < 0.05$ (Student's t probabilities calculated within GLM; $n = 60$, $F_{3,237} = 6.051$). [See online article for color version of this figure.]

(Miya et al., 2007; Wan et al., 2008), which is abundant in the aphid cytoskeleton, including the aphid mouthparts that are in contact with the plant during feeding. Nonetheless, the response to GPA-derived extract was not reduced in an Arabidopsis *fls2 efr cerk1* triple mutant (Fig. 5B). Thus, aphid elicitor-induced ROS burst is dependent on BAK1 and a thus-far unknown PRR.

We also investigated whether BAK1 was involved in the induced resistance to the larger-than-10-kD eliciting fraction. Induced resistance was observed on Col-0 Arabidopsis plants but not on the *bak1-5* mutant plants for the 3- to 10-kD and larger-than-10-kD fractions (Fig. 5D). Therefore, BAK1 is involved in the signaling pathways to both of these eliciting fractions.

Elicitors perceived by PRRs are often conserved among groups of pathogens (Medzhitov and Janeway, 1997). To investigate if this is also the case for aphids, we examined the expression levels of the PTI marker genes *FRK1*, *CYP81F2*, and *PAD3* in Arabidopsis plants treated with extracts of various aphid species (pea aphid, cabbage aphid, and English grain aphid). The expression of these genes were induced to similar levels after treatment with aphid-derived extracts from the three other species tested, although the induction of *FRK1* and *CYP81F2* was not statistically significant upon treatment with English grain aphid-derived extract (Fig. 6A). These results provide evidence that aphid-derived elicitors perceived by Arabidopsis are potentially conserved among different aphid genera/species.

The pea aphid host range is mostly restricted to plants of the legume family; these insects do not like to feed on brassicas, such as Arabidopsis. Because PRRs regulate the first active line of plant defense response and are proposed to be involved in nonhost resistance in plant species distantly related to the natural host (Schulze-Lefert and Panstruga, 2011), we investigated if the pea aphid survives better on Arabidopsis *bak1-5* mutant plants. About 50% of the pea aphids on Arabidopsis Col-0 are still alive between 3 and 4 d (Fig. 6B). Remarkably, at this time, the survival rates of pea aphids were significantly higher, about 75%, on

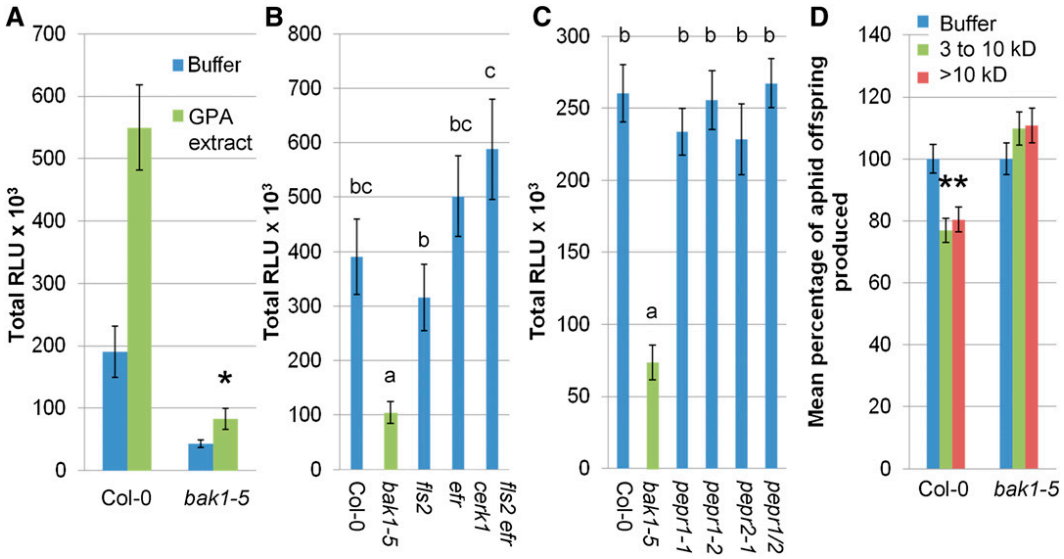


Figure 5. Plant immune responses to individual GPA-derived elicitor fractions are BAK1 dependent. A, BAK1 is involved in Arabidopsis ROS burst to GPA-derived elicitors. ROS bursts were measured in response to buffer and 2.5 mg mL^{-1} 3- to 10-kD GPA-derived extract over an 800-min period. Bars show means ± SE of n = eight leaf discs per replicate. Data of one representative experiment are shown. The experiment was repeated three times with similar results. Asterisk indicates significant differences at $P < 0.05$ between GPA-derived extract ROS burst in Col-0 and *bak1-5* using Student's t test. B and C, The ROS burst of Arabidopsis to GPA-derived elicitors is not reduced in mutants of known PRR genes. ROS bursts were measured in response to 2.5 mg mL^{-1} 3- to 10-kD GPA-derived extract over an 800-min period. Bars show means ± SE of $n = 16$ leaf discs per replicate. Data of one representative experiment are shown. The experiment was repeated three times with similar results. Letters indicates significant differences at $P < 0.05$ using ANOVA. D, Induced resistance to GPA 3- to 10-kD and larger-than-10-kD fractions is dependent on BAK1. Bars show the means ± SE of total nymphs produced per plant of four independent experiments (n = eight per experiment). Nymph counts were normalized with the buffer control set at 100%. Asterisks indicate significant differences at $P < 0.05$ (Student's t probabilities calculated within GLM; Col-0, $n \geq 28$, $F_{2,86} = 8.14$; *bak1-5*, $n \geq 25$, $F_{2,80} = 1.53$). [See online article for color version of this figure.]

the Arabidopsis *bak1-5* mutant plants (Fig. 6C). Thus, nonhost resistance of Arabidopsis to the pea aphid appears compromised in the *bak1-5* background, further reflecting an important contribution of BAK1 (and by extension PRR-mediated immunity) to plant immunity against aphids.

DISCUSSION

Our research provides an increased understanding of plant perception of insects, by showing that BAK1 is required for the ROS burst, callose deposition, and induced resistance triggered by GPA-derived elicitors. GPA-derived elicitors trigger plant immunity characteristic of PTI, including the induction of PTI marker genes, AtRbohD-dependent ROS burst, PEN2-dependent callose deposition, and induced resistance. The GPA-derived eliciting fractions are likely to contain heat-sensitive peptides of 3 to 10 kD and larger than 10 kD in which the 3- to 10-kD fraction induces the ROS burst and both 3- to 10-kD and larger-than-10-kD fractions elicit induced resistance to GPA. Induced resistance is also dependent on PAD3, the expression of which is induced upon Arabidopsis perception of aphid-derived elicitors and is independent of BAK1 and ROS. Finally, the legume specialist pea aphid survives better on the Arabidopsis *bak1-5* mutant than on wild-type Col-0 plants.

Our results are in agreement with those of De Vos and Jander (2009), who found that the 3- to 10-kD GPA saliva fraction generates induced resistance, which is lost upon boiling and proteinase K treatments of the fraction (De Vos and Jander, 2009). In addition, Arabidopsis colonization by another aphid species, the cabbage aphid, triggers an ROS burst and the expression of *PAD3*, *CYP81F2*, and *FRK1* genes (Kuśnierczyk et al., 2008; Barah et al., 2013). These findings and our observation that multiple aphids induce *PAD3*, *CYP81F2*, and *FRK1* expression (Fig. 5A) suggest that the eliciting components are conserved among aphids. Our study shows evidence that there are at least two eliciting fractions derived from aphids: the GPA 3- to 10-kD fraction that triggers an ROS burst and induced resistance and the larger than 10-kD fraction that does not induce ROS burst but nonetheless triggers induced resistance. The eliciting activities of both fractions require BAK1 and are lost upon boiling and proteinase K treatments, indicating that the elicitors are likely proteins with enzymatic activities. It is possible that the two eliciting fractions contain different concentrations of the same elicitor due to incomplete separation by

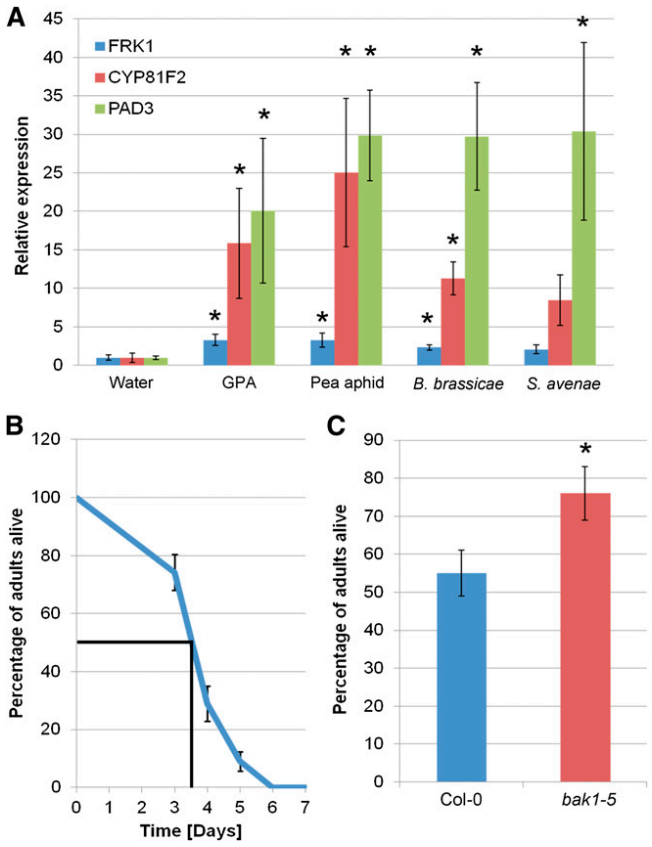


Figure 6. BAK1 is involved in pea aphid resistance. A, Elicitors derived from several aphid species trigger up-regulation of PTI marker genes. Bars show the means ± SE of target gene expression levels of four biological replicates (n = three per replicate). Asterisks indicate significant differences in aphid-derived extracts compared with water (Student's t probabilities calculated within GLM) with $^*P < 0.05$. B, Pea aphids do not survive beyond 6 d on Col-0 Arabidopsis. Data show the percentage of aphids alive at a given time point with means ± SE of four biological replicates with n = five per replicate. The time point at which 50% of pea aphids are still alive is indicated. C, Pea aphids survive better on Arabidopsis *bak1-5* plants. Bars show the percentage of aphids alive between days 3 and 4 with means ± SE of six biological replicates with n = five per replicate. Asterisk indicates significant difference in aphid survival (Student's t probabilities calculated within GLM; $n = 30$, $F_{1,59} = 5.028$; $^*P = 0.025$). [See online article for color version of this figure.]

the M_r cutoff columns. Therefore, the elicitor may be in sufficient quantity to trigger an ROS burst in the 3- to 10-kD fraction but not the larger-than-10-kD fraction. It is important to note that the elicitors perceived by Arabidopsis are either derived directly from aphids or from their endosymbionts. However, the possibility remains that elicitors in GPA-derived extract may not normally come into contact with plants. Further investigation is required to identify the elicitors and their origin. This will then allow the availability of the GPA-derived elicitors to be perceived by the plant during the plant-aphid interaction to be assessed.

The ROS burst triggered by flg22 is an early transient response, which starts very soon after addition of the PAMP and finishes within 30 min. By contrast, the ROS burst triggered by the GPA-derived 3- to 10-kD fraction occurs much later, starting more than an hour after addition of the extract. Its duration is also longer compared with flg22, as the burst takes nearly 9 h to reach basal level again. These kinetics are consistent with potential enzymatic activities of the GPA-derived elicitors. However, the kinetics of plant immune responses triggered by distinct elicitors can be highly variable. For example, *Phytophthora infestans* elicitin INF1 triggers a BAK1-dependent ROS burst in *Nicotiana benthamiana* that is also much longer than that of flg22 (Chaparro-Garcia et al., 2011). While there is a delay in the GPA-derived elicitor ROS burst compared with that of flg22, there is no delay in GPA-derived gene expression of *PAD3*, *CYP81F2*, and *FRK1*. We show that *PAD3* expression to GPA-derived elicitors does not require ROS (Fig. 3D). *CYP81F2* and *FRK1* are MAPK-activated genes (Boudsocq et al., 2010), and MAPK activation in PTI does not require ROS (Ranf et al., 2011; Segonzac et al., 2011). Consistent with this, *FRK1* expression upon flg22 treatment is not reduced in *AtrbohD* (Macho et al., 2012).

GPA elicitation is specific, as proteinase K triggers an ROS burst in Arabidopsis that is lost upon boiling, but this ROS burst does not generate induced resistance to GPA. Arabidopsis can generate induced resistance to GPA without a measurable ROS burst, as evidenced by the induced resistance triggered by the larger-than-10-kD GPA fraction. Nonetheless, the ROS burst plays a role in Arabidopsis innate immunity to GPA given that Arabidopsis mutants in RbohD, which is required for PTI- and effector-triggered immunity ROS bursts (Torres et al., 2002; Zhang et al., 2007), are more susceptible to GPA (Miller et al., 2009). Thus, aphid-derived elicitors are likely to trigger different immune pathways in plants, some of which involve ROS bursts and others that do not. All these pathways together likely contribute to an effective immunity against aphids.

BAK1 is required for the establishment of PTI by ligand-induced heteromerization with surface-localized PRRs. Characterized PRRs that require BAK1 for signaling include FLS2, EFR, and PEPR1/PEPR2 (Chinchilla et al., 2007; Heese et al., 2007; Postel et al., 2010; Roux et al., 2011). However, Arabidopsis mutants for FLS2, EFR, PEPR1, and PEPR2 are not affected in ROS bursts to the 3- to 10-kD GPA fraction. Therefore, elicitors in the 3- to 10-kD GPA fraction are likely to interact with thus-far unknown Arabidopsis PRRs, which form ligand-induced heteromers with BAK1 for triggering an ROS burst upon perception of aphid-derived elicitors.

The involvement of BAK1 in plant-herbivore interactions was previously investigated in *N. attenuata* (Yang et al., 2011a). Plants are likely to perceive insect elicitors, often referred to as herbivory-associated molecular patterns, in insect OS and egg-associated molecular patterns in egg fluid (Wu and Baldwin, 2010; Gouhier-Darimont et al., 2013). Application of OS into wounds activates two MAPKs, salicylic acid

(SA)-induced protein kinase and wound-induced protein kinase, which are required for the accumulation of JA, JA-Ile, and ethylene (ET), phytohormones that are important for mediating plant immunity to insects (Wu and Baldwin, 2010). The LECTIN-RECEPTOR KINASES LecRK1 and LecRK-I.8 act upstream or downstream of phytohormone signaling events (Gilardoni et al., 2011; Gouhier-Darimont et al., 2013). While silencing of BAK1 in *N. attenuata* leads to attenuated JA and JA-Ile levels in wounded and OS-treated plants, activities of the two MAPKs were not impaired (Yang et al., 2011a). This indicated that BR signaling but not innate immunity may be compromised in these *BAK1*-silenced plants (Yang et al., 2011b). The Arabidopsis *bak1-5* mutant used in our study is severely compromised in PTI signaling but is not impaired in BR signaling and cell death control (Schwessinger et al., 2011). In addition, the saliva-induced resistance to GPA in Arabidopsis is not dependent on JA, SA, and ET signaling (De Vos and Jander, 2009). This is in agreement with a study of Arabidopsis responses to the necrotrophic fungus *Botrytis cinerea* showing that plant-derived oligogalacturonides induce a resistance that is not dependent on JA, SA, and ET (Ferrari et al., 2007). Similarly to aphids, the induction of resistance to *B. cinerea* requires PAD3 (Ferrari et al., 2007). Thus, BAK1 contributes most likely to innate immunity to GPA in a manner that is independent of BR, JA, SA, and ET signaling in Arabidopsis.

Arabidopsis is a nonhost to the pea aphid. We observed that these aphids nonetheless attempt to feed on Arabidopsis leaves but do not adopt a settled feeding behavior and often walk to the top of the leaf cages, where they die within 6 d. Notably, pea aphids survive longer on Arabidopsis *bak1-5* plants compared with Col-0, indicating that they may obtain more nutrition from the mutant plant or receive fewer toxic compounds. While BAK1 has a role in plant immune signaling upon pea aphid perception, the observation that pea aphids do not fully survive on Arabidopsis *bak1-5* plants suggests that other BAK1-independent receptor complexes and/or additional downstream components also contribute to the triggering of plant immunity to aphids. Studying of pea aphid-Arabidopsis interactions will be useful for the identification of such components. Aphids that use brassicas, including Arabidopsis, as hosts, such as GPA and the cabbage aphid, are likely to possess specific effectors that suppress the PTI-like plant immune responses. We identified about 50 candidate effectors in GPA (Bos et al., 2010) and found that three promote GPA colonization on Arabidopsis, whereas the pea aphid homologs of these three effectors do not promote GPA colonization on this plant (Pitino and Hogenhout, 2013). It remains to be investigated if the GPA effectors, but not pea aphid effectors, suppress PTI-like plant defenses.

In summary, we identified an upstream (BAK1) and downstream (camalexin) component of two independent pathways in plant innate immunity to aphids. This is in agreement with earlier findings that camalexin is involved in plant defense to aphids (Kuśnierczyk et al., 2008; Kettles et al., 2013). Aphids are likely to suppress innate immunity to colonize plants. This is in agreement with the identification of a GPA effector that suppress PTI (Bos et al., 2010) and aphid effectors that promote colonization of the plant (Atamian et al., 2013; Pitino and Hogenhout, 2013).

MATERIALS AND METHODS

Aphids

GPAs (*Myzus persicae*; Rothamsted Research genotype O; Bos et al., 2010) were reared on Chinese cabbage (*Brassica rapa*, subspecies *chinensis*), and pea aphids (*Acyrthosiphon pisum*) were reared on broad bean (*Vicia faba*) in 52-cm × 52-cm × 50-cm cages. Cabbage aphids (*Brevicoryne brassicae*) were reared on Chinese cabbage, and English grain aphids (*Sitobion avenae*) were reared on oat (*Avena sativa*) in 24-cm × 54-cm × 47-cm cages. All species were reared in controlled-environment conditions with a 14-h-day (90 μmol m^{-2} s^{-1} at 18°C) and a 10-h-night (15°C) photoperiod.

Plant Growth Conditions

All plants were germinated and grown in Scotts Levington F2 compost. Arabidopsis (*Arabidopsis thaliana*) seeds were vernalized for 1 week at 5°C to 6°C and then grown in a controlled-environment room (CER) with a 10-h-day (90 μmol m^{-2} s^{-1}) and a 14-h-night photoperiod and at a constant temperature of 22°C.

All Arabidopsis mutants used in this study were generated in Col-0 background, except *pen2-1*, which is in the *glabrous1* background. The *bak1-5*, *bak1-4*, *bkk1-1*, *efr-1* (*efr*), *fls2c* (*fls2*), and *fls2 efr cerk1* mutants were previously described (Zipfel et al., 2004, 2006; He et al., 2007; Gimenez-Ibanez et al., 2009; Schwessinger et al., 2011). The *pepr1-1*, *pepr1-2*, and *pepr2-1* mutants (Yamaguchi et al., 2010) were obtained from the Nottingham Arabidopsis Stock Centre. The *pepr1/pepr2* double mutant (Krol et al., 2010) was obtained from Dirk Becker (Department of Molecular Plant Physiology and Biophysics, University of Wuerzburg). The *pen2-1* (Lipka et al., 2005) and *AtrbohD* (Torres et al., 2002) mutants were obtained from Jonathan Jones (The Sainsbury Laboratory). The *pad3* and *cyp79b2/cyb79b3* double mutants (Glazebrook and Ausubel, 1994; Zhao et al., 2002) were used in a previous study (Kettles et al., 2013).

Preparation of Aphid-Derived Extract and Fractions for Elicitation Experiments

Apterous late instar and adult aphids were collected using a moist paintbrush, placed in a 2-mL Eppendorf tube, and snap frozen in liquid nitrogen. The aphids were ground to a fine powder using a prechilled mortar and pestle. The powder was then transferred to a 50-mL Corning tube on ice using a prechilled spoon. Sterile, distilled water was added to the ground powder and thoroughly mixed with a pipette to generate 20 mg (wet weight) mL^{-1} of whole aphid-derived extract.

GPA-derived extracts were further processed as described (De Vos and Jander, 2009; Schäfer et al., 2011). The ground aphid powder was resuspended in sterile 0.025 M potassium phosphate buffer (KH_2PO_4, pH 6.8). The extract was centrifuged at 13,200 rpm for 15 min at 4°C, and the supernatant was collected. For fractionation of GPA-derived extract, the supernatant was filtered by centrifuging at 13,200 rpm for 15 min at 4°C using a 10-kD cutoff column (Ultracel 10K membrane, Millipore). The fraction remaining in the upper part of the column was the larger-than-10-kD fraction. The fraction that passed through the column was retrieved by placing the column upside down in a fresh centrifuge tube and centrifuging it at 1,000*g* for 2 min. It was then filtered by centrifuging at 13,200 rpm for 15 min at 4°C using a 3-kD cutoff column (Ultracel 3K membrane, Millipore). The fraction that passed through the column was the smaller-than-3-kD fraction, while the fraction that remained in upper part of the column was the 3- to 10-kD fraction. The 3- to 10-kD fraction was retrieved by placing the column upside down in a fresh

centrifuge tube at centrifuging at 1,000*g* for 2 min. After filtering, all fractions were adjusted to their original volume using potassium phosphate buffer.

GPA-derived extract was denatured by boiling for 10 min or degraded in a final concentration of 0.2 μg μL^{-1} of proteinase K (Sigma-Aldrich) at 37°C for 30 min.

Saliva Collection

GPA saliva was collected using a Parafilm sachet. Two 500-mL plastic tumblers (Sainsbury's Supermarkets) had several small holes pierced in them with a hot syringe (Terumo). Approximately 1,000 adult GPA from the Chinese cabbage stock cage, amounting to a weight of 0.2 g (50 adult GPA weighed 0.01 g), were added to one of the tumblers. The other tumbler served as a no-aphid control. A thin layer of Parafilm (Brand GMBH) was stretched over each tumbler, and 1 mL of sterile, distilled water was pipetted onto the Parafilm. A second layer of Parafilm was then stretched over each tumbler. The tumblers were placed underneath a sheet of yellow plastic (Lincoln Polythene) to enhance feeding activity in a CER with a 14-h-day (90 μmol m^{-2} s^{-1} at 18°C) and 10-h-night (15°C) photoperiod. After 24 h, the saliva/water was collected from both tumblers under sterile conditions. The 3- to 10-kD fraction of the saliva and control was obtained using centrifugal filters as described above. After filtering, the saliva and control were adjusted to their original volume using sterile, distilled water.

Induced Resistance Assays

Induced-resistance fecundity assays were carried out using a modified protocol as described (De Vos and Jander, 2009). Experiments were conducted in a CER with an 8-h-day (90 μmol m^{-2} s^{-1} at 18°C) and 16-h-night (16°C) photoperiod. To obtain aphids of the approximately the same age, 5-week old Col-0 Arabidopsis plants were potted into 1-L round black pots (13-cm diameter, 10 cm tall) that were caged inside clear plastic tubing (10-cm diameter, 15 cm tall; Jetran tubing, Bell Packaging), which was pushed inside the soil of the pot and capped at the top with a white gauze-covered plastic lid. Each plant was seeded with 20 adult GPA. After 24 h, all adults were removed from the Col-0 plants, while the nymphs remained on the plants for 10 d.

For treatment of plants with aphid elicitors, 5-week old Arabidopsis plants in black plastic pots (base measurement, 3.5 cm × 3.5 cm; top measurement, 5.5 cm × 5.5 cm; height, 5.5 cm) were infiltrated with the GPA-derived extracts on the first fully expanded leaf using a needleless 1-mL syringe (Terumo). The extracts being tested were diluted 1:10 with distilled water or potassium phosphate buffer as appropriate. The 3- to 10-kD fraction of GPA saliva was diluted 1:2 with distilled water. Control plants were infiltrated with distilled water or potassium phosphate buffer without GPA-derived extract. The infiltrated leaves were marked. The plants were used for aphid reproduction assays after 24 h.

To assay aphid reproduction on the infiltrated leaves, one aged adult of 10 d was placed in a clip cage using a moist paintbrush, and the cage was placed on the infiltrated leaf at one aphid per plant. Plants were returned to the experimental CER and left for 10 d. After 10 d, the number of aphids in each clip cage was counted. Each experiment included 10 plants per condition and/or genotype unless otherwise stated. Each plant was randomly placed in a tray of 42 cm × 52 cm × 9 cm. Each experiment was repeated at least three times on different days to generate data from at least three independent biological replicates. Leaves that had shriveled up and died, thus killing all the aphids, were removed from the analysis.

GPA Whole-Plant Fecundity Assays

GPA whole-plant fecundity assays were carried out as previously described (Kettles et al., 2013). Experiments were conducted in a CER with an 8-h-day (90 μmol m^{-2} s^{-1} at 18°C) and 16-h-night (16°C) photoperiod. Four-week-old Arabidopsis plants were potted into 1-L round black pots and caged in clear plastic tubing as described above. Each plant was seeded with five adult GPA. After 48 h, all adults were removed from test plants, while the nymphs remained at five nymphs per plant. These nymphs developed into adults and started producing their own nymphs at about day 8. The number of nymphs and surviving adults were counted on days 11 and 14, in which the nymphs were removed at each count. The total number of nymphs produced per live adult was calculated for each time point and combined. Each experiment included five plants per genotype, and each plant was randomly placed in a tray of 42 cm × 52 cm × 9 cm. Each experiment was repeated three times on different days to generate data from three independent biological replicates.

Pea Aphid Survival Assays

To obtain pea aphid adults of the same age, 50 adult pea aphids were transferred to three mature broad bean plants between 3 and 4 weeks old and placed in 24-cm × 54-cm × 47-cm cages. Each cage was placed in a CER with a 14-h-day (90 μmol m^{-2} s^{-1} at 18°C) and 10-h-night (15°C) photoperiod. After 24 h, all adults were removed from the plants, while the nymphs remained. Pea aphid adults 10 to 14 d old were used for survival experiments on Arabidopsis. The survival experiments on Arabidopsis were conducted in a CER with an 8-h-day (90 μmol m^{-2} s^{-1} at 18°C) and 16-h-night (16°C) photoperiod. Five 10- to 14-d adult pea aphids were placed in one clip cage using a moist paintbrush. The clip cages were clipped on one leaf per plant of 7-week-old Arabidopsis plants potted in black plastic pots (base measurement, 3.5 cm × 3.5 cm; top measurement, 5.5 cm × 5.5 cm; height, 5.5 cm). To ascertain pea aphid survival on Col-0 Arabidopsis, the number of aphids remaining alive on days 3 to 7 was counted. To compare survival on Col-0 and *bak1-5* Arabidopsis, the number of adult aphids remaining alive on days 3 and 4 were recorded, and the average of these two readings were taken. Each experiment consisted of five plants per genotype. Each plant was randomly placed in a tray of 42 cm × 52 cm × 9 cm. The experiments were repeated at least four times on different days to generate data from at least four independent biological replicates.

Measurements of ROS Bursts

Measurements of ROS bursts to the peptide flg22 (QRLSTGSRINSAKDDAAGLQIA; Felix et al., 1999; Peptron) and GPA-derived extracts were carried out as previously described (Bos et al., 2010). One leaf disc was taken from each of the two youngest fully expanded leaves of 5-week-old Arabidopsis plants using a circular cork borer (diameter, 4 mm). The leaf discs were floated on water overnight in 96-well plates (Grenier Bio-One). Flg22 (final concentration 100 nM unless stated otherwise) or GPA-derived extract (final concentration, 5 mg mL^{-1} unless otherwise stated) were added to a solution containing 20 μg mL^{-1} horseradish peroxidase (Sigma-Aldrich) and 21 nM of the luminol derivative 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione (Nishinaka et al., 1993; Wako). Before the experiment began, the water was removed from the wells and replaced with 100 μL of horseradish peroxidase and 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione solution containing flg22, GPA-derived extract, or water/buffer controls. ROS burst assays to proteinase K were conducted with 100 μg of proteinase K (Sigma-Aldrich) or 100 μg of proteinase K boiled for 10 min. Luminescence was captured using a Photek camera system and analyzed using company software and Microsoft Office Excel. Experiments were repeated at least three times on different days to generate independent biological replicates.

Quantitative Reverse Transcriptase (qRT)-PCR Assays

Two Arabidopsis leaf discs were taken from each of the two youngest fully expanded leaves of the 5-week-old Col-0 plant using a circular cork borer with a diameter of 6 mm. The leaf discs were floated on water overnight in 96-well plates (Grenier Bio-One). Before the experiment began, the water was removed, and leaf discs were exposed to 100 μL of water (control), 100 nM flg22 (in water), and 20 mg mL^{-1} GPA-derived extract (in water) for 1 h. Eight leaf discs under the same treatment were pooled generating one sample. Samples were ground in chilled 1.5-mL Eppendorf tubes using disposable pellet pestles (Sigma-Aldrich). Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) and included a DNase I treatment (RQ1 DNase set; Promega). Complementary DNA (cDNA) was synthesized from 1 μg RNA using the M-MLV-RT Kit (Invitrogen) and oligo(dT) primer, following the manufacturer's instructions. cDNA from these reactions was diluted 1:10 with distilled water before qRT-PCR.

Each reaction consisted of 20 μL containing 25 ng of cDNA and 0.5 μM of each primer (Supplemental Table S1) added to SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in a single well of a 96-well plate white ABgene PCR plate (Thermo Scientific). Reactions for the target and reference genes and corresponding controls were combined in one 96-well plate, which was placed

in a CFX96 Real-Time System with a C1000 Thermal Cycler (Bio-Rad). PCRs were carried out using the following thermocycle: 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C and melt curve analysis for 30 s at 50°C (65°C–95°C at 0.5°C increments, 5 s for each).

Using a selection of candidates previously identified as superior reference genes (Czechowski et al., 2005), we selected Arabidopsis genes *GLYCERAL DEHYDE-3-PHOSPHATE DEHYDROGENASE C2* (*At1g13440*) and *TWO A AND RELATED PHOSPHATASE-ASSOCIATED PROTEIN42-INTERACTING PROTEIN OF 41 KD* (*At4g34270*) as the most stable across a range of mock, flg22, and GPA-derived extract-exposed Arabidopsis leaf disc RNA samples by geNORM analysis (Vandesompele et al., 2002). All primers are listed in Supplemental Table S1.

To calculate the relative expression levels of target genes, mean cycle threshold (C_t) values for each sample-primer pair combination were calculated from three replicate reaction wells. Mean C_t values were then converted to relative expression values using efficiency of primer pair $^{-\Delta Ct}$. The geometric mean of the relative expression values of the reference genes was calculated to produce a normalization factor unique to each sample that was used to calculate the relative expression values for each gene of interest in each sample. These values from independent biological replicates were compared using a described method (Willems et al., 2008).

Callose Staining

The first two fully expanded leaves of 5-week-old Arabidopsis plants were infiltrated using a 1-mL syringe with buffer (control), 100 nM flg22 (in buffer), and 20 mg mL^{-1} GPA-derived extract (in buffer). After 24 h, one leaf disc was taken from each infiltrated leaf using a circular cork borer with a diameter of 5 mm. To remove chlorophyll from the leaf discs, the discs were placed in 70% (v/v) ethanol for 1 h, 95% (v/v) ethanol with chloroform overnight (18 h), and 100% (v/v) ethanol for 2 h. The discs were then rehydrated for 30 min in 70% (v/v) ethanol, 30 min in 50% (v/v) ethanol, and 30 min in 67 mM K_2HPO_4 at pH 9.5. Staining with 0.1% (w/v) aniline blue in 67 mM K_2HPO_4 at pH 9.5 was carried out for 1 h. Leaf discs were mounted in glycerol and viewed under a Nikon Eclipse 800 microscope using a UV filter (Bandpass, 340–380 nm; Longpass, 425 nm). An image was taken of the entire field of view of the center of each leaf disc under 10× magnification (1.34 mm^2–1.34 mm by 1 mm). The images were analyzed using ImageJ (National Institutes of Health) to count the number of callose deposits.

Statistical Analyses

Statistical analyses were conducted using Genstat version 12 (VSN International). Aphid survival or fecundity assays and callose deposition were analyzed by classical linear regression analysis using a Poisson distribution within a generalized linear model (GLM). ROS burst assays comparing two conditions were analyzed with Student's *t* tests, and those comparing more than two conditions were analyzed with ANOVA. The qRT-PCR data were analyzed using classical linear regression analysis within a GLM in which the means were compared by calculating Student's *t* probabilities within the GLM.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. GPA reproduction on *bak1* and *bkk1* Arabidopsis mutants.

Supplemental Figure S2. Induced resistance in Arabidopsis to the 3-10 kD fraction of GPA saliva is BAK1 dependent.

Supplemental Figure S3. Proteinase K triggers an ROS burst in Arabidopsis.

Supplemental Table S1. Primers used in this study.

ACKNOWLEDGMENTS

We thank Simon Lloyd and Lucy Gannon for help with the callose assays, members of the Hogenhout and Zipfel laboratories for materials and useful discussions, Graham McGrann and Alexander Coleman for help with the qRT-PCR experiments, Ian Bedford, Gavin Hatt, and Anna Jordan for rearing the insects, and the John Innes Horticultural Services for taking care of plants.

Received January 11, 2014; accepted February 27, 2014; published February 28, 2014.

LITERATURE CITED

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature **415:** 977–983

Atamian HS, Chaudhary R, Cin VD, Bao E, Girke T, Kaloshian I (2013) In planta expression or delivery of potato aphid *Macrosiphum euphorbiae* effectors Me10 and Me23 enhances aphid fecundity. Mol Plant Microbe Interact **26:** 67–74

Balmer D, Planchamp C, Mauch-Mani B (2013) On the move: induced resistance in monocots. J Exp Bot **64:** 1249–1261

Barah P, Winge P, Kusnierczyk A, Tran DH, Bones AM (2013) Molecular signatures in *Arabidopsis thaliana* in response to insect attack and bacterial infection. PLoS ONE **8:** e58987

Bednarek P, Piślewska-Bednarek M, Svatoš A, Schneider B, Doubský J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, et al (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science **323:** 101–106

Blackman RL, Eastop VF (2000) Aphids on the World's Crops: An Identification and Information Guide. John Wiley and Sons, Chichester, UK

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol **60:** 379–406

Bos JIB, Hogenhout SA (2011) Effectors in Plant-Insect Interactions. *In* F Martin, S Kamoun, eds, Effectors in Plant–Microbe Interactions. Wiley-Blackwell, Oxford, pp 355–375

Bos JIB, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA (2010) A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (green peach aphid). PLoS Genet **6:** e1001216

Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng SH, Sheen J (2010) Differential innate immune signalling via Ca^{2+} sensor protein kinases. Nature **464:** 418–422

Bruce TJA, Pickett JA (2011) Perception of plant volatile blends by herbivorous insects—finding the right mix. Phytochemistry **72:** 1605–1611

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux JP, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores. Plant J **62:** 876–885

Carolan JC, Caragea D, Reardon KT, Mutti NS, Dittmer N, Pappan K, Cui F, Castaneto M, Poulain J, Dossat C, et al (2011) Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): a dual transcriptomic/proteomic approach. J Proteome Res **10:** 1505–1518

Chaparro-Garcia A, Wilkinson RC, Gimenez-Ibanez S, Findlay K, Coffey MD, Zipfel C, Rathjen JP, Kamoun S, Schornack S (2011) The receptor-like kinase SERK3/BAK1 is required for basal resistance against the late blight pathogen *Phytophthora infestans* in *Nicotiana benthamiana*. PLoS ONE **6:** e16608

Cherqui A, Tjallingii WF (2000) Salivary proteins of aphids, a pilot study on identification, separation and immunolocalisation. J Insect Physiol **46:** 1177–1186

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JDG, Felix G, Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448:** 497–500

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. Science **323:** 95–101

Cooper WR, Dillwith JW, Puterka GJ (2010) Salivary proteins of Russian wheat aphid (Hemiptera: Aphididae). Environ Entomol **39:** 223–231

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol **139:** 5–17

de Jonge R, van Esse HP, Maruthachalam K, Bolton MD, Santhanam P, Saber MK, Zhang Z, Usami T, Lievens B, Subbarao KV, et al (2012) Tomato immune receptor Ve1 recognizes effector of multiple fungal

pathogens uncovered by genome and RNA sequencing. Proc Natl Acad Sci USA **109:** 5110–5115

De Vos M, Jander G (2009) *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. Plant Cell Environ **32:** 1548–1560

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Métraux JP, Van Loon LC, Dicke M, et al (2005) Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Mol Plant Microbe Interact **18:** 923–937

Denoux C, Galletti R, Mammarella N, Gopalan S, Werck D, De Lorenzo G, Ferrari S, Ausubel FM, Dewdney J (2008) Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. Mol Plant **1:** 423–445

Dixon AFG (1998) Aphid Ecology: An Optimization Approach, Ed 2. Chapman and Hall, London

Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J **18:** 265–276

Ferrari S, Galletti R, Denoux C, De Lorenzo G, Ausubel FM, Dewdney J (2007) Resistance to *Botrytis cinerea* induced in Arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires *PHYTOALEXIN DEFICIENT3*. Plant Physiol **144:** 367–379

Filichkin SA, Brumfield S, Filichkin TP, Young MJ (1997) In vitro interactions of the aphid endosymbiotic SymL chaperonin with barley yellow dwarf virus. J Virol **71:** 569–577

Fradin EF, Zhang Z, Juarez Ayala JC, Castroverde CDM, Nazar RN, Robb J, Liu CM, Thomma BPHJ (2009) Genetic dissection of *Verticillium* wilt resistance mediated by tomato Ve1. Plant Physiol **150:** 320–332

Gilardoni PA, Hettenhausen C, Baldwin IT, Bonaventure G (2011) *Nicotiana attenuata LECTIN RECEPTOR KINASE1* suppresses the insect-mediated inhibition of induced defense responses during *Manduca sexta* herbivory. Plant Cell **23:** 3512–3532

Gimenez-Ibanez S, Ntoukakis V, Rathjen JP (2009) The LysM receptor kinase CERK1 mediates bacterial perception in Arabidopsis. Plant Signal Behav **4:** 539–541

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc Natl Acad Sci USA **91:** 8955–8959

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol Cell **5:** 1003–1011

Gouhier-Darimont C, Schmiesing A, Bonnet C, Lassueur S, Reymond P (2013) Signalling of *Arabidopsis thaliana* response to *Pieris brassicae* eggs shares similarities with PAMP-triggered immunity. J Exp Bot **64:** 665–674

Gust AA, Biswas R, Lenz HD, Rauhut T, Ranf S, Kemmerling B, Götz F, Glawischnig E, Lee J, Felix G, et al (2007) Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in Arabidopsis. J Biol Chem **282:** 32338–32348

Harmel N, Létocart E, Cherqui A, Giordanengo P, Mazzucchelli G, Guillonneau F, De Pauw E, Haubruge E, Francis F (2008) Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*. Insect Mol Biol **17:** 165–174

He K, Gou X, Yuan T, Lin H, Asami T, Yoshida S, Russell SD, Li J (2007) BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. Curr Biol **17:** 1109–1115

Heese A, Hann DR, Gimenez-Ibanez S, Jones AME, He K, Li J, Schroeder JI, Peck SC, Rathjen JP (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proc Natl Acad Sci USA **104:** 12217–12222

Hogenhout SA, Bos JIB (2011) Effector proteins that modulate plant—insect interactions. Curr Opin Plant Biol **14:** 422–428

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol **59:** 41–66

Kemmerling B, Schwedt A, Rodriguez P, Mazzotta S, Frank M, Qamar SA, Mengiste T, Betsuyaku S, Parker JE, Müssig C, et al (2007) The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. Curr Biol **17:** 1116–1122

Kettles GJ, Drurey C, Schoonbeek HJ, Maule AJ, Hogenhout SA (2013) Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs. New Phytol **198:** 1178–1190

Kørner CJ, Klauser D, Niehl A, Domínguez-Ferreras A, Chinchilla D, Boller T, Heinlein M, Hann DR (2013) The immunity regulator BAK1 contributes to resistance against diverse RNA viruses. Mol Plant Microbe Interact **26:** 1271–1280

Krol E, Mentzel T, Chinchilla D, Boller T, Felix G, Kemmerling B, Postel S, Arents M, Jeworutzki E, Al-Rasheid KAS, et al (2010) Perception of the Arabidopsis danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2. J Biol Chem **285:** 13471–13479

Kuśnierczyk A, Winge P, Jørstad TS, Troczyńska J, Rossiter JT, Bones AM (2008) Towards global understanding of plant defence against aphids—timing and dynamics of early Arabidopsis defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. Plant Cell Environ **31:** 1097–1115

Li J, Wen J, Lease KA, Doke JT, Tax FE, Walker JC (2002) BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell **110:** 213–222

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, et al (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science **310:** 1180–1183

Luna E, Pastor V, Robert J, Flors V, Mauch-Mani B, Ton J (2011) Callose deposition: a multifaceted plant defense response. Mol Plant Microbe Interact **24:** 183–193

Ma R, Chen JL, Cheng DF, Sun JR (2010) Activation of defense mechanism in wheat by polyphenol oxidase from aphid saliva. J Agric Food Chem **58:** 2410–2418

Macho AP, Boutrot F, Rathjen JP, Zipfel C (2012) Aspartate oxidase plays an important role in Arabidopsis stomatal immunity. Plant Physiol **159:** 1845–1856

Medzhitov R, Janeway CA Jr (1997) Innate immunity: the virtues of a nonclonal system of recognition. Cell **91:** 295–298

Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, Dangl JL, Mittler R (2009) The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Sci Signal **2:** ra45

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc Natl Acad Sci USA **104:** 19613–19618

Monaghan J, Zipfel C (2012) Plant pattern recognition receptor complexes at the plasma membrane. Curr Opin Plant Biol **15:** 349–357

Nam KH, Li J (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell **110:** 203–212

Nam KJ, Hardie J (2012) Host acceptance by aphids: probing and larviposition behaviour of the bird cherry-oat aphid, *Rhopalosiphum padi* on host and non-host plants. J Insect Physiol **58:** 660–668

Nishinaka Y, Aramaki Y, Yoshida H, Masuya H, Sugawara T, Ichimori Y (1993) A new sensitive chemiluminescence probe, L-012, for measuring the production of superoxide anion by cells. Biochem Biophys Res Commun **193:** 554–559

Nühse TS, Bottrill AR, Jones AME, Peck SC (2007) Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. Plant J **51:** 931–940

Pitino M, Hogenhout SA (2013) Aphid protein effectors promote aphid colonization in a plant species-specific manner. Mol Plant Microbe Interact **26:** 130–139

Postel S, Küfner I, Beuter C, Mazzotta S, Schwedt A, Borlotti A, Halter T, Kemmerling B, Nürnberger T (2010) The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in Arabidopsis development and immunity. Eur J Cell Biol **89:** 169–174

Ranf S, Eschen-Lippold L, Pecher P, Lee J, Scheel D (2011) Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. Plant J **68:** 100–113

Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, Holton N, Malinovsky FG, Tör M, de Vries S, Zipfel C (2011) The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. Plant Cell **23:** 2440–2455

Sabri A, Vandermoten S, Leroy PD, Haubruge E, Hance T, Thonart P, De Pauw E, Francis F (2013) Proteomic investigation of aphid honeydew reveals an unexpected diversity of proteins. PLoS ONE **8:** e74656

Schäfer M, Fischer C, Meldau S, Seebald E, Oelmüller R, Baldwin IT (2011) Lipase activity in insect oral secretions mediates defense responses in Arabidopsis. Plant Physiol **156:** 1520–1534

Schoonhoven L, van Loon JJ, Dicke M (2005) Insect-Plant Biology, Ed 2. Oxford University Press, Oxford

Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatoš A, Halkier BA, Glawischnig E (2006) CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. Plant Physiol **141:** 1248–1254

Schulze B, Mentzel T, Jehle AK, Mueller K, Beeler S, Boller T, Felix G, Chinchilla D (2010) Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. J Biol Chem **285:** 9444–9451

Schulze-Lefert P, Panstruga R (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. Trends Plant Sci **16:** 117–125

Schwessinger B, Roux M, Kadota Y, Ntoukakis V, Sklenar J, Jones A, Zipfel C (2011) Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. PLoS Genet **7:** e1002046

Segonzac C, Feike D, Gimenez-Ibanez S, Hann DR, Zipfel C, Rathjen JP (2011) Hierarchy and roles of pathogen-associated molecular pattern-induced responses in *Nicotiana benthamiana*. Plant Physiol **156:** 687–699

Sun Y, Li L, Macho AP, Han Z, Hu Z, Zipfel C, Zhou JM, Chai J (2013) Structural basis for flg22-induced activation of the Arabidopsis FLS2-BAK1 immune complex. Science **342:** 624–628

Torres MA, Dangl JL, Jones JDG (2002) Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci USA **99:** 517–522

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol **3:** H0034

Wan J, Zhang XC, Neece D, Ramonell KM, Clough S, Kim SY, Stacey MG, Stacey G (2008) A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. Plant Cell **20:** 471–481

Whippo CW, Hangarter RP (2005) A brassinosteroid-hypersensitive mutant of BAK1 indicates that a convergence of photomorphogenic and hormonal signaling modulates phototropism. Plant Physiol **139:** 448–457

Willems E, Leyns L, Vandesompele J (2008) Standardization of real-time PCR gene expression data from independent biological replicates. Anal Biochem **379:** 127–129

Wu J, Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. Annu Rev Genet **44:** 1–24

Yamaguchi Y, Huffaker A, Bryan AC, Tax FE, Ryan CA (2010) PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in *Arabidopsis*. Plant Cell **22:** 508–522

Yamaguchi Y, Pearce G, Ryan CA (2006) The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells. Proc Natl Acad Sci USA **103:** 10104–10109

Yang DH, Hettenhausen C, Baldwin IT, Wu J (2011a) BAK1 regulates the accumulation of jasmonic acid and the levels of trypsin proteinase inhibitors in *Nicotiana attenuata*'s responses to herbivory. J Exp Bot **62:** 641–652

Yang DH, Hettenhausen C, Baldwin IT, Wu J (2011b) The multifaceted function of BAK1/SERK3: plant immunity to pathogens and responses to insect herbivores. Plant Signal Behav **6:** 1322–1324

Zhang J, Shao F, Li Y, Cui H, Chen L, Li H, Zou Y, Long C, Lan L, Chai J, et al (2007) A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. Cell Host Microbe **1:** 175–185

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL (2002) Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev **16:** 3100–3112

Zhou N, Tootle TL, Glazebrook J (1999) *Arabidopsis PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. Plant Cell **11:** 2419–2428

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell **125:** 749–760

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428:** 764–767

Supplemental data

Figure S1

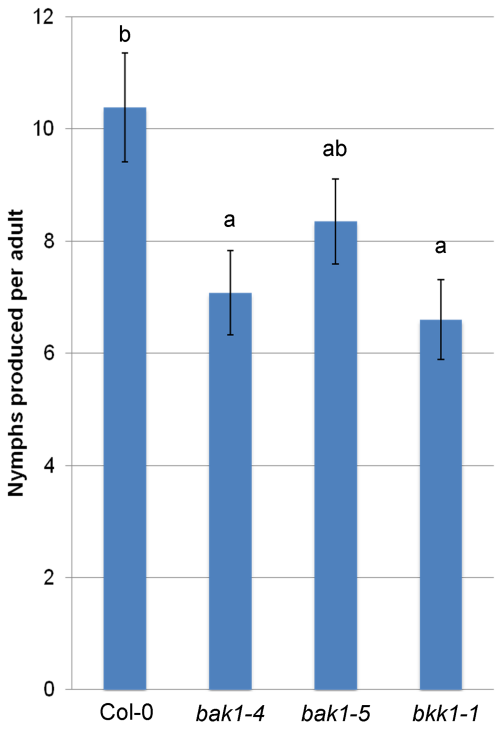


Figure S1. GPA reproduction on *bak1* and *bkk1* Arabidopsis mutants.

Bars show the means ± SE of GPA nymph produced over a 14-day period on 5 plants per genotype of three independent experiments. Different letters above the bars indicate significant differences at $P < 0.05$ (n = 15, $F_{3,59} = 3.998$; t probabilities calculated within GLM).

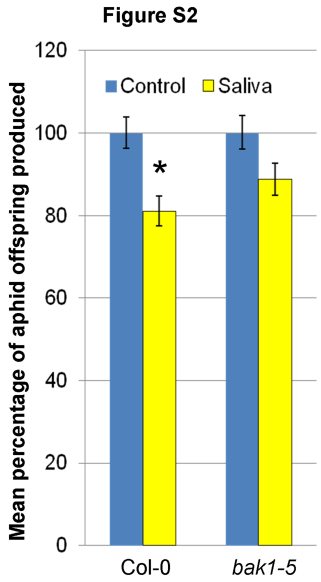


Figure S2. Induced resistance in Arabidopsis to the 3-10 kDa fraction of GPA saliva is BAK1 dependent. Bars show the means ± SE of total nymphs produced per plant of three independent experiments (n = 10 per experiment). Nymph counts were normalized with water (control) set at 100%. *P < 0.001 (wild type Col-0 $F_{1,59}$ = 12.224) and P = 0.052 (*bak1-5* mutant, $F_{1,59}$ = 3.773).

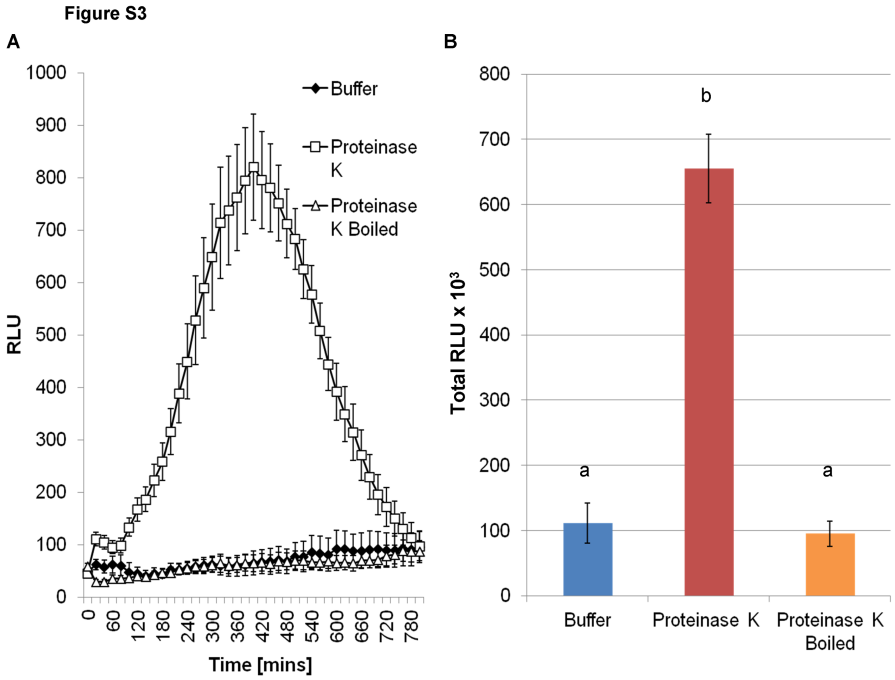


Figure S3. Proteinase K triggers a ROS burst in Arabidopsis.

ROS bursts were measured over a 600 min period. Bars show means ± SE of n = 16 leaf discs per replicate. Data of one representative experiment is shown. The experiment was repeated three times with similar results. Letters indicates significant differences at $P < 0.01$ using ANOVA.

Table S1. Primers used in this study.

Gene name	Identifier	Use in study	Sequence (5' -> 3')
GAPDH	At1g13440	Reference gene	F AGGTCAAGCATTTTCGATGC
			R AACGATAAGGTCAACGACACG
TIP41	At4g34270	Reference gene	F TCCATCAGTCAGAGGCTTCC
			R AAGAAAGCTCATCGGTACGC
FRK1	At2g19190	Marker gene	F ATCTTCGCTTGGAGCTTCTC
			R TGCAGCGCAAGGACTAGAG
CYP81F2	At5g57220	Marker gene	F AATGGAGAGAGCAACACAATG
			R ATACTGAGCATGAGCCCTTTG
PAD3	At3g26830	Marker gene	F TGCTCCCAAGACAGACAATG
			R GTTTTGGATCACGACCCATC

Appendix B

Resistance of Arabidopsis thaliana to the green peach aphid, Myzus persicae, involves camalexin and is regulated by microRNAs. Kettles et al., 2013

New Phytologist

Research

Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs

Graeme J. Kettles[1], Claire Drurey[1], Henk-jan Schoonbeek[2], Andy J. Maule[1] and Saskia A. Hogenhout[1]

[1]Department of Cell and Developmental Biology and [2]Department of Crop Genetics, John Innes Centre, Norwich Research Park, Norwich, Norfolk, NR4 7UH, UK

Author for correspondence:
Saskia A. Hogenhout
Tel: +44 1603 450393
Email: saskia.hogenhout@jic.ac.uk

Received: *11 December 2012*
Accepted: *31 January 2013*

New Phytologist (2013)
doi: 10.1111/nph.12218

Key words: aphid resistance, camalexin, glucosinolates, microRNA, *PAD3*.

Summary

- Small RNAs play important roles in resistance to plant viruses and the complex responses against pathogens and leaf-chewing insects. We investigated whether small RNA pathways are involved in *Arabidopsis* resistance against a phloem-feeding insect, the green peach aphid (*Myzus persicae*).
- We used a 2-wk fecundity assay to assess aphid performance on *Arabidopsis* RNA silencing and defence pathway mutants. Quantitative real-time polymerase chain reaction was used to monitor the transcriptional activity of defence-related genes in plants of varying aphid susceptibility. High-performance liquid chromatography-mass spectrometry was employed to measure the accumulation of the antimicrobial compound camalexin. Artificial diet assays allowed the assessment of the effect of camalexin on aphid performance.
- *Myzus persicae* produces significantly less progeny on *Arabidopsis* microRNA (miRNA) pathway mutants. Plants unable to process miRNAs respond to aphid infestation with increased induction of *PHYTOALEXIN DEFICIENT3* (*PAD3*) and production of camalexin. Aphids ingest camalexin when feeding on *Arabidopsis* and are more successful on *pad3* and *cyp79b2/cyp79b3* mutants defective in camalexin production. Aphids produce less progeny on artificial diets containing camalexin.
- Our data indicate that camalexin functions beyond antimicrobial defence to also include hemipteran insects. This work also highlights the extensive role of the miRNA-mediated regulation of secondary metabolic defence pathways with relevance to resistance against a hemipteran pest.

Introduction

The green peach aphid (GPA), *Myzus persicae*, is one of the most destructive pests on cultivated crops worldwide (Blackman & Eastop, 2000). GPA causes feeding damage and, more importantly, is the vector of many different plant viruses (Ng & Perry, 2004; Hogenhout *et al.*, 2008). Insect herbivores, including aphids, have often specialized to colonize one or a few related plant species, whereas only a few herbivores, such as GPA, can colonize diverse plant species. Therefore, most plants can defend themselves effectively against the majority of insect herbivores. Moreover, insects are probably required to modulate a variety of plant processes to facilitate colonization. However, the mechanisms by which plants defend themselves against insect colonization and how aphids modulate plant processes are not fully understood.

Aphids possess specialized mouthparts, named stylets, which are developed for the piercing of plant tissues and the ingestion of sap, and allow them to feed from phloem tissue (Tjallingii, 2006). Access to this tissue is gained following extensive probing by the stylets of epidermal and parenchymal cell layers, before the establishment of a successful feeding site in the phloem sieve element (Tjallingii & Esch, 1993). Once established, feeding can be maintained for several hours (Tjallingii, 1995).

In plants, small RNAs (sRNAs) regulate changes in gene expression in response to a variety of biotic and abiotic stimuli (Sunkar & Zhu, 2004; Fujii *et al.*, 2005; Ruiz-Ferrer & Voinnet, 2009; Katiyar-Agarwal & Jin, 2010). It has long been known that components of sRNA pathways play an extensive role in antiviral defence (Ding & Voinnet, 2007). More recently, sRNA pathways have been implicated in resistance to bacteria, fungi, nematodes and insects (Navarro *et al.*, 2006; Pandey & Baldwin, 2007; Hewezi *et al.*, 2008; Pandey *et al.*, 2008; Ellendorff *et al.*, 2009). sRNAs modify gene expression by acting at both the transcriptional and post-transcriptional levels (Voinnet, 2009). RNA-induced silencing is initiated by double-stranded RNA (dsRNA), which can occur as a stem-loop precursor, or a longer dsRNA molecule generated by either bidirectional transcription or the action of an RNA-dependent RNA polymerase (RDR) on a single-stranded RNA (ssRNA) template (Ruiz-Ferrer & Voinnet, 2009). In Arabidopsis, segments of dsRNA are cleaved into 18–24-nucleotide (nt) sRNA duplexes by one or a

combination of four Dicer-like (DCL) endoribonucleases. Following methylation of the 2-nt 3′ overhang by the methyltransferase HUA ENHANCER1 (HEN1; Yu *et al.*, 2005), sRNA can be exported from the nucleus before incorporation into an RNA-induced silencing complex (RISC) containing one of 10 Argonaute (AGO) proteins (Vazquez *et al.*, 2010). The sRNA guides the RISC to either cleave or repress the translation of target transcripts bearing sufficient homology to the loaded sRNA.

sRNAs can be divided into subgroups depending on their source and mode of processing (Vazquez *et al.*, 2010). Small interfering RNA (siRNA) is processed from segments of long, perfectly complementary dsRNA, which may be derived from pathogens (e.g. viruses) or generated from loci throughout the genome, but especially from highly repetitive regions (Rabinowicz *et al.*, 2003; Matzke *et al.*, 2007). The latter is consistent with the known role for siRNAs in directing heterochromatic silencing of genomic regions harbouring mobile genetic elements (Matzke *et al.*, 2007). MicroRNAs (miRNAs) are a class of largely 21-nt sRNAs derived from imperfectly complementary stem-loop precursors. miRNAs are excised from their precursors by DCL1 (Park *et al.*, 2002; Kurihara & Watanabe, 2004), although the rate and fidelity of this excision is dependent on the cofactors SERRATE (SE) and HYPONASTIC LEAVES 1 (HYL1; Dong *et al.*, 2008). miRNAs are subject to methylation by HEN1 and are exported from the nucleus via both HASTY (HST)-dependent and independent mechanisms (Park *et al.*, 2005). At some point, there is unravelling of the duplex into its component miR and complementary miR* strands, before one strand is selectively incorporated into RISC. AGO1 is the dominant slicer of the miRNA pathway (Baumberger & Baulcombe, 2005), although a proportion is reported to act through AGO7 or AGO10 (Brodersen *et al.*, 2008; Montgomery *et al.*, 2008).

The miRNA pathway is known to play a significant role in the regulation of the defence response that occurs following challenge by the bacterial biotroph *Pseudomonas syringae* (Navarro *et al.*, 2006; Zhang *et al.*, 2011) and the pathogen-associated molecular pattern (PAMP) flg22 (Li *et al.*, 2010). The defence pathways activated in response to attack from chewing herbivores are also governed by sRNAs. The growth of *Manduca sexta* (tobacco hornworm) larvae is enhanced on *Nicotiana attenuata* lacking RDR1 (Pandey & Baldwin, 2007). In this interaction, RDR1-dependent siRNAs are required to coordinate a defence response involving nicotine biosynthesis and the jasmonic acid (JA) and ethylene (ET) signalling pathways (Pandey *et al.*, 2008).

Aphid infestations elicit transcriptional reprogramming in host plants, despite causing little visible feeding damage (Moran *et al.*, 2002; Couldridge *et al.*, 2007; Kusnierczyk *et al.*, 2007, 2008; Gao *et al.*, 2010). In one study, these changes were more pronounced than those elicited by fungal or bacterial pathogens, or a leaf-chewing lepidopteran pest (De Vos *et al.*, 2005). miRNAs, in particular, are known to target large families of transcription factors. Infestation by several aphid species also results in large-scale changes in the transcription factor profile of infested tissue (Kusnierczyk *et al.*, 2008; Gao *et al.*, 2010; Sattar *et al.*, 2012). Given these observations and the known involvement of sRNAs in defence responses against pathogens and a chewing herbivore, we speculated that sRNAs may play a similarly important role in coordinating the complex and large-scale response to aphids.

GPA effectively colonizes members of the family Brassicaceae, including the model plant *Arabidopsis thaliana*. Here, we report that Arabidopsis plants deficient in miRNA processing show increased resistance to GPA. This resistance is partly a result of the enhanced production of the phytoalexin camalexin, which is known to play a role in plant defence against bacterial and fungal microbial pathogens. Camalexin is produced at GPA stylet penetration sites, and this plant compound accumulates in aphids fed on plants and an artificial diet containing camalexin. Progeny production is reduced in aphids exposed to camalexin, whereas aphids produce more progeny on plants compromised in camalexin production. Together, this work uncovers a novel role for camalexin in modifying insect reproductive ability.

Materials and Methods

Aphids

Stock colonies of *M. persicae* (Sulzer) (RRes genotype O; GPA; Bos *et al.*, 2010) were reared in 52 × 52 × 50-cm^3 cages containing up to six Chinese cabbage (*Brassica rapa*, subspecies *chinensis*) plants with a 14-h day (90 μmol m^{-2} s^{-1} at 18°C) and a 10-h night (15°C) cycle.

Plant growth conditions

All plants used in this investigation belong to the Arabidopsis Col-0 ecotype. The *ago1-25*, *ago1-26* and *ago1-27* mutants (Morel *et al.*, 2002) were supplied by Hervé Vaucheret (Laboratoire de Biologie Cellulaire, INRA Centre de Versailles, Versailles Cedex, France). The *dcl1-9*, *hen1-5*, *rdr1-1*, *rdr2-1* and *rdr6* mutants (Jacobsen *et al.*, 1999; Mourrain *et al.*, 2000; Vazquez *et al.*, 2004b; Xie *et al.*, 2004) were kindly provided by Fuquan Liu (Queen's University, Belfast, UK). The *dcl2*, *dcl3*, *dcl4*, *dcl2/3*, *dcl2/4* and *dcl2/3/4* mutants (Xie *et al.*, 2004, 2005; Henderson *et al.*, 2006) were obtained from Olivier Voinnet (Swiss Federal Institute of Technology, Zurich, Switzerland). The *hst*, *se1*, *ago2*, *ago4*, *ago7*, *cyp81f2*, 35S:LOX2 and 35S:LOX2 antisense lines (Bell *et al.*, 1995; Bollman *et al.*, 2003; Zilberman *et al.*, 2003; Vazquez *et al.*, 2004a; Lobbes *et al.*, 2006; Pfalz *et al.*, 2009) were provided by the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK). *dcl1.fwf2* and *fwf2* (Katiyar-Agarwal *et al.*, 2007) were kindly provided by Rebecca Mosher (University of Arizona, Tucson, AZ, USA). The *phytoalexin deficient3* (*pad3*), *nonexpressor of pathogenesis-related genes1* (*npr1*) and *salicylic acid induction-deficient2* (*sid2*) mutants (Cao *et al.*, 1994; Glazebrook & Ausubel, 1994; Nawrath & Metraux, 1999) were obtained from Alexandre Robert-Seilaniantz (Sainsbury Laboratory, Norwich, Norfolk, UK). The *cyp79b2/cyp79b3* double mutant (Zhao *et al.*, 2002) was obtained from Jean-Pierre Métraux (University of Fribourg, Fribourg, Switzerland). The *coronatine insensitive1* (*coi1-35*) and *jasmonate*

resistant1 (*jar1*) mutants (Staswick *et al.*, 1992) were provided by Jonathan Jones (Sainsbury Laboratory). The *ethylene insensitive2* (*ein2-5*) and *ethylene resistant1* (*etr1-1*) mutants (Bleecker *et al.*, 1988; Alonso *et al.*, 1999) were from Freddy Boutrot (Sainsbury Laboratory). The *CYP71B15p::GUS* (*PAD3p::GUS*) transgenic lines (Schuhegger *et al.*, 2006) were supplied by Erich Glawischnig (Technische Universität München, Munich, Germany).

All Arabidopsis plants used in the aphid fecundity experiments were germinated and maintained on Scotts Levington F2 compost. Seeds of the Arabidopsis sRNA mutants were vernalized at 4°C for 72 h and grown in a controlled environment room (CER) with an 8-h day (90 μmol m^{-2} s^{-1} at 18°C) and 16-h night (16°C) cycle. Two-week-old seedlings were transferred to seedling trays containing 24 modules. Plants were used for experiments after a further 2 wk when they were 4 wk old.

Seeds of the Arabidopsis hormone/secondary metabolite pathway mutants were vernalized for 1 wk at 5–6°C and grown in a CER with a 10-h day (90 μmol m^{-2} s^{-1} at 22°C) and a 14-h night (22°C) cycle. Plants were used for experiments at 4 wk old.

Aphid fecundity assays

All fecundity assays were carried out in a CER with an 8-h day (90 μmol m^{-2} s^{-1} at 18°C) and a 16-h night (16°C) cycle. Four-week-old plants were potted into 1-l round black pots (diameter, 13 cm; height, 10 cm) containing fresh compost, and were caged in clear plastic tubing (diameter, 10 cm; height, 15 cm; Jetran tubing; Bell Packaging Ltd, Luton, UK) capped at the top with white gauze-covered plastic lids. Each plant was seeded with four adult GPA from the stock colony, and the plants were returned to the CER. After 48 h, all adults were removed from the test plants (day 0) and the plants were returned to the growth room. On day 3, excess nymphs were removed, leaving five nymphs per plant. On day 11, when most nymphs had reached adulthood and started to produce their own offspring, the numbers of these new nymphs were counted. The newly produced nymphs were removed and the adults remained on the plant. On day 14, a second nymph count was carried out, together with a count of the surviving adults. Experiments were terminated on day 14. The total number of nymphs produced was calculated by combining the day 11 and day 14 nymph counts. Each experiment included five plants per genotype that were arranged in trays using a randomized block design, and each experiment was repeated at least twice. The experiment to assess aphid performance over a shorter period was performed following a method described previously (Pegadaraju *et al.*, 2005).

All statistical analyses were conducted using the GenStat 11 statistical package (VSNi Ltd, Hemel Hempstead, Hertfordshire, UK). Data were checked for approximate normal distribution by visualizing residuals. Classical linear regression analysis using a generalized linear model (GLM) with Poisson distributions was applied to analyse the GPA fecundity on plants with 'nymphs' as a response variable. The aphid nymph production on five plants per genotype was used as an independent data point in statistical analyses in which the biological replicate was used as a variable.

Single-leaf aphid infestations

Thirty GPA nymphs from the stock cage were transferred to a single clip-cage and confined to a single mature rosette leaf of a 5-wk-old plant at one clip-cage per plant. Plants were returned to the CER for the appropriate infestation period. Two to four aphid-exposed leaves per treatment were pooled to produce each sample, and the leaves caged with aphid-free clip-cages were used as controls. For the 12-h infestations of the RNA silencing mutants, three independent experiments were conducted containing three, four and two biological replicates, respectively. This gave nine biological replicates in total, which were statistically analysed together. The 24- and 48-h infestations of the RNA silencing mutants contained four biological replicates. For the 6-, 12-, 24- and 48-h Col-0 infestation time courses, four biological replicates of each treatment were analysed.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Pooled leaf samples were ground in chilled 1.5-ml Eppendorf tubes using disposable pellet pestles (Sigma-Aldrich, St Louis, MO, USA). Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) and included a DNaseI treatment (RQ1 DNase set; Promega, Madison, WI, USA). RNA was purified using the RNA cleanup protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 500 ng RNA using the MMLV-RT Kit (Invitrogen, Carlsbad, CA, USA) and oligo dT primer, following the manufacturer's instructions. cDNA from these reactions was diluted 1 : 20 with distilled H_2O before qRT-PCR.

Twenty-microlitre reactions were set up in 96-well white ABgene PCR plates (Thermo Scientific, Loughborough, Leicestershire, UK) in a CFX96 Real-Time System with a C1000 Thermal Cycler (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich).

All reactions were carried out using the following thermocycle: 3 min at 95°C, followed by 40 cycles of (30 s at 95°C, 30 s at 60°C, 30 s at 72°C), followed by melt curve analysis: 30 s at 50°C (65–95°C at 0.5°C increments, 5 s for each).

Reference genes for this study were chosen from a selection of candidates previously identified as superior reference genes (Czechowski *et al.*, 2005). Using geNORM (Vandesompele *et al.*, 2002), it was established that ACT2 (At3g18780), Clathrin adapter complex subunit (At5g46630) and PEX4 (At5g25760) were the most stable across a range of mock and GPA-exposed Arabidopsis rosette leaf RNA samples. Mean C_t values for each sample–primer pair combination were calculated from two or three replicate reaction wells. Mean C_t values were then converted to relative expression values using the formula $2^{-\Delta C_t}$. The geometric mean of the relative expression values of the three reference genes was calculated to produce a normalization factor unique to each sample. Relative expression values for each gene of interest were normalized using the normalization

factor for each sample. The normalized expression values for each gene of interest were then compared between mock and aphid-exposed samples across all plant lines tested in the experiment. Analysis of variance (ANOVA) was performed to assign variance attributable to plant genotype, block and replicate using a GLM in GenStat. Means were compared by calculating *t* probabilities within the GLM. Primer sequences for both reference and target genes are available in Supporting Information Table S1.

Camalexin extraction and measurement

For plant samples, single leaves from 5-wk-old Arabidopsis were infested with 30 GPA nymphs and the leaves were confined with clip-cages. Leaves treated with empty clip-cages were used as controls. Both mock and aphid-infested leaves were harvested after 48 h. Camalexin extractions were carried out using a method based on work described previously (Meuwly & Metraux, 1993). Samples were analysed by high-performance liquid chromatography (HPLC) on a Surveyor instrument (Thermo Scientific) attached to a DecaXP^{plus} ion trap mass spectrometer (Thermo Scientific). Camalexin and *o*-anisic acid were separated on a Luna C18(2) column (50 mm × 2 mm, 3 μm; Phenomenex, Macclesfield, UK). All peak areas were integrated using the Xcalibur software Genesis algorithm (Thermo Scientific). Each experiment contained three biological replicates of each genotype–treatment combination and the experiment was conducted twice.

For camalexin measurements in aphids, 120 nymphs were used to infest whole 5-wk-old Arabidopsis plants. After 48 h, aphids were harvested and camalexin was extracted using the same protocol as described for plant samples. Each experiment contained three biological replicates of each treatment and the experiment was conducted twice.

Artificial diet experiments

Aphid feeders were constructed by cutting the top 2-cm portion of a 50-ml Corning tube and reattaching the lid. Parafilm was stretched over the open end to form a feeding sachet containing 100 μl of artificial diet. We used an artificial diet previously described for these experiments (Kim & Jander, 2007). Aphids were fed diet alone, dimethylsulfoxide (DMSO)-spiked (0.1%) diet or diet containing the indicated concentration of camalexin. Synthetic camalexin was provided by Jean-Pierre Métraux (University of Fribourg, Fribourg, Switzerland; Stefanato *et al.*, 2009). Ten adult aphids from the stock cage were added to each feeder. Feeders were inverted, covered with a yellow plastic sheet and placed in a CER with an 8-h day (90 μmol m^{-2} s^{-1} at 18°C) and 16-h night (16°C) cycle. The number of surviving adults (from 10) and the number of nymphs produced were assessed after 48 h. Each experiment contained five feeders per treatment and the experiment was conducted twice. ANOVA was performed to assign variance attributable to diet treatment and replicate using a GLM in GenStat. Means were compared by calculating *t* probabilities within the GLM.

β-Glucuronidase (GUS) staining

Leaves of 4-wk-old transgenic Arabidopsis lines expressing *CYP71B15p::GUS* (*PAD3p::GUS*) were infested with 30 GPA nymphs contained within clip-cages. Leaves with empty clip-cages were used as negative controls and leaves treated with *Botrytis cinerea* (B05.10) were used as positive controls. After 48 h, aphids were carefully removed and leaves were immediately submerged in GUS staining solution (0.2 M Na_2HPO_4, 0.2 M $NaH_2PO_4{\cdot}2H_2O$, 10% Triton X-100, 10 mM EDTA, pH 7) containing 50 mg ml^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) and 0.3% H_2O_2. Leaves were vacuum infiltrated with staining solution and returned to normal atmospheric pressure. This was repeated three times. Leaves were incubated in staining solution for 16 h at 37°C in the dark before destaining in 70% ethanol. Leaves were mounted on glass microscope slides in 40% glycerol and viewed under a Nikon Eclipse 800 light microscope (Nikon UK Ltd) attached to a Pixera Pro ES600 digital camera (Pixera UK Ltd).

Results

Aphid fecundity is reduced on Arabidopsis miRNA mutants

To determine whether sRNAs are involved in Arabidopsis resistance to GPA, aphid performance was assessed on a collection of RDR, DCL and AGO mutants and wild-type Col-0 Arabidopsis. In our assay, 4-wk-old plants were seeded with five nymphs aged < 48 h. These nymphs were allowed 14 d to develop to adulthood and to produce offspring. The number of offspring produced was recorded as fecundity. In our initial experiment, fecundity was unchanged among three RDR mutants (*rdr1*, *rdr2*, *rdr6*) compared with Col-0 (Fig. 1a). This indicates that RDRs are not involved in *Arabidopsis* resistance to GPA, unlike the *rdr1* mutant of *N. attenuate*, which shows decreased resistance to the herbivore *Manduca sexta* (Pandey & Baldwin, 2007). By contrast, aphids produced significantly fewer offspring on *dcl1* mutants relative to Col-0 (*t* probabilities within GLM, $P < 0.001$, $n = 5$), but were not affected on *dcl2*, *dcl3* or *dcl4* mutants (Fig. 1b). In addition, aphid fecundity was significantly lower on the *ago1-25* mutant (GLM, $P < 0.001$, $n = 5$), but was unchanged on *ago2*, *ago4* and *ago7* mutants (Fig. 1c). Aphid performance was also not affected on the *dcl2/3* and *dcl2/4* double mutants or the *dcl2/3/4* triple mutant (Fig. 1d). Because DCL1 and AGO1 both process sRNAs in the miRNA pathway, these data suggest that the miRNA pathway is involved in Arabidopsis resistance to GPA, whereas other sRNA processing pathways do not appear to play a significant role.

To investigate this further, we conducted GPA fecundity assays on other mutants in the miRNA pathway. In addition, to determine whether the smaller stature of *dcl1* and *ago1* mutants affects aphid fecundity, we included the Arabidopsis Plasmodesmata Located Protein 1 (PDLP1) overexpression line 35S::PDLP1a:GFP (Thomas *et al.*, 2008) as a control, as this line exhibits a dwarfing phenotype similar to the miRNA mutants (Fig. S1). We observed that aphid fecundity was not

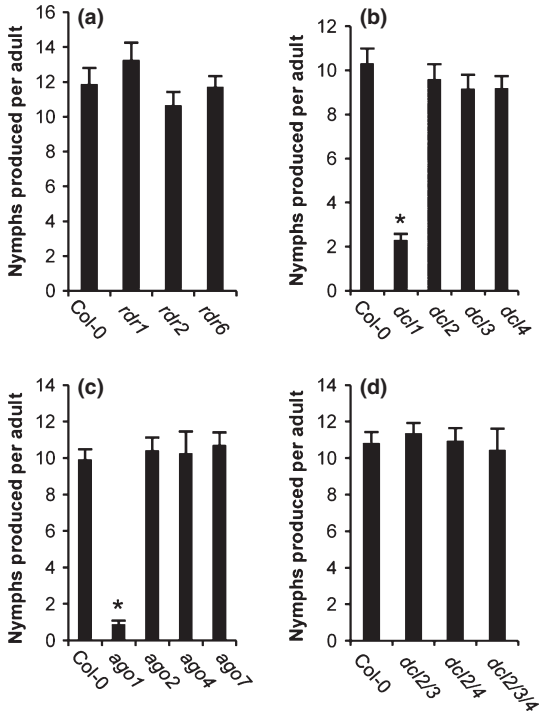


Fig. 1 The *Arabidopsis* microRNA (miRNA) pathway is involved in aphid resistance. Aphid fecundity is reduced on miRNA pathway mutants (*dcl1*, *ago1*) (b, c), but not on mutants in other small interfering RNA (siRNA) pathways (a–d). Each plant was seeded with five nymphs, and the average fecundity of these nymphs as they progressed to adulthood was recorded. Bars represent the mean (± SE) of five plants of each genotype. Each experiment was repeated at least twice with similar results. Asterisks represent $P < 0.001$ as determined by analysis of deviance (ANODE; GenStat).

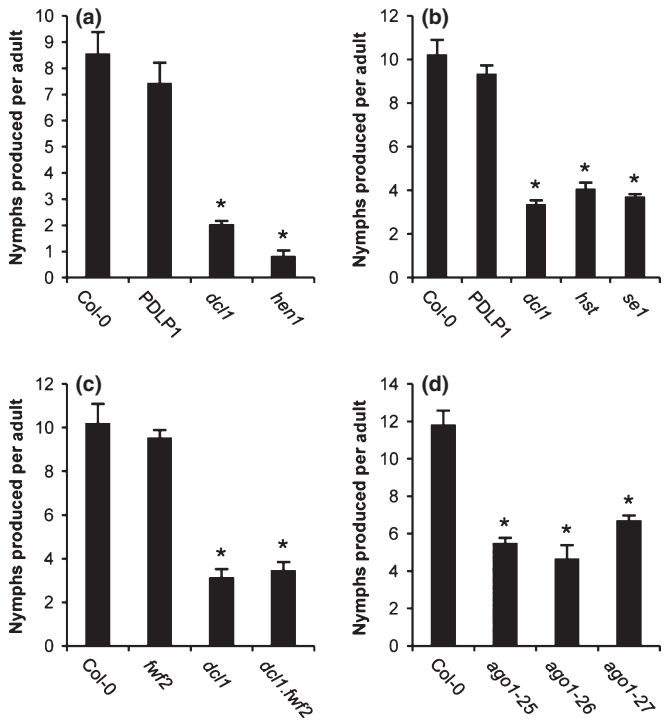


Fig. 2 Plant stature has no effect on aphid fecundity. Aphid fecundity is reduced on Arabidopsis lines that aberrantly process microRNA (miRNA) (*hen1*, *hst*, *se1*), but remains high on the unrelated dwarf Plasmodesmata Located Protein 1 (PDLP1) line (a, b). Reduced fecundity is also observed on the partial *dcl1* rescue line *dcl1.fwf2* (c) and across several *ago1* alleles (d). Bars represent the mean (± SE) of five plants of each genotype. Each experiment was repeated at least twice with similar results. Asterisks represent $P < 0.001$ as determined by analysis of deviance (ANODE).

significantly different between PDLP1 and Col-0, whereas aphids produced significantly fewer nymphs on the miRNA mutant *dcl1* and the *hen1* mutant, which is deficient in all sRNA pathways (GLM, $P < 0.001$, $n = 5$; Fig. 2a). Similarly, aphids were significantly less fecund on *hst* and *se* mutants compared with both Col-0 and PDLP1 (GLM, $P < 0.001$, $n = 5$; Fig. 2b). SE is a zinc finger protein that assists DCL1 in the accurate excision of miRNAs from their precursors, and HST is involved in the export of miRNAs from the nucleus (Park *et al.*, 2005; Dong *et al.*, 2008). To provide additional evidence that plant stature does not affect aphid fecundity, we also assessed aphid performance on the partial *dcl1* rescue line *dcl1.fwf2*, which retains impaired miRNA processing, but exhibits a less dwarf phenotype (Katiyar-Agarwal *et al.*, 2007; Fig. S1). Fecundity on these plants matched that of *dcl1*-raised aphids (Fig. 2c). We also obtained other *ago1* alleles reported to have various degrees of dwarfism (Morel *et al.*, 2002). Aphid fecundity was comparable across all of these lines (Fig. 2d), although, in our growth conditions, the *ago1-26* and *ago1-27* mutants were similar in size and stature to the *ago1-25* mutant analysed in Fig. 1(c) (Fig. S1). Nonetheless, these results suggest that the miRNA pathway is involved in the regulation of the plant resistance response to GPA, whereas other siRNA pathways are not involved. Furthermore, the resistance exhibited by miRNA pathway mutants is independent of the dwarfism phenotype.

Camalexin, ET and JA pathway transcripts are upregulated in aphid-exposed *dcl1* mutants

Arabidopsis responses to aphid attack have been investigated extensively and involve the salicylic acid (SA), JA, ET, glucosinolate and camalexin pathways (Moran *et al.*, 2002; De Vos *et al.*, 2005; Couldridge *et al.*, 2007; Kusnierczyk *et al.*, 2007, 2008). We investigated whether the induction of these pathways was altered in an miRNA mutant by comparing the expression levels of a range of marker genes illustrative of these pathways by qRT-PCR. To assess the temporal aspect of the response, we measured defence induction in Col-0 at 6, 12, 24 and 48 h post-inoculation (hpi), and found that defence gene inductions were first reliably detected at 12 hpi, and were higher and did not change dramatically between the 24- and 48-hpi time points (Fig. S2). Therefore, we selected the 12-hpi time point as it would be possible to detect a decrease as well as an increase in gene expression levels.

PAD3 (*CYP71B15*), a marker for the camalexin biosynthetic pathway (Chassot *et al.*, 2008; Xu *et al.*, 2008), was most strikingly induced on exposure to aphids in the *dcl1* mutant compared with Col-0 and the *dcl2/3/4* triple mutant among all the genes tested (Figs 3a, S4). In addition, *CYP81F2*, a gene involved in the indolic glucosinolate pathway, was induced significantly in aphid-infested *dcl1* plants compared with Col-0 and *dcl2/3/4*

New Phytologist

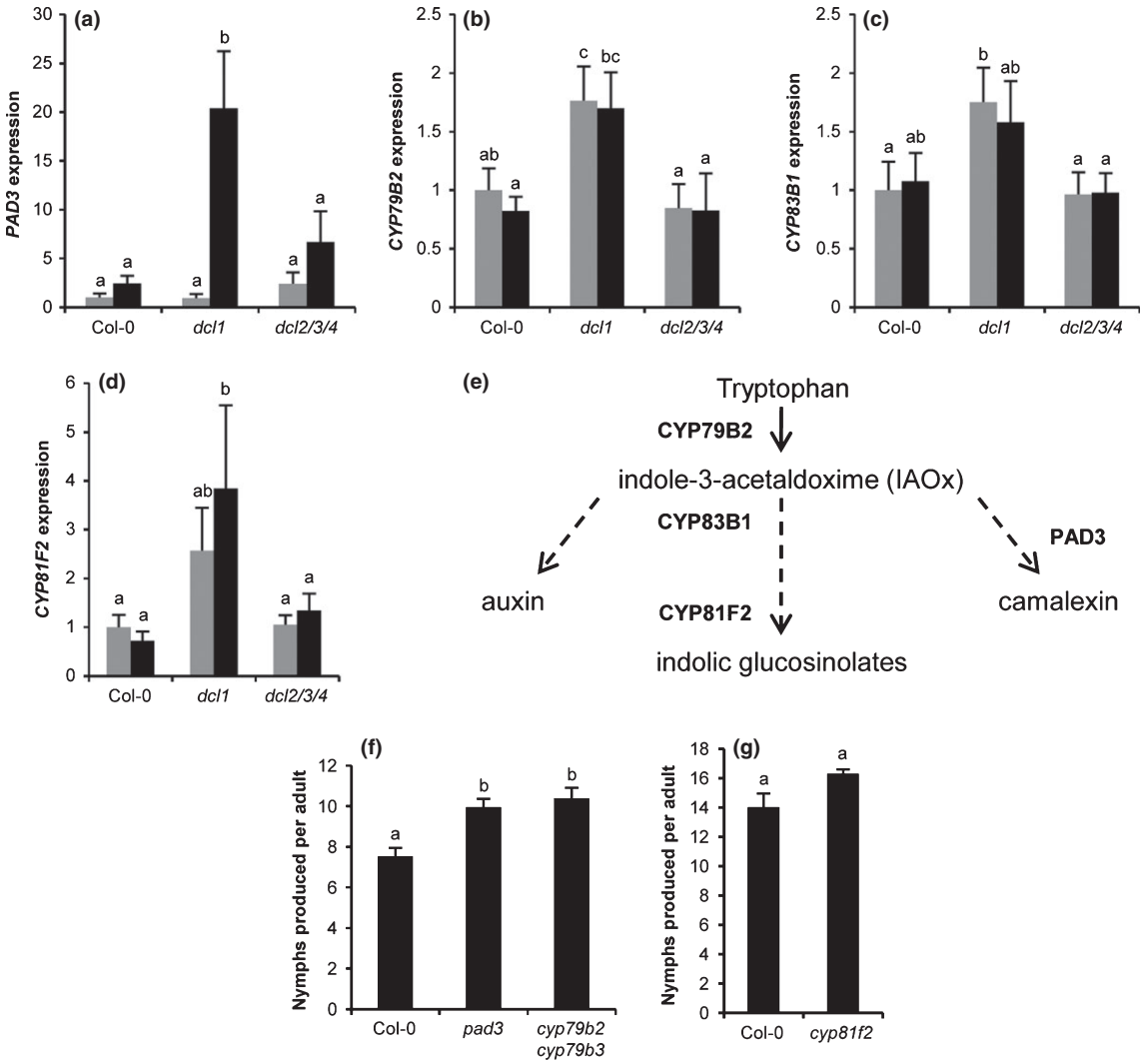


Fig. 3 MicroRNA (miRNA) mutants show differential expression of enzymes involved in tryptophan-derived secondary metabolism. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of transcripts involved in the production of (a) camalexin (*PAD3*), (b) camalexin/indole glucosinolates (*CYP79B2*) and (c, d) indole glucosinolates (*CYP83B1*, *CYP81F2*) following 12 h of aphid infestation. miRNA mutants (*dcl1*) show greater induction of *PAD3* and *CYP81F2* relative to Col-0 and *dcl2/3/4*, and also show increased basal expression of *CYP79B2* and *CYP83B1*. Mock, grey bars; aphids, black bars. Bars represent the mean expression levels (± SE) across nine biological replicates from three independent experiments. Letters indicate differences at $P < 0.05$ as determined by *t* probabilities within a generalized linear model (GLM). (e) Position of *PAD3*, CYP79B2, CYP83B1 and CYP81F2 in the camalexin and indole glucosinolate biosynthetic pathways. (f) Aphid fecundity is similarly increased on camalexin-deficient (*pad3*) and camalexin/indole glucosinolate-deficient (*cyp79b2/cyp79b3*) mutants, indicating that camalexin production is the major resistance factor. (g) Aphid fecundity is unchanged on *cyp81f2* mutants. Bars represent the mean (± SE) of 10 plants of each genotype from two experiments. Letters indicate differences at $P < 0.05$ as determined by analysis of deviance (ANODE).

(Fig. 3d). The JA biosynthetic gene *LIPOXYGENASE2* (*LOX2*) was also upregulated significantly in aphid-exposed *dcl1* compared with aphid-exposed Col-0 and *dcl2/3/4* (Fig. 4b). The defence-related gene *MITOGEN-ACTIVATED PROTEIN KINASE3* (*MPK3*) was most strongly induced in *dcl1*, although the increase was not significantly different from aphid-exposed Col-0 or *dcl2/3/4* (Fig. S3). *PATHOGENESIS-RELATED1* (*PR1*), which has been used as a marker for SA signalling (De Vos *et al.*, 2005; Kusnierczyk *et al.*, 2007), is upregulated on aphid exposure; however, its induction was not significantly different among the Col-0, *dcl1* and *dcl2/3/4* plants (Fig. 4a). The basal expression levels of some genes, such as *CYP79B2* and *CYP83B1* of the indole glucosinolate/camalexin pathways, were greater in *dcl1* compared with Col-0 and *dcl2/3/4*, but did not alter significantly in any line on exposure to aphids (Fig. 3b,c). *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) and *PLANT DEFENSIN1.2* (*PDF1.2*) have been used as downstream markers of the JA and ET pathways (De Vos *et al.*, 2005). We found that the expression of these genes was either stable or repressed following aphid treatment, and did not differ significantly across any of the lines tested (Fig. 4c,d). By contrast, the ET-responsive transcript *HEVEIN-LIKE* (*HEL*) (*PR4*) was induced significantly in aphid-exposed *dcl1* plants compared with aphid-exposed Col-0 and *dcl2/3/4* (Figs 4e, S4). As genes involved in glucosinolate and camalexin biosynthesis and the JA and ET signalling pathways were differentially regulated in *dcl1* plants, we predicted that

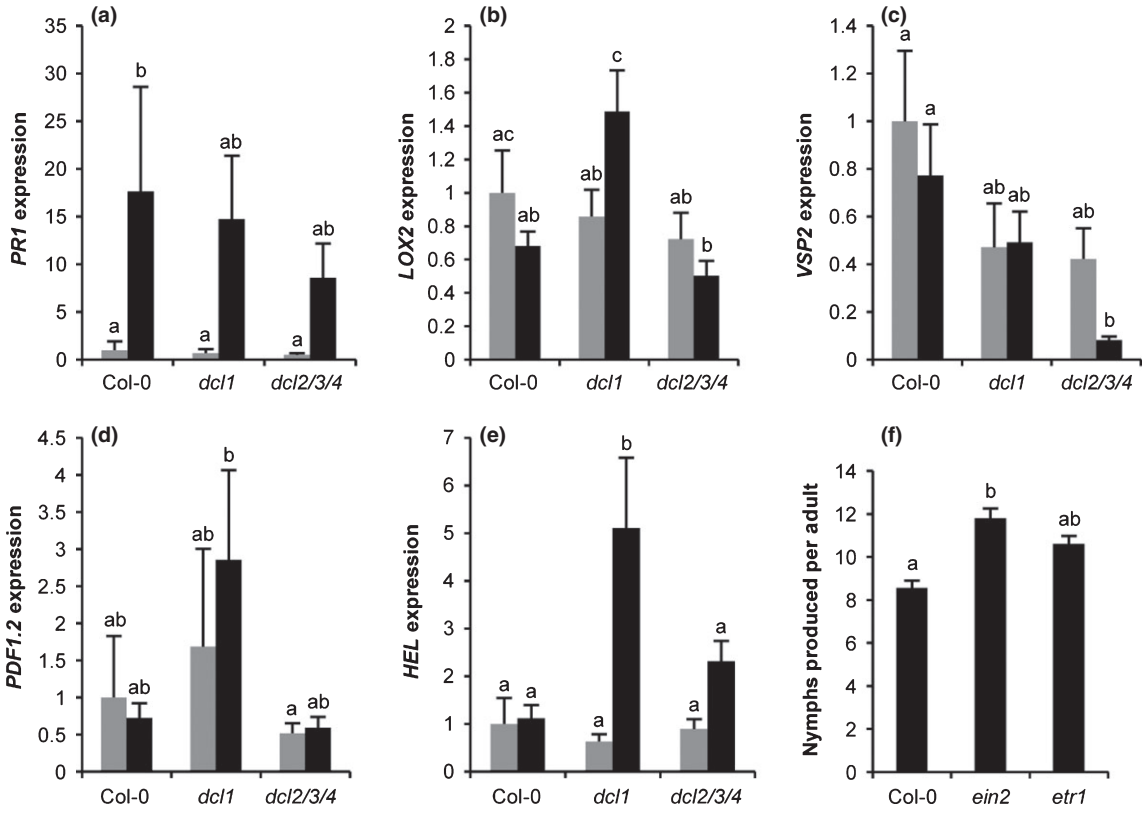


Fig. 4 MicroRNA (miRNA) mutants show altered expression of genes involved in jasmonic acid (JA) synthesis and ethylene (ET) response. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of transcripts involved in (a) salicylic acid (SA; *PR1*), (b, c) JA (*LOX2*, *VSP2*), (d) JA/ET (*PDF1.2*) and (e) ET (*HEL*) pathways following 12 h of aphid infestation. The expression of *LOX2* and *HEL* was increased in *dcl1* relative to both Col-0 and *dcl2/3/4*. Bars represent the mean expression levels (± SE) across nine biological replicates from three independent experiments. Letters indicate differences at $P < 0.05$ as determined by *t* probabilities within a generalized linear model (GLM). Mock, grey bars; aphids, black bars. (f) Aphid fecundity is increased on *ethylene-insensitive2* (*ein2*) mutants. Bars represent the mean (± SE) of 10 plants of each genotype from two experiments. The experiment was repeated with similar results. Letters indicate differences at $P < 0.05$ as determined by analysis of deviance (ANODE).

these pathways may be responsible for the aphid-resistant phenotype exhibited by Arabidopsis miRNA pathway mutants.

GPA fecundity is increased on camalexin-deficient plants

The cytochrome P450 *PAD3* catalyses the conversion of dihydrocamalexic acid to camalexin, the major Arabidopsis phytoalexin (Schuhegger *et al.*, 2006; Fig. 3e). CYP81F2 is involved in a downstream part of the indolic glucosinolate pathway that has been shown to have relevance to aphid resistance (Pfalz *et al.*, 2009; Fig. 3e). To investigate the contribution of *PAD3*, CYP81F2 and CYP79B2/CYP79B3 (which act upstream of the glucosinolate and camalexin pathways), we exposed the *pad3* (camalexin-deficient), *cyp81f2* (aphid-relevant glucosinolate-deficient) and *cyp79b2/cyp79b3* (camalexin and indole glucosinolate-deficient) mutants to insects. Aphid fecundity was significantly higher on both *pad3* and *cyp79b2/cyp79b3* mutants compared with Col-0 (GLM, $P < 0.05$, $n = 10$; Fig. 3f). However, aphid fecundity was not significantly different on *cyp79b2/cyp79b3* plants compared with *pad3*. It is possible that the aphid reproduction activity is maximized on each of the mutant plants to the degree that the absence of both camalexin and indole glucosinolates adds relatively little to aphid reproduction. Nonetheless, this indicates that the blocking of the camalexin pathway has a negative effect on aphid reproduction. The increased aphid performance on *pad3* mutants agreed with the finding that *PAD3* expression was highly induced in aphid-resistant *dcl1* plants. We found that aphid fecundity was increased on the *cyp81f2* mutant, but not significantly relative to Col-0 (Fig. 3g). Together, these data indicate that camalexin plays a substantial role in the aphid resistance exhibited by Arabidopsis miRNA pathway mutants.

Aphid fecundity is unaffected on JA and SA pathway mutants, but is increased on *ein2* plants

Our qRT-PCR data indicated that, in *dcl1* plants, the JA pathway transcript *LOX2* is induced following aphid infestation (Fig. 4b). This is in contrast with infested Col-0 and *dcl2/3/4*, where this transcript is not induced. This suggests that an aspect of JA signalling may be involved in miRNA mutant resistance. To assess this possibility, we exposed plants defective in JA signalling (*coi1*, *jar1*, 35S:*LOX2*) to aphids. Aphid fecundity was increased slightly on these lines relative to controls (Fig. S5); however, the increase was not statistically significant. This indicates that, in *dcl1* plants, there is differential regulation of the JA

pathway relative to Col-0 and *dcl2/3/4*, but this has little bearing on the ability of these plants to resist aphid infestation. Aphid performance was also unchanged on plants deficient in SA signalling (Fig. S5).

As *dcl1* plants show increased induction of the ET-responsive *HEL* transcript following infestation (Figs 4e, S4), we investigated whether ET signalling affects aphid performance by assessing aphid performance on the ET-insensitive *etr1-1* and *ein2-5* mutants. Aphid fecundity was significantly higher on *ein2* plants relative to Col-0 (GLM, $P < 0.05$, $n = 10$) and was also higher on the *etr1* mutant, albeit not significantly, compared with Col-0 (Fig. 4f).

Camalexin accumulation is increased in miRNA mutants

To assess whether increased *PAD3* expression in *dcl1* plants led to increased levels of camalexin, we exposed plants to 48 h of aphid infestation and measured camalexin content by HPLC and mass spectrometry (MS). We found that camalexin was present in similarly small quantities in Col-0, *dcl1* and *dcl2/3/4* plants without aphid challenge (Fig. 5a). However, on aphid exposure, there was increased camalexin accumulation in all plant genotypes, particularly in aphid-exposed *dcl1* compared with aphid-exposed Col-0 or *dcl2/3/4* (Fig. 5a). This result mirrors our previous data, which showed increased levels of *PAD3* mRNA in aphid-exposed *dcl1* plants relative to aphid-exposed Col-0 or *dcl2/3/4* (Figs 3a, S4). This indicates that elevated levels of *PAD3* expression correlate with increased camalexin accumulation during aphid attack.

Camalexin is present in the phloem and is ingested by aphids during feeding

Camalexin is produced in significant quantities in aphid-challenged leaves; however, it is unknown whether camalexin is present in the Arabidopsis phloem stream and whether it is ingested by aphids on feeding. We obtained plants expressing a *PAD3p:GUS* transgene (Schuhegger *et al.*, 2006) and exposed leaves to aphid infestation (Fig. S6). Leaves exposed to spores of the necrotrophic fungus *B. cinerea* showed GUS staining in a localized circular pattern surrounding the edge of the *B. cinerea* lesion (Kliebenstein *et al.*, 2005; Fig. S6b). GUS staining was also observed in leaves exposed to GPA, although the pattern of staining differed considerably from that of *B. cinerea*-exposed leaves. The staining patterns on aphid-exposed leaves were much less uniform than those for *B. cinerea*, and were localized at aphid stylet penetration sites on the midveins of infested leaves (Fig. S6c–h). At the majority of feeding sites, GUS staining was observed in small patches around stylet penetrations (Fig. S6e,f). In a smaller proportion of feeding sites, stylet tracks were observed without any GUS staining (Fig. S6c,d), indicating that aphids had either abandoned probing, or had established a successful feeding site without activating a defence response involving *PAD3* induction. Third, on some leaves, GUS staining was observed in an extremely localized fashion (Fig. S6g,h), appearing to be confined to the vasculature tissue running perpendicular to the aphid feeding tracks. These data suggest that *PAD3* is expressed in the vasculature, and raises the

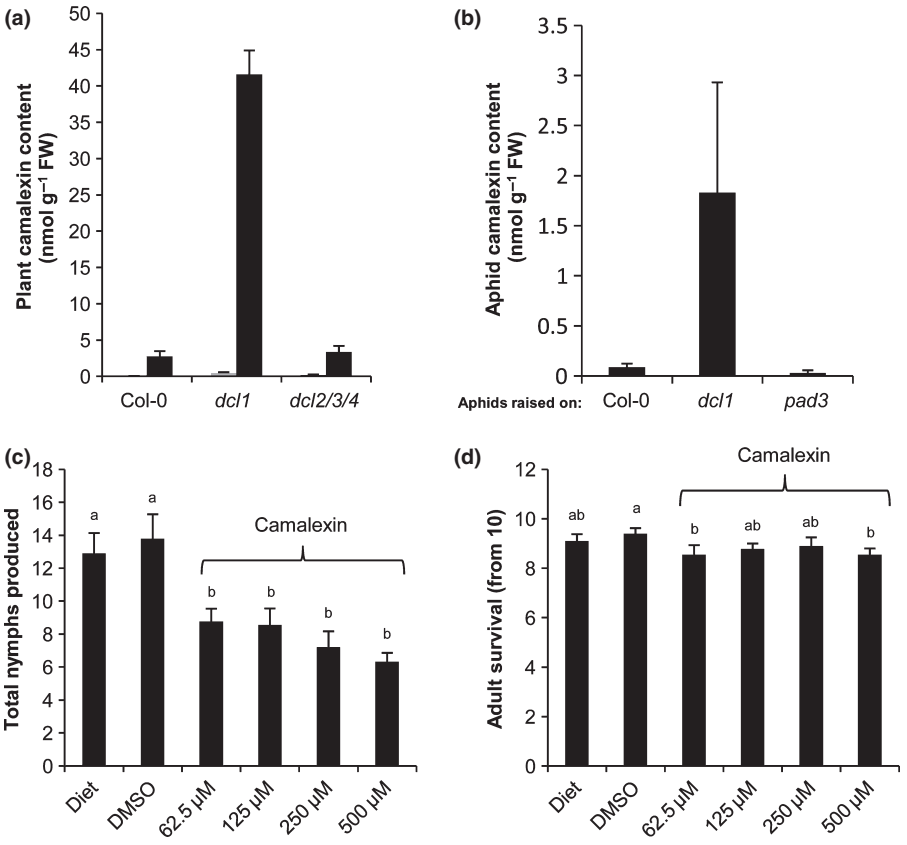


Fig. 5 Camalexin accumulates in *dcl1* plants and *dcl1*-raised aphids and affects aphid reproductive development. (a) High-performance liquid chromatography (HPLC) (+MS) analysis of mock (grey bars) and aphid-infested (black bars) Col-0, *dcl1* and *dcl2/3/4* indicates that *dcl1* accumulates more camalexin when exposed to aphids. Bars represent mean camalexin content (± SE) from six biological replicates from two independent experiments. (b) Camalexin is detected in aphids and at higher levels in insects raised on *dcl1* than in Col-0- or *pad3*-raised aphids. Bars represent mean camalexin content (± SE) from six biological replicates from two independent experiments. (c, d) Feeding camalexin by artificial diet retards aphid fecundity (c), but has no effect on adult aphid survival (d). Dimethylsulfoxide (DMSO) (0.1%) served as a negative control. Each experiment contained five feeders at each condition. Bars represent the mean number of nymphs produced (c) or surviving adults (d) (± SE) from two independent experiments. Letters indicate differences at $P < 0.05$ as determined by *t* probabilities within a generalized linear model (GLM).

possibility that camalexin is present in the phloem stream and is ingested by aphids when they feed.

To confirm that aphids ingest camalexin during feeding, we raised insects on plants considered to be high-camalexin-producing (*dcl1*), low-camalexin-producing (Col-0) and nonproducing (*pad3*). Aphids were harvested after 48 h of feeding and camalexin was quantified using the same methods as described for plant tissue samples. We were able to detect camalexin in aphids raised on all three plant genotypes (Fig. 5b), indicating that aphids are able to ingest this metabolite when feeding from Arabidopsis. In addition, we found that aphids raised on high-camalexin-producing hosts (*dcl1*) contained more camalexin than aphids raised on low-camalexin-producing hosts (Col-0; Fig. 5b). By contrast, there was little difference in the amount of camalexin detected in aphids raised on low-producing plants (Col-0) when compared with nonproducing plants (*pad3*; Fig. 5b). These data show that aphids ingest camalexin when feeding from Arabidopsis, and that a relationship exists between the quantity produced *in planta* and the quantity that accumulates in aphids.

Camalexin inhibits adult aphid reproduction, but not survival

We next investigated the effects of supplying camalexin to aphids via an artificial diet. Ten adult aphids were transferred to parafilm sachet feeders containing a complex artificial diet used previously to examine aphid performance (Kim & Jander, 2007). Following 2 d of feeding, the numbers of remaining live adults were recorded as adult survival, and the total number of nymphs produced was recorded as fecundity. We found that, at all camalexin concentrations tested, fecundity was reduced significantly compared with both diet-only (Diet) and DMSO (0.1%) controls (GLM; $P < 0.01$, $n = 10$; Fig. 5c). By contrast, we found that adult survival was unchanged at all camalexin doses relative to the diet-only control (Fig. 5d). However, at camalexin doses of 62.5 and 500 μM, adult survival was significantly lower than that of the DMSO control (GLM; $P < 0.05$, $n = 10$; Fig. 5c). These data illustrate that camalexin can limit the number of individuals present within an aphid colony, predominantly through a deleterious effect on adult reproductive success.

Aphid performance is partially restored on a *dcl1/pad3* double mutant

Finally, to confirm that *PAD3* and camalexin production are involved in the *dcl1* resistance phenotype, we introduced the *pad3* mutation into a *dcl1* genetic background. We isolated *dcl1/pad3* double mutants and tested aphid performance on these plants. We found that aphids reproduced significantly better on *dcl1/pad3* than on *dcl1* (GLM; $P < 0.01$, $n = 18$); however, fecundity was not fully restored to the levels observed on Col-0 plants (Fig. 6). This indicates that the camalexin pathway is responsible for a significant portion of the *dcl1* aphid-resistant phenotype.

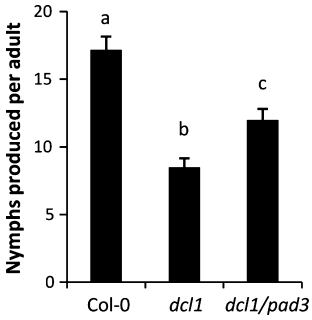


Fig. 6 Aphid fecundity is partially restored on a *dcl1/pad3* double mutant. Aphid fecundity is higher on *dcl1/pad3* than on *dcl1* single mutants, but is not fully restored to wild-type levels. Bars represent the mean (± SE) of 18 plants of each genotype from three independent experiments. Letters indicate differences at $P < 0.01$ as determined by *t*-probabilities within a generalized linear model (GLM).

Discussion

In this study, we have shown that GPA produces significantly less progeny on Arabidopsis plants that aberrantly process miRNAs. Plants unable to process miRNAs respond to aphid infestation with increased induction of *PAD3* and production of camalexin. Aphids are more successful on the Arabidopsis *pad3* and *cyp79b2/cyp79b3* mutants defective in camalexin production. In addition, camalexin is present in the phloem stream and aphids raised on miRNA pathway mutants accumulate more camalexin than aphids raised on control plants. Aphids produce less progeny on artificial diets containing camalexin, indicating that this phytoalexin reduces the reproductive ability of GPA. Finally, aphid fecundity is partially restored for aphids raised on *dcl1/pad3* mutants relative to *dcl1*.

Our finding that aphids were less successful on *dcl1* plants was initially unexpected, as pathogen and insect performances have been shown to increase on silencing-deficient hosts (Deleris *et al.*, 2006; Pandey & Baldwin, 2007). Indeed, type III secretion system (T3SS)-deficient *P. syringae* (which normally reproduces poorly on Arabidopsis) shows increased proliferation on Arabidopsis miRNA pathway mutants, but not on Arabidopsis plants defective in other silencing pathways (Navarro *et al.*, 2008). Similarly, *Pseudomonas fluorescens* and *Escherichia coli*, which do not normally infect Arabidopsis, can multiply on Arabidopsis miRNA pathway mutants (Navarro *et al.*, 2008). In addition, some RNA silencing mutants are hypersusceptible to infection by the vascular fungus *Verticillium* (Ellendorff *et al.*, 2009). More specifically, for insects, an RDR1-silenced line of *Nicotiana attenuata* (irRdR1) is more susceptible to larvae of the solanaceous specialist *Manduca sexta* (Pandey & Baldwin, 2007). Nonetheless, there are several examples of increased resistance of Arabidopsis miRNA mutants to pathogens and pests. Both Arabidopsis miRNA and siRNA pathway mutants exhibit increased resistance to the cyst nematode *Heterodera schachtii* (Hewezi *et al.*, 2008), and *dcl1* plants are resistant to tumour formation following stab inoculation with tumorigenic *Agrobacterium* (Dunoyer *et al.*, 2006). This may be expected, as miRNAs are integral players in plant development, and cyst

nematodes and *Agrobacterium* reprogramme plant development to generate cysts and galls, respectively, which provide feeding and replication sites for these plant colonizers. Thus, our observation that aphids do less well on Arabidopsis miRNA mutants may be a consequence of the highly specialized feeding mode of aphids. GPA does not form noticeable galls, but may still need to modulate specific developmental or basic plant defence processes that are regulated by miRNAs in order to establish long-term feeding sites. The salivary components that aphids release into cells whilst they navigate to the phloem and during phloem feeding (Will *et al.*, 2007; Mutti *et al.*, 2008; De Vos & Jander, 2009; Bos *et al.*, 2010; Pitino & Hogenhout, 2013) may induce these modulations. We propose that the GPA colonization efficiency of Arabidopsis is enhanced by the ability of this aphid to modulate specific plant processes that are regulated by miRNAs.

dcl1 plants display greater resistance to GPA infestation, and our data suggest that this is a result, in part, of the hyperactivation of the camalexin defence pathway. By contrast, this pathway is only modestly induced in aphid-susceptible Col-0 and *dcl2/3/4* plants. One possibility is that factors that act as brakes or suppressors of defence hyperactivation in Col-0 or *dcl2/3/4* are ineffective or absent in *dcl1* plants. Suppressors of hyperactivation may be protein effectors present in aphid saliva that can modify aspects of host physiology and suppress defensive mechanisms. Therefore, host proteins involved in camalexin production or specific miRNAs involved in the management of this pathway may be targets for as yet uncharacterized aphid salivary effectors. Indeed, effectors from a plant pathogen are capable of interfering with host miRNA processing (Navarro *et al.*, 2008). Another possibility is that plants actively manage their response through the induction of specific miRNA species that target transcripts involved in the camalexin pathway. This control mechanism would be largely disabled in *dcl1* plants. As large quantities of camalexin are toxic to Arabidopsis cells in culture (Rogers *et al.*, 1996), this dampening effect may represent a form of plant self-defence.

In Arabidopsis, some miRNAs target transcripts related to secondary metabolism. One group of miRNAs (miR160, miR167, miR390, miR393) is specifically related to auxin signalling (Zhang *et al.*, 2011), which is linked to camalexin and glucosinolate biosynthesis. In addition, miR393 has a role in the plant immune response as it is induced following exposure to the PAMP flg22 (Navarro *et al.*, 2006; Li *et al.*, 2010), and following inoculation of both virulent and avirulent strains of *P. syringae* pv. *tomato* (*Pst*; Zhang *et al.*, 2011). It has also been reported that miR393 has a role in resource allocation between the glucosinolate and camalexin pathways (Robert-Seilaniantz *et al.*, 2011).

Aphids transmit one-third of *c.* 800 described plant viruses (Ng & Perry, 2004; Hogenhout *et al.*, 2008). Many of these viruses encode suppressor molecules which block antiviral RNA silencing (Ding & Voinnet, 2007) and can interfere with the miRNA pathway during infection (Chapman *et al.*, 2004). Silencing suppression is crucial to promote virus infectivity; however, suppression of the miRNA pathway might have a negative impact on the fecundity of the aphid vectors through the mechanisms described here. The relationship between virus and insect will strongly determine the outcome of this tritrophic interaction. Viruses that are acquired rapidly and transmitted by aphids will benefit from plant behaviour that discourages aphid settling (Mauck *et al.*, 2010). By contrast, viruses that require longer acquisition times, such as those that are phloem limited, may act to extend aphid feeding time at a particular feeding site (Eigenbrode *et al.*, 2002).

Our qRT-PCR assays indicated that aphid-resistant *dcl1* plants increase transcription of an ET-responsive gene relative to susceptible Col-0 and *dcl2/3/4* plants following aphid colonization. Fecundity assays confirmed the involvement of ET signalling in resistance, as aphid performance was improved significantly on *ein2* mutants. Our result, showing no change in aphid fecundity on *etr1*, is consistent with previous studies in which the performances of GPA and *Brevicoryne brassicae* were either unaffected or reduced on *etr1* mutants (Mewis *et al.*, 2005, 2006). Other laboratories have demonstrated that saliva-induced aphid resistance is independent of EIN2 and ET signalling (De Vos & Jander, 2009), whereas EIN2 is known to be critical for resistance to GPA following treatment with the bacterial protein harpin (Dong *et al.*, 2004; Liu *et al.*, 2011). It remains a possibility that altered regulation of this signalling mechanism contributes to the *dcl1* resistance phenotype.

Aphid fecundity was increased on the *pad3* and *cyp79b2/cyp79b3* mutants relative to Col-0. By contrast, aphid performance was unchanged on the *cyp81f2* mutant. Taken together, these results indicate that, under our experimental conditions, the production of camalexin is a major resistance factor. This is in contrast with the observations of Pegadaraju *et al.* (2005), who found no statistically significant increase in GPA colonization ability on *pad3* mutants. In addition, Kim *et al.* (2008) found no change in fecundity of aphids raised on *cyp79b2/cyp79b3* mutants relative to wild-type plants. However, in both cases, nonaged aphids were exposed to the mutant plants for a relatively short period, that is 2–5 d, whereas, in the experiments reported herein, the nymphs were born on the mutant plants and reared on these plants to adulthood (*c.* 16 d), during the course of which they began to produce nymphs themselves. Thus, differences in the experimental procedures may account for the different outcomes. Indeed, the *dcl1* resistance phenotype was absent when experiments were carried out following a previously published protocol (Pegadaraju *et al.*, 2005; Fig. S7). It is also possible that the aphid colonies maintained by different laboratories have varying susceptibilities to different phytochemicals. Our results are in agreement with those of Kusnierczyk *et al.* (2008), who found that *B. brassicae* (cabbage aphid) is more successful on *pad3* relative to wild-type Arabidopsis when both plants are pretreated with UV light to induce camalexin production. In these experiments, aged nymphs were raised on test plants for 13 d, a protocol very similar to our own assay. Furthermore, aphids produce less progeny on artificial diets containing camalexin compared with control diets, confirming that camalexin has a negative impact on GPA performance. This indicates an unsuspected depth to camalexin function beyond antifungal and antibacterial defence. This work also highlights the extensive role of the miRNA-mediated regulation of secondary metabolic defence pathways with relevance to resistance against an aphid pest.

Acknowledgements

We thank Akiko Sugio, Francesca Stefanato and all members of the Hogenhout laboratory for useful discussions. We also thank Alexandre Robert-Seilaniantz for discussions and advice regarding *PAD3*, and Graham McGrann for his expertise with qRT-PCR. We are grateful to Chris Burt for help with statistical analysis, Lionel Hill for developing the camalexin detection protocol and Ian Bedford, Gavin Hatt and Anna Jordan for rearing the insects. Plants were cared for by John Innes Horticultural Services. This work was supported by BB/J004553/1 from the Biotechnology and Biological Sciences Research Council (BBSRC) and the John Innes Foundation. G.J.K. and C.D. were funded from BBSRC studentships.

References

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR. 1999. EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* **284**: 2148–2152.

Baumberger N, Baulcombe DC. 2005. Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proceedings of the National Academy of Sciences, USA* **102**: 11928–11933.

Bell E, Creelman RA, Mullet JE. 1995. A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **92**: 8675–8679.

Blackman R, Eastop V. 2000. *Aphids on the World's crops: an identification and information guide*. Chichester, UK: John Wiley & Sons.

Bleecker AB, Estelle MA, Somerville C, Kende H. 1988. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* **241**: 1086–1089.

Bollman KM, Aukerman MJ, Park M-Y, Hunter C, Berardini TZ, Poethig RS. 2003. HASTY, the Arabidopsis ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. *Development* **130**: 1493–1504.

Bos JIB, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA. 2010. A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). *PLoS Genetics* **6**: e1001216.

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320**: 1185–1190.

Cao H, Bowling SA, Gordon AS, Dong X. 1994. Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* **6**: 1583–1592.

Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC. 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes & Development* **18**: 1179–1186.

Chassot C, Buchala A, Schoonbeek H-J, Métraux J-P, Lamotte O. 2008. Wounding of Arabidopsis leaves causes a powerful but transient protection against *Botrytis* infection. *Plant Journal* **55**: 555–567.

Couldridge C, Newbury HJ, Ford-Lloyd B, Bale J, Pritchard J. 2007. Exploring plant responses to aphid feeding using a full Arabidopsis microarray reveals a small number of genes with significantly altered expression. *Bulletin of Entomological Research* **97**: 523–532.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology* **139**: 5–17.

De Vos M, Jander G. 2009. *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. *Plant, Cell & Environment* **32**: 1548–1560.

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Metraux J-P, Van Loon LC, Dicke M, *et al.* 2005. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. *Molecular Plant–Microbe Interactions* **18**: 923–937.

Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O. 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313**: 68–71.

Ding S, Voinnet O. 2007. Antiviral immunity directed by small RNAs. *Cell* **130**: 413–426.

Dong Z, Han M, Fedoroff N. 2008. The RNA-binding proteins HYL1 and SE promote accurate *in vitro* processing of pri-miRNA by DCL1. *Proceedings of the National Academy of Sciences, USA* **105**: 9970–9975.

Dong H-P, Peng J, Bao Z, Meng X, Bonasera JM, Chen G, Beer SV, Dong H. 2004. Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. *Plant Physiology* **136**: 3628–3638.

Dunoyer P, Himber C, Voinnet O. 2006. Induction, suppression and requirement of RNA silencing pathways in virulent *Agrobacterium tumefaciens* infections. *Nature Genetics* **38**: 258–263.

Eigenbrode S, Ding H, Shiel P, Berger P. 2002. Volatiles from potato plants infected with potato leafroll virus attract and arrest the virus vector, *Myzus persicae* (Homoptera: Aphididae). *Proceedings of the Royal Society B* **269**: 455–460.

Ellendorff U, Fradin EF, de Jonge R, Thomma BPHJ. 2009. RNA silencing is required for Arabidopsis defence against Verticillium wilt disease. *Journal of Experimental Botany* **60**: 591–602.

Fujii H, Chiou T-J, Lin S-I, Aung K, Zhu J-K. 2005. A miRNA involved in phosphate-starvation response in Arabidopsis. *Current Biology* **15**: 2038–2043.

Gao L-L, Kamphuis LG, Kakar K, Edwards OR, Udvardi MK, Singh KB. 2010. Identification of potential early regulators of aphid resistance in *Medicago truncatula* via transcription factor expression profiling. *New Phytologist* **186**: 980–994.

Glazebrook J, Ausubel FM. 1994. Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. *Proceedings of the National Academy of Sciences, USA* **91**: 8955–8959.

Henderson IR, Zhang X, Lu C, Johnson L, Meyers BC, Green PJ, Jacobsen SE. 2006. Dissecting *Arabidopsis thaliana* DICER function in small RNA processing, gene silencing and DNA methylation patterning. *Nature Genetics* **38**: 721–725.

Hewezi T, Howe P, Maier TR, Baum TJ. 2008. Arabidopsis small RNAs and their targets during cyst nematode parasitism. *Molecular Plant–Microbe Interactions* **21**: 1622–1634.

Hogenhout SA, Ammar ED, Whitfield AE, Redinbaugh MG. 2008. Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology, vol 46*. Palo Alto: Annual Reviews, 327–359.

Jacobsen SE, Running MP, Meyerowitz EM. 1999. Disruption of an RNA helicase/RNAse III gene in Arabidopsis causes unregulated cell division in floral meristems. *Development* **126**: 5231–5243.

Katiyar-Agarwal S, Gao S, Vivian-Smith A, Jin H. 2007. A novel class of bacteria-induced small RNAs in Arabidopsis. *Genes & Development* **21**: 3123–3134.

Katiyar-Agarwal S, Jin H. 2010. Role of small RNAs in host–microbe interactions. *Annual Review of Phytopathology* **48**: 225–246.

Kim JH, Jander G. 2007. *Myzus persicae* (green peach aphid) feeding on Arabidopsis induces the formation of a deterrent indole glucosinolate. *Plant Journal* **49**: 1008–1019.

Kim JH, Lee BW, Schroeder FC, Jander G. 2008. Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid). *Plant Journal* **54**: 1015–1026.

Kliebenstein DJ, Rowe HC, Denby KJ. 2005. Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. *Plant Journal* **44**: 25–36.

Kurihara Y, Watanabe Y. 2004. *Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions. *Proceedings of the National Academy of Sciences, USA* **101**: 12753–12758.

Kusnierczyk A, Winge PER, Jørstad TS, Troczynska J, Rossiter JT, Bones AM. 2008. Towards global understanding of plant defence against aphids – timing and dynamics of early Arabidopsis defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. *Plant, Cell & Environment* **31**: 1097–1115.

Kusnierczyk A, Winge P, Midelfart H, Armbruster WS, Rossiter JT, Bones AM. 2007. Transcriptional responses of *Arabidopsis thaliana* ecotypes with different

glucosinolate profiles after attack by polyphagous *Myzus persicae* and oligophagous *Brevicoryne brassicae*. *Journal of Experimental Botany* **58**: 2537–2552.

Li Y, Zhang Q, Zhang J, Wu L, Qi Y, Zhou J-M. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiology* **152**: 2222–2231.

Liu R, Chen L, Jia Z, Lü B, Shi H, Shao W, Dong H. 2011. Transcription factor AtMYB44 regulates induced expression of the *ETHYLENE INSENSITIVE2* gene in Arabidopsis responding to a harpin protein. *Molecular Plant–Microbe Interactions* **24**: 377–389.

Lobbes D, Rallapalli G, Schmidt DD, Martin C, Clarke J. 2006. SERRATE: a new player on the plant microRNA scene. *EMBO Reports* **7**: 1052–1058.

Matzke M, Kanno T, Huettel B, Daxinger L, Matzke AJ. 2007. Targets of RNA-directed DNA methylation. *Current Opinion in Plant Biology* **10**: 512–519.

Mauck KE, De Moraes CM, Mescher MC. 2010. Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proceedings of the National Academy of Sciences, USA* **107**: 3600–3605.

Meuwly P, Metraux JP. 1993. Ortho-anisic acid as internal standard for the simultaneous quantitation of salicylic acid and its putative biosynthetic precursors in cucumber leaves. *Analytical Biochemistry* **214**: 500–505.

Mewis I, Appel HM, Hom A, Raina R, Schultz JC. 2005. Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiology* **138**: 1149–1162.

Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J. 2006. Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* **67**: 2450–2462.

Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, Chapman EJ, Fahlgren N, Allen E, Carrington JC. 2008. Specificity of ARGONAUTE7–miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell* **133**: 128–141.

Moran PJ, Cheng Y, Cassell JL, Thompson GA. 2002. Gene expression profiling of *Arabidopsis thaliana* in compatible plant–aphid interactions. *Archives of Insect Biochemistry and Physiology* **51**: 182–203.

Morel J-B, Godon C, Mourrain P, Beclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H. 2002. Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**: 629–639.

Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, Jouette D, Lacombe A-M, Nikic S, Picault N, *et al.* 2000. Arabidopsis *SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**: 533–542.

Mutti NS, Louis J, Pappan LK, Pappan K, Begum K, Chen M-S, Park Y, Dittmer N, Marshall J, Reese JC, *et al.* 2008. A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. *Proceedings of the National Academy of Sciences, USA* **105**: 9965–9969.

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* **312**: 436–439.

Navarro L, Jay F, Nomura K, He SY, Voinnet O. 2008. Suppression of the microRNA pathway by bacterial effector proteins. *Science* **321**: 964–967.

Nawrath C, Metraux J-P. 1999. Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* **11**: 1393–1404.

Ng JCK, Perry KL. 2004. Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology* **5**: 505–511.

Pandey SP, Baldwin IT. 2007. RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature. *Plant Journal* **50**: 40–53.

Pandey SP, Shahi P, Gase K, Baldwin IT. 2008. Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in *Nicotiana attenuata*. *Proceedings of the National Academy of Sciences, USA* **105**: 4559–4564.

Park W, Li J, Song R, Messing J, Chen X. 2002. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Current Biology* **12**: 1484–1495.

Park MY, Wu G, Gonzalez-Sulser A, Vaucheret H, Poethig RS. 2005. Nuclear processing and export of microRNAs in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **102**: 3691–3696.

Pegadaraju V, Knepper C, Reese J, Shah J. 2005. Premature leaf senescence modulated by the Arabidopsis *PHYTOALEXIN DEFICIENT4* gene is associated with defense against the phloem-feeding green peach aphid. *Plant Physiology* **139**: 1927–1934.

Pfalz M, Vogel H, Kroymann J. 2009. The gene controlling the indole glucosinolate modifier1 quantitative trait locus alters indole glucosinolate structures and aphid resistance in *Arabidopsis*. *Plant Cell* **21**: 985–999.

Pitino M, Hogenhout SA. 2013. Aphid protein effectors promote aphid colonization in a plant species-specific manner. *Molecular Plant–Microbe Interactions* **26**: 130–139.

Rabinowicz PD, Palmer LE, May BP, Hemann MT, Lowe SW, McCombie WR, Martienssen RA. 2003. Genes and transposons are differentially methylated in plants, but not in mammals. *Genome Research* **13**: 2658–2664.

Robert-Seilaniantz A, MacLean D, Jikumaru Y, Hill L, Yamaguchi S, Kamiya Y, Jones JDG. 2011. The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates. *Plant Journal* **67**: 218–231.

Rogers EE, Glazebrook J, Ausubel FM. 1996. Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in Arabidopsis–pathogen interactions. *Molecular Plant–Microbe Interactions* **9**: 748–757.

Ruiz-Ferrer V, Voinnet O. 2009. Roles of plant small RNAs in biotic stress responses. *Annual Review of Plant Biology* **60**: 485–510.

Sattar S, Song Y, Anstead JA, Sunkar R, Thompson GA. 2012. *Cucumis melo* microRNA expression profile during aphid herbivory in a resistant and susceptible interaction. *Molecular Plant–Microbe Interactions* **25**: 839–848.

Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatos A, Halkier BA, Glawischnig E. 2006. CYP71B15 (*PAD3*) catalyzes the final step in camalexin biosynthesis. *Plant Physiology* **141**: 1248–1254.

Staswick PE, Su W, Howell SH. 1992. Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proceedings of the National Academy of Sciences, USA* **89**: 6837–6840.

Stefanato FL, Abou-Mansour E, Buchala A, Kretschmer M, Mosbach A, Hahn M, Bochet CG, Métraux J-P, Schoonbeek H-J. 2009. The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*. *Plant Journal* **58**: 499–510.

Sunkar R, Zhu J-K. 2004. Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell* **16**: 2001–2019.

Thomas CL, Bayer EM, Ritzenthaler C, Fernandez-Calvino L, Maule AJ. 2008. Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. *PLoS Biology* **6**: e7.

Tjallingii WF. 1995. Regulation of phloem sap feeding by aphids. In: Chapman RF, de Boer G. eds, *Regulatory mechanisms in insect feeding*. New York, NY, USA: Chapman & Hall, 190–209.

Tjallingii WF. 2006. Salivary secretions by aphids interacting with proteins of phloem wound responses. *Journal of Experimental Botany* **57**: 739–745.

Tjallingii WF, Esch T. 1993. Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiological Entomology* **18**: 317–328.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**: RESEARCH0034.

Vazquez F, Gasciolli V, Crete P, Vaucheret H. 2004a. The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Current Biology* **14**: 346–351.

Vazquez F, Legrand S, Windels D. 2010. The biosynthetic pathways and biological scopes of plant small RNAs. *Trends in Plant Science* **15**: 337–345.

Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, Hilbert J-L, Bartel DP, Crete P. 2004b. Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. *Molecular Cell* **16**: 69–79.

Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. *Cell* **136**: 669–687.

Will T, Tjallingii WF, Thonnessen A, van Bel AJE. 2007. Molecular sabotage of plant defense by aphid saliva. *Proceedings of the National Academy of Sciences, USA* **104**: 10536–10541.

Xie Z, Allen E, Wilken A, Carrington JC. 2005. DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **102**: 12984–12989.

Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC. 2004. Genetic and functional diversification of small RNA pathways in plants. *PLoS Biology* **2**: e104.

Xu J, Li Y, Wang Y, Liu H, Lei L, Yang H, Liu G, Ren D. 2008. Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in Arabidopsis. *Journal of Biological Chemistry* **283**: 26996–27006.

Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, Steward R, Chen X. 2005. Methylation as a crucial step in plant microRNA biogenesis. *Science* **307**: 932–935.

Zhang W, Gao S, Zhou X, Chellappan P, Chen Z, Zhou X, Zhang X, Fromuth N, Coutino G, Coffey M, *et al.* 2011. Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. *Plant Molecular Biology* **75**: 93–105.

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL. 2002. Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. *Genes & Development* **16**: 3100–3112.

Zilberman D, Cao X, Jacobsen SE. 2003. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299**: 716–719.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phenotypes of all plants used in the fecundity screen of the silencing mutants.

Fig. S2 Investigation into the temporal response of Col-0 plants to *Myzus persicae* (green peach aphid, GPA) infestation.

Fig. S3 Induction of the pathogen response-related gene *MITOGEN-ACTIVATED PROTEIN KINASE3* (MPK3) is highest in *dcl1* plants.

Fig. S4 Expression levels of *PHYTOALEXIN DEFICIENT3* (*PAD3*) and *HEVEIN-LIKE* (*HEL*) at 24 and 48 h post-inoculation (hpi) in Col-0, *dcl1* and *dcl2/3/4* plants.

Fig. S5 Aphid fecundity trials on Arabidopsis salicylic acid (SA) and jasmonic acid (JA) pathway mutants.

Fig. S6 Spatial induction of a *CYP71B15p::GUS* (*PAD3p::GUS*) transgene following aphid feeding.

Fig. S7 Aphid fecundity trial on Arabidopsis Col-0, *dcl1* and *dcl2/3/4* using a previously described fecundity/survival assay protocol.

Table S1 Oligonucleotide primers used for quantitative real-time polymerase chain reaction (qRT-PCR) experiments

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

Supporting Information Figs S1-S7, Table S1

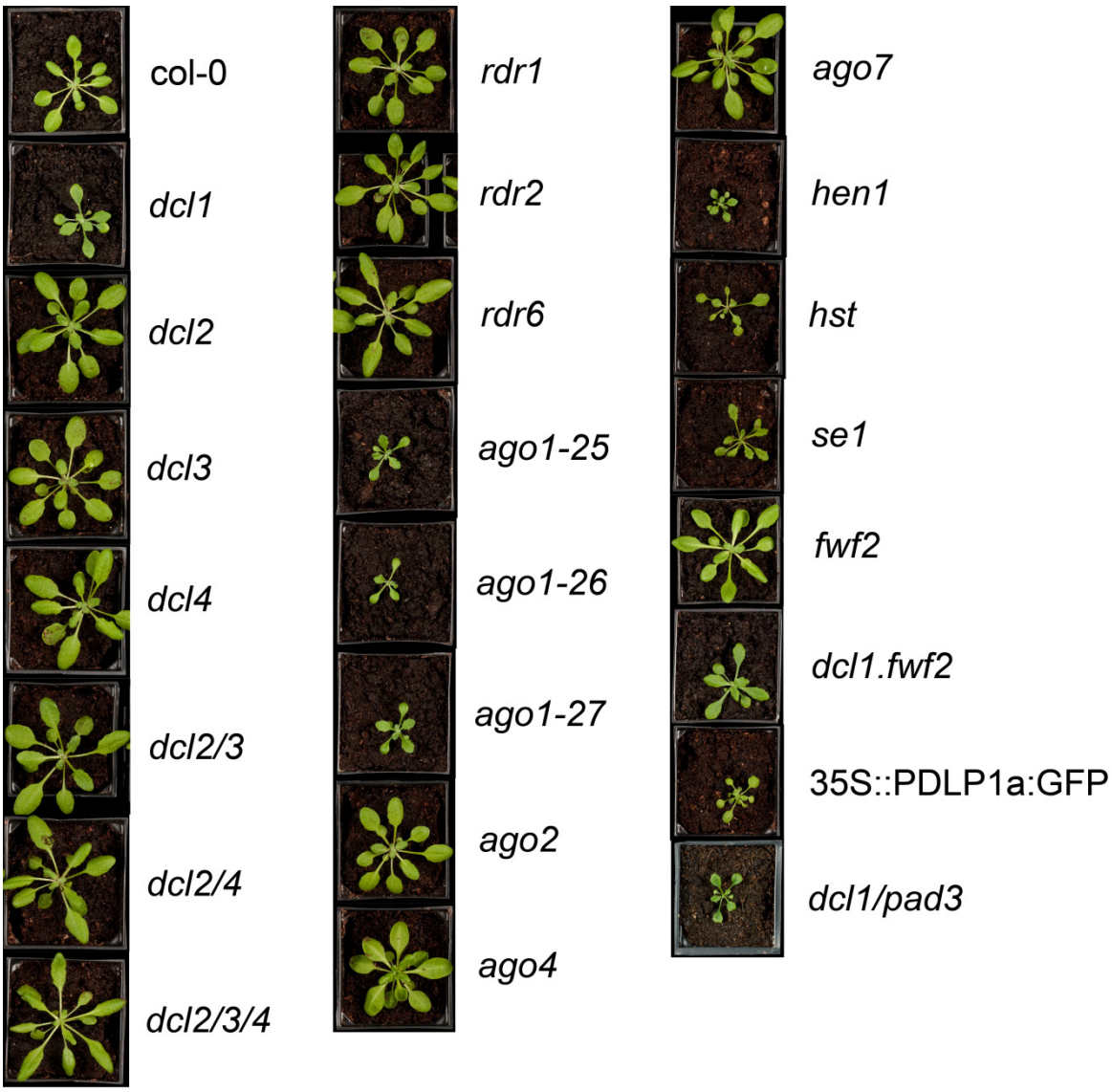


Fig. S1 Phenotypes of all plants used in the silencing mutants fecundity screen.

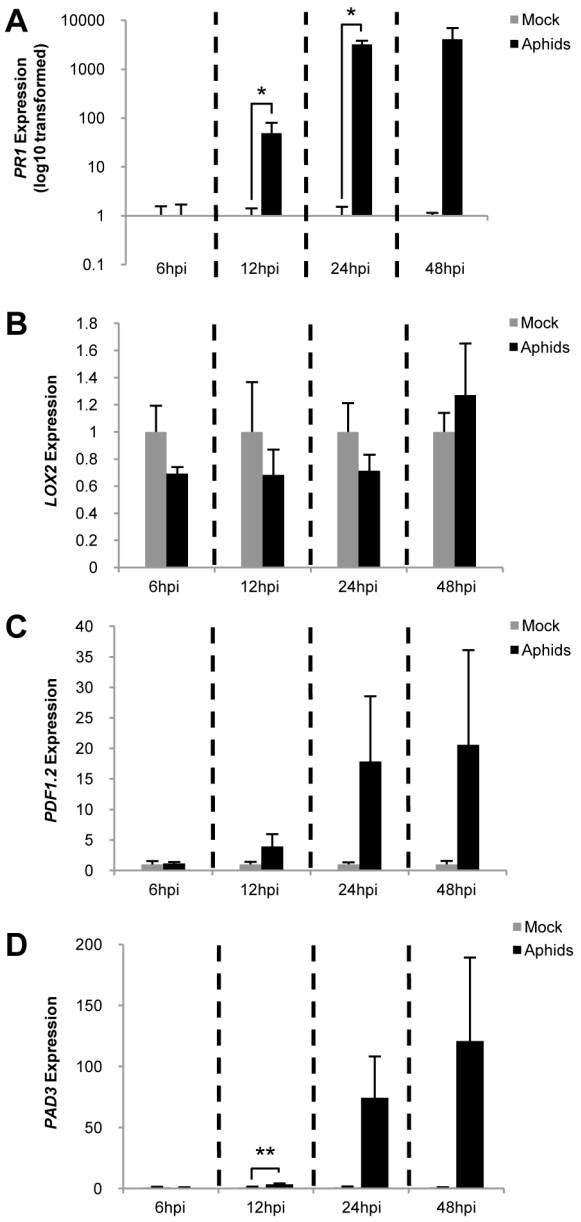


Fig. S2 Investigation into temporal response of Col-0 plants to *M. persicae* (GPA) infestation.

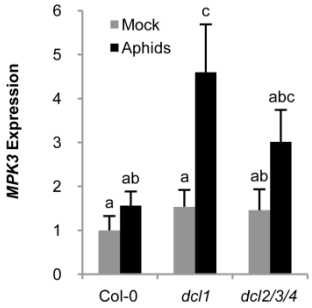


Fig. S3 Induction of the pathogen response-related gene MPK3 is highest in *dcl1* plants.

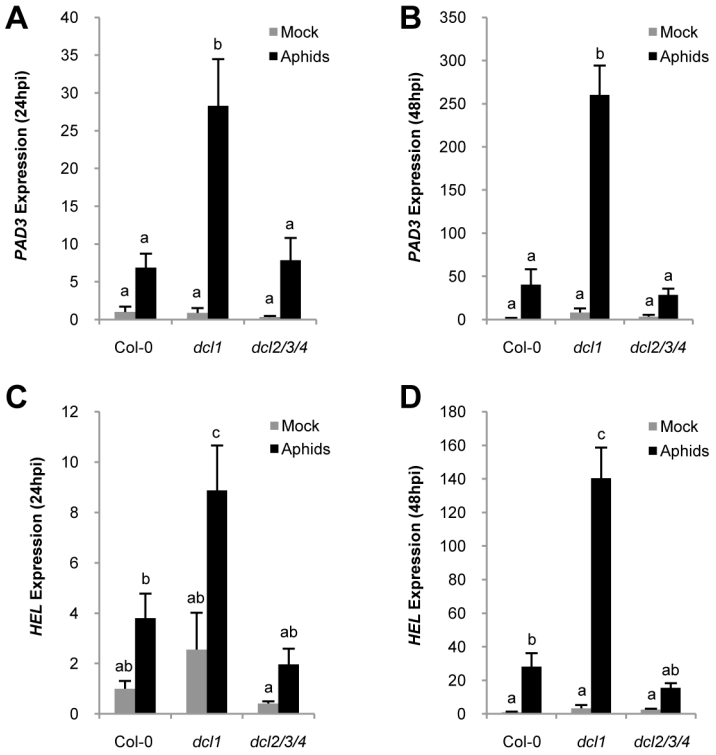


Fig. S4 Expression levels of *PAD3* and *HEL* at 24hpi and 48hpi in Col-0, *dcl1* and *dcl2/3/4* plants.

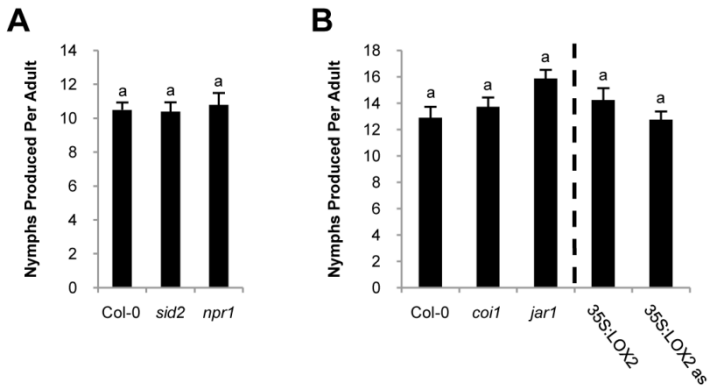


Fig. S5 Aphid fecundity trials on Arabidopsis SA and JA pathway mutants.

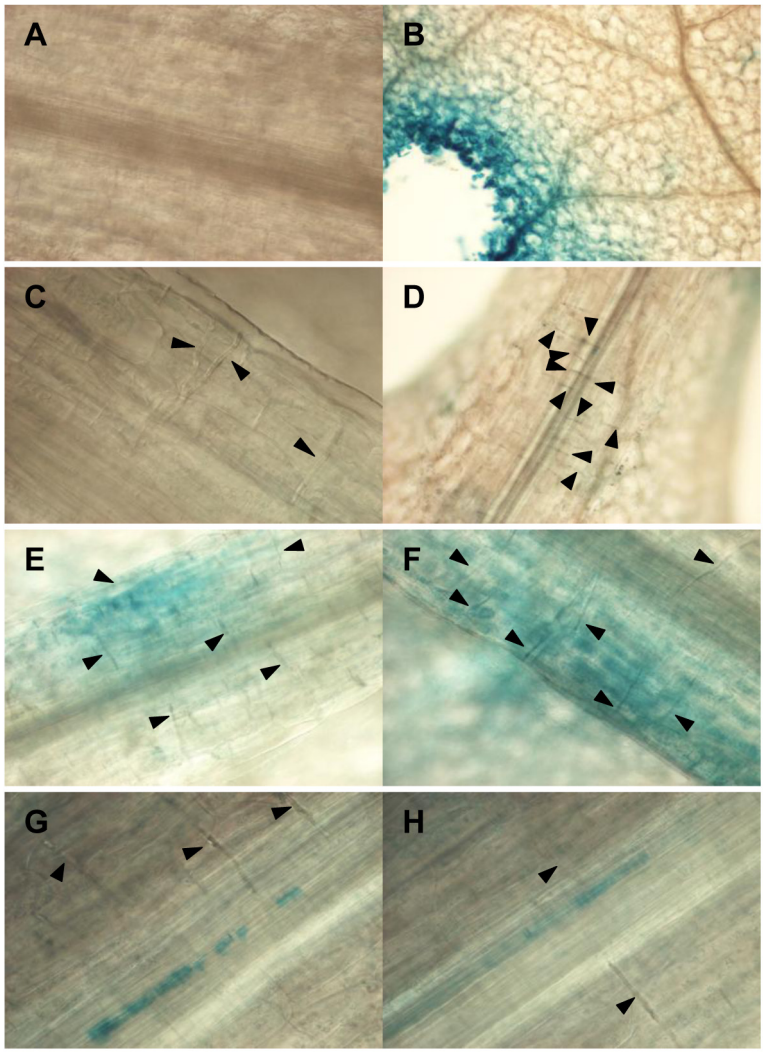


Fig. S6 Spatial induction of a *CYP71B15p::GUS* (*PAD3p::GUS*) transgene following aphid feeding. (a) no treatment negative control, (b) Botrytis lesion positive control, (c-h) aphid feeding sites on leaf midveins with varying degrees and patterns of transgene induction.

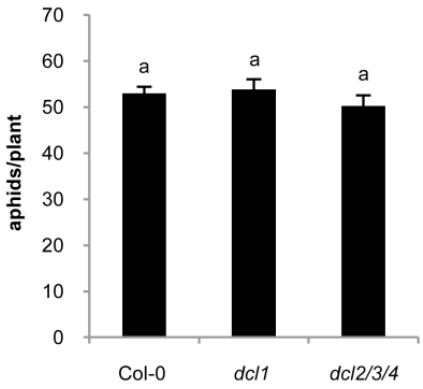


Fig. S7 Aphid fecundity trial on Arabidopsis Col-0, *dcl1* and *dcl2/3/4* using a previously described fecundity/survival assay protocol.

Reference Genes

Gene Name	Identifier	Sequence (5' -> 3')
ACT2	At3g18780	F GATGAGGCAGGTCCAGGAATC
		R GTTTGTCACACACAAGTGCATC
At5g46630	At5g46630	F TGCGTTTTGGTTAATCTGTCTC
		R CCGTGTTGTAACCGCTCTTC
PEX4	At5g25760	F TGCAACCTCCTCAAGTTCG
		R CACAGACTGAAGCGTCCAAG

Target Genes

Gene Name	Identifier	Sequence (5' -> 3')
PR1	At2g14610	F GTTGCAGCCTATGCTCGGAG
		R CCGCTACCCCAGGCTAAGTT
LOX2	At3g45140	F GCAAGCTCCAATATCTAGAAGGAGTG
		R CGGTAACACCATGCTCAGAGGTAG
VSP2	At5g24770	F GTTAGGGACCGGAGCATCAA
		R AACGGTCACTGAGTATGATGGGT
PDF1.2	At5g44420	F CCATCATCACCCTTATCTTCGC
		R TGTCCCACTTGGCTTCTCG
HEL/PR4	At3g04720	F TGTGAGAATAGTGGACCAATGC
		R ATGAGATGGCCTTGTTGATAGC
PAD3	At3g26830	F TGCTCCCAAGACAGACAATG
		R GTTTTGGATCACGACCCATC
CYP79B2	At4g39950	F TCTCCGGTTTATCTCGTTCAGTA
		R CGTGTCTCATTCTCAGGTAGCTT
CYP83B1	At4g31500	F TGCTGGTAGATATGGCGTGAC
		R AAGGGACCCGAATATTAAACATC
CYP81F2	At5g57220	F TGGCTATGCGTAAACTCGTG
		R GGTAAACTTCAAAATGGTGGTCA
MPK3	At3g45640	F TCCGAATGGCTACTTAGTATCTTTG
		R TGGAGCTACACTTAATCACTAGCAG

Table S1 Oligonucleotide primers used for qRT-PCR experiments

Appendix C

List of plasmids

Name	Insert	Backbone	Resistance (bacterial)	Other	Source
Plant Expression Vectors					
35s:AvrPtoB	AvrPtoB	pCB302-3	Kanamycin		J. Bos (JIC, UK)
35s: Mp10	Mp10	pCB302-3	Kanamycin		J. Bos
35s:EV	Empty vector	pCB302-3	Kanamycin		J. Bos
GFP-PVX	GFP	pGR106	Kanamycin		J. Bos
Mp10-PVX	Mp10	pGR106	Kanamycin		J. Bos
Mp10 Ax2-PVX	Mp10 Y40 W120A	pGR106	Kanamycin		C. Drurey
Mp10->D1 x2-PVX	Mp10 Y40F W120Y	pGR106	Kanamycin		C. Drurey
MpOS-D1-PVX	MpOS-D1	pGR106	Kanamycin		C. Drurey
dsMp10	Mp10	pJawohl8	Ampicillin	BASTA	D. Prince (JIC, UK)
dsMpOS-D1	MpOS-D1	pJawohl8	Ampicillin	BASTA	D. Prince
FLAG-Mp10	Mp10	pTRBO	Kanamycin		D. Prince
FLAG-MpOS-D1	MpOS-D1	pTRBO	Kanamycin		D. Prince
GFP-Mp10	Mp10	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-MpOS-D1	MpOS-D1	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-Mp10 Y40A	Mp10 Y40A	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-Mp10 Y40F	Mp10 Y40F	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-Mp10 W120A	Mp10 W120A	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-Mp10 W120Y	Mp10 W120Y	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-Mp10 x2	Mp10 Y40A W120A	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-Mp10->D1 x2	Mp10 Y40F W120Y	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-OS-D1 F28Y	MpOS-D1 F28Y	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-OS-D1 Y108W	MpOS-D1 Y108W	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP-OS-D1 x2	MpOS-D1 F28Y Y108W	pB7WGF2	Spectinomycin	BASTA	C. Drurey
GFP	GFP	pB7WG2	Spectinomycin	BASTA	A.Sugio (JIC, UK)
GFP-AMSH1	AtAMSH1	pB7WGF2	Spectinomycin	BASTA	C.Drurey/C. Wilson
GFP-AMSH2	AtAMSH2	pB7WGF2	Spectinomycin	BASTA	C.Drurey/C. Wilson
GFP-AMSH3	AtAMSH3	pB7WGF2	Spectinomycin	BASTA	C.Drurey/C. Wilson
GFP-NbAMSH2	NbAMSH2	pB7WGF2	Spectinomycin	BASTA	F. Bernsdorff (JIC, UK)
AMSH2 RNAi	NbAMSH2	pK7GWIWG2 (II)	Spectinomycin	Kanamycin	F. Bernsdorff
GUS RNAi	GUS	pK7GWIWG2 (II)	Spectinomycin	Kanamycin	S. Mugford
RFP-ARA6	ARA6	pUBQ10	Spectinomycin	BASTA	S. Robatzek (TSL, UK)
RFP-GUS	GUS	pUBQ10	Spectinomycin	BASTA	V. Thole (JIC, UK)
RFP-Mp10	Mp10	pUBQ10	Spectinomycin	BASTA	C. Drurey
RFP-Mp10	MpOS-D1	pUBQ10	Spectinomycin	BASTA	C. Drurey
AtFLS2-GFP	AtFLS2	pCAMBIA2300	Kanamycin		J. Loiseau (TSL, UK)
Sl-FLS2-GFP	SlFLS2	pCAMBIA2300	Kanamycin		J. Loiseau
BAK1-GFP	StBAK1/SERK3A	pK7FWG2	Spectinomycin	Kanamycin	T. Bozkurt (Imperial, UK)
AvrRps4-GFP	AvrRps4	pK7FWG2	Spectinomycin	Kanamycin	P. Sarris (TSL, UK)
E187A-GFP	AvrRps4 E187A	pK7FWG2	Spectinomycin	Kanamycin	P. Sarris
RPS4-HA	AtRPS4	pBIN19	Kanamycin		P. Sarris
RRS1-His-FLAG	AtRRS1	pICH86988	Kanamycin		P. Sarris
Mp19-FLAG	Mp19	pGWB11	Kanamycin		C. Drurey
Mp21-FLAG	Mp21	pGWB11	Kanamycin		C. Drurey
Mp44.1-FLAG	Mp44.1	pGWB11	Kanamycin		C. Drurey
Mp44.3-FLAG	Mp44.3	pGWB11	Kanamycin		C. Drurey
Yeast expression vectors					
BD-Mp10 (screen)	Mp10	pLex-AN	Ampicillin	Tryptophan	C. Wilson
AD-EV	Empty vector	pDEST22	Ampicillin	Tryptophan	Invitrogen
AD-Mp10	Mp10	pDEST22	Ampicillin	Tryptophan	C. Drurey
AD-MpOS-D1	MpOS-D1	pDEST22	Ampicillin	Tryptophan	C. Drurey
AD-Mp10 x2	Mp10 Y40F W120Y	pDEST22	Ampicillin	Tryptophan	C. Caceres
AD-Mp2	Mp2	pDEST22	Ampicillin	Tryptophan	C. Caceres
AD-VPS2.1	AtVPS2.1	pDEST22	Ampicillin	Tryptophan	C. Caceres
AD-VPS24.1	AtVPS24.1	pDEST22	Ampicillin	Tryptophan	C. Caceres
AD-AMSH2	AtAMSH2.1	pDEST22	Ampicillin	Tryptophan	F. Bernsdorff
AD-AMSH3	AtAMSH3	pDEST22	Ampicillin	Tryptophan	F. Bernsdorff
BD-EV	Empty vector	pDEST32	Gentamicin	Leucine	Invitrogen

Name	Insert	Backbone	Resistance (bacterial)	Other	Source
BD-AMSH1	AtAMSH1	pDEST32	Gentamicin	Leucine	C. Wilson
BD-AMSH2.1	AtAMSH2.1	pDEST32	Gentamicin	Leucine	C. Wilson
BD-AMSH2.2	AtAMSH2.2	pDEST32	Gentamicin	Leucine	C. Wilson
BD-AMSH2.3	AtAMSH2.3	pDEST32	Gentamicin	Leucine	C. Wilson
BD-AMSH3	AtAMSH3	pDEST32	Gentamicin	Leucine	C. Wilson
BD-Mp2	Mp2	pDEST32	Gentamicin	Leucine	C. Caceres
BD-VPS24.1	VPS24.1	pDEST32	Gentamicin	Leucine	F. Bernsdorff
BD-Mp10	Mp10	pDEST32	Gentamicin	Leucine	C. Drurey
pGAD	Empty vector	pGAD-HA	Ampicillin	Leucine	Dualsystems
pGAD Large T	SV40 large T antigen	pGAD-HA	Ampicillin	Leucine	Dualsystems
pGAD-C002	MpC002	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp1	Mp1	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp2	Mp2	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Ap2	Ap2	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp10	Mp10	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp17	Mp17	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp19	Mp19	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp23	Mp23	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp43.1	Mp43.1	pGAD-HA	Ampicillin	Leucine	C. Caceres
pGAD-Mp44.3	Mp44.3	pGAD-HA	Ampicillin	Leucine	C. Caceres
pLEX	Empty vector	pLEX-AN	Ampicillin	Tryptophan	Dualsystems
p53	Tumour protein p53	pLEX-AN	Ampicillin	Tryptophan	Dualsystems
pLEX-Mp1	Mp1	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
pLEX-Mp2	Mp2	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
pLEX-Mp10	Mp10	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
pLEX-Mp17	Mp17	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
pLEX-Mp23	Mp23	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
pLEX-Mp43.1	Mp43.1	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
pLEX-Mp44.1	Mp44.1	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
pLEX-Mp44	Mp44.3	pLEX-AN	Ampicillin	Tryptophan	C. Caceres
10-Mp1	Mp1	pLAW10	Ampicillin	Tryptophan	C. Drurey
10-Mp2	Mp2	pLAW10	Ampicillin	Tryptophan	C. Drurey
10-Mp3	Mp3	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp4	Mp4	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp5	Mp5	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp6	Mp6	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp8	Mp8	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp10	Mp10	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp11	Mp11	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp12	Mp12	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp14	Mp14	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp15	Mp15	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp16	Mp16	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp17	Mp17	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp19	Mp19	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp20	Mp20	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp21	Mp21	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp22	Mp22	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp23	Mp23	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp24	Mp24	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp28	Mp28	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp29	Mp29	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp30	Mp30	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp31	Mp31	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp32	Mp32	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp33.2	Mp33.2	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp33.3	Mp33.3	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp35	Mp35	pLAW10	Kanamycin	Tryptophan	C. Drurey

Name	Insert	Backbone	Resistance (bacterial)	Other	Source
10-Mp36	Mp36	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp37	Mp37	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp39	Mp39	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp40	Mp40	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp41	Mp41	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp42	Mp42	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp43	Mp43	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp44.1	Mp44.1	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp44.3	Mp44.3	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp45	Mp45	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp46	Mp46	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp47	Mp47	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp49	Mp49	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp50	Mp50	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp51	Mp51	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp53	Mp53	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Mp54	Mp54	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-C002	MpC002	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Ap1	Ap1	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-Ap2	Ap2	pLAW10	Kanamycin	Tryptophan	C. Drurey
10-ApC002	ApC002	pLAW10	Kanamycin	Tryptophan	C. Drurey
11-Mp1	Mp1	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp2	Mp2	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp3	Mp3	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp4	Mp4	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp5	Mp5	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp6	Mp6	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp8	Mp8	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp10	Mp10	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp11	Mp11	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp12	Mp12	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp14	Mp14	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp15	Mp15	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp16	Mp16	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp17	Mp17	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp19	Mp19	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp20	Mp20	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp21	Mp21	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp22	Mp22	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp23	Mp23	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp24	Mp24	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp28	Mp28	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp29	Mp29	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp30	Mp30	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp31	Mp31	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp32	Mp32	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp33.2	Mp33.2	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp33.3	Mp33.3	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp35	Mp35	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp36	Mp36	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp37	Mp37	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp39	Mp39	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp40	Mp40	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp41	Mp41	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp42	Mp42	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp43	Mp43	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp44.1	Mp44.1	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp44.3	Mp44.3	pLAW11	Ampicillin	Leucine	C. Drurey

Name	Insert	Backbone	Resistance (bacterial)	Other	Source
11-Mp45	Mp45	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp46	Mp46	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp47	Mp47	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp49	Mp49	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp50	Mp50	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp51	Mp51	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp53	Mp53	pLAW11	Ampicillin	Leucine	C. Drurey
11-Mp54	Mp54	pLAW11	Ampicillin	Leucine	C. Drurey
11-C002	MpC002	pLAW11	Ampicillin	Leucine	C. Drurey
11-Ap1	Ap1	pLAW11	Ampicillin	Leucine	C. Drurey
11-Ap2	Ap2	pLAW11	Ampicillin	Leucine	C. Drurey
11-ApC002	ApC002	pLAW11	Ampicillin	Leucine	C. Drurey
		Cloning Vectors			
GW-Mp10	Mp10	pDONR207	Gentamicin		D. Prince
GW-OSD1	MpOS-D1	pDONR207	Gentamicin		D. Prince
10 Y40A	Mp10 Y40A	pDONR207	Gentamicin		C. Drurey
10 Y40F	Mp10 Y40F	pDONR207	Gentamicin		C. Drurey
10 W120A	Mp10 W120A	pDONR207	Gentamicin		C. Drurey
10 W120W	Mp10 W120Y	pDONR207	Gentamicin		C. Drurey
10 Ax2	Mp10 Y40A W120A	pDONR207	Gentamicin		C. Drurey
10 ->D1 x2	Mp10 Y40F W120Y	pDONR207	Gentamicin		C. Drurey
OSD1 F28Y	MpOS-D1 F28Y	pDONR207	Gentamicin		C. Drurey
OSD1 Y108W	MpOS-D1 Y108W	pDONR207	Gentamicin		C. Drurey
OSD1 x2	MpOS-D1 F28Y Y108W	pDONR207	Gentamicin		C. Drurey
AMSH1	AtAMSH1	pDONR207	Gentamicin		C. Wilson
AMSH2.1	AtAMSH2.1	pDONR207	Gentamicin		C. Wilson
AMSH2.2	AtAMSH2.2	pDONR207	Gentamicin		C. Wilson
AMSH2.3	AtAMSH2.3	pDONR207	Gentamicin		C. Wilson
AMSH3	AtAMSH3	pDONR207	Gentamicin		C. Wilson
221-AMSH1	AtAMSH1	pDONR221	Kanamycin		C. Wilson
221-AMSH2	AtAMSH2.1	pDONR221	Kanamycin		C. Wilson
221-AMSH2.2	AtAMSH2.2	pDONR221	Kanamycin		C. Wilson
221-AMSH2.3	AtAMSH2.3	pDONR221	Kanamycin		C. Wilson
221-AMSH3	AtAMSH3	pDONR221	Kanamycin		C. Wilson
Mp1 NoSTOP	Mp1	pDONR207	Gentamicin		C. Drurey
Mp2 NoSTOP	Mp2	pDONR207	Gentamicin		C. Drurey
Mp3 NoSTOP	Mp3	pDONR207	Gentamicin		C. Drurey
Mp4 NoSTOP	Mp4	pDONR207	Gentamicin		C. Drurey
Mp5 NoSTOP	Mp5	pDONR207	Gentamicin		C. Drurey
Mp6 NoSTOP	Mp6	pDONR207	Gentamicin		C. Drurey
Mp8 NoSTOP	Mp8	pDONR207	Gentamicin		C. Drurey
Mp10 NoSTOP	Mp10	pDONR207	Gentamicin		C. Drurey
Mp11 NoSTOP	Mp11	pDONR207	Gentamicin		C. Drurey
Mp12 NoSTOP	Mp12	pDONR207	Gentamicin		C. Drurey
Mp14 NoSTOP	Mp14	pDONR207	Gentamicin		C. Drurey
Mp15 NoSTOP	Mp15	pDONR207	Gentamicin		C. Drurey
Mp16 NoSTOP	Mp16	pDONR207	Gentamicin		C. Drurey
Mp17 NoSTOP	Mp17	pDONR207	Gentamicin		C. Drurey
Mp19 NoSTOP	Mp19	pDONR207	Gentamicin		C. Drurey
Mp20 NoSTOP	Mp20	pDONR207	Gentamicin		C. Drurey
Mp21 NoSTOP	Mp21	pDONR207	Gentamicin		C. Drurey
Mp22 NoSTOP	Mp22	pDONR207	Gentamicin		C. Drurey
Mp23 NoSTOP	Mp23	pDONR207	Gentamicin		C. Drurey
Mp24 NoSTOP	Mp24	pDONR207	Gentamicin		C. Drurey
Mp28 NoSTOP	Mp28	pDONR207	Gentamicin		C. Drurey
Mp29 NoSTOP	Mp29	pDONR207	Gentamicin		C. Drurey
Mp30 NoSTOP	Mp30	pDONR207	Gentamicin		C. Drurey

Name	Insert	Backbone	Resistance (bacterial)	Other	Source
Mp31 NoSTOP	Mp31	pDONR207	Gentamicin		C. Drurey
Mp32 NoSTOP	Mp32	pDONR207	Gentamicin		C. Drurey
Mp33.2 NoSTOP	Mp33.2	pDONR207	Gentamicin		C. Drurey
Mp33.3 NoSTOP	Mp33.3	pDONR207	Gentamicin		C. Drurey
Mp35 NoSTOP	Mp35	pDONR207	Gentamicin		C. Drurey
Mp36 NoSTOP	Mp36	pDONR207	Gentamicin		C. Drurey
Mp37 NoSTOP	Mp37	pDONR207	Gentamicin		C. Drurey
Mp39 NoSTOP	Mp39	pDONR207	Gentamicin		C. Drurey
Mp40 NoSTOP	Mp40	pDONR207	Gentamicin		C. Drurey
Mp41 NoSTOP	Mp41	pDONR207	Gentamicin		C. Drurey
Mp42 NoSTOP	Mp42	pDONR207	Gentamicin		C. Drurey
Mp43 NoSTOP	Mp43	pDONR207	Gentamicin		C. Drurey
Mp44.1 NoSTOP	Mp44.1	pDONR207	Gentamicin		C. Drurey
Mp44.3 NoSTOP	Mp44.3	pDONR207	Gentamicin		C. Drurey
Mp45 NoSTOP	Mp45	pDONR207	Gentamicin		C. Drurey
Mp46 NoSTOP	Mp46	pDONR207	Gentamicin		C. Drurey
Mp47 NoSTOP	Mp47	pDONR207	Gentamicin		C. Drurey
Mp49 NoSTOP	Mp49	pDONR207	Gentamicin		C. Drurey
Mp50 NoSTOP	Mp50	pDONR207	Gentamicin		C. Drurey
Mp51 NoSTOP	Mp51	pDONR207	Gentamicin		C. Drurey
Mp53 NoSTOP	Mp53	pDONR207	Gentamicin		C. Drurey
Mp54 NoSTOP	Mp54	pDONR207	Gentamicin		C. Drurey
MpC002 NoSTOP	MpC002	pDONR207	Gentamicin		C. Drurey
Ap1 NoSTOP	Ap1	pDONR207	Gentamicin		C. Drurey
Ap2 NoSTOP	Ap2	pDONR207	Gentamicin		C. Drurey
ApC002 NoSTOP	ApC002	pDONR207	Gentamicin		C. Drurey

Appendix D

Alignment of aphid CSPs

CSP alignment used for creation of phylogenetic tree in Chapter 5. CSPs from pea aphid (*Acyrthosiphon pisum*), cotton aphid (*Aphis gossypii*), and GPA (*Myzus persicae*).

CSP1Apisum

----------ATGAATTTGTTAGCGATTTTTTGTTATATAACCATGATGTGTG
ACTCTCAA---TTTAGGCGACTTGAGCAACCGACTGCGATCCCTCAAGTCAAGCGAATTGAGC
AACCGGCTACGATCGCAACACGGATCGGACAGGCTACGATCGCACCTAAGTTCGGACAGCCT
ACGATCGCACCTCGGTTTGGCCAAGCTACGGTCGCACCTCAGATAGGAGAGGCTGCGATCGG
TCCT----------
----------CGGATCGGACAAAGTTTTCAGAGT
GTGAATAACAGCGTGTCACCAACCACCGATGGACGTAAAACCACCAGAGAAACGTCGTCATA
TCCAACTCGATACGATTTC----------ATTGATAT
TGAAGCTGTTATGAACAATGAACGAATCATAAAAATTCTTTTCAATTGTGTTATGAACCAA
GGGCCGTGTACCAGAGAGGGTTTGGAGCTCAAAAGG---ATTGTTCCGGATGCAATACAAACA
GAATGTGCTAAATGTAATGATAGACAAAGAAAACAAGCAGGGAAAGTTTTGGCTCATTTAC
TACAGTATAAACCAGAGTATTGGAACATGTTGGTGAAAAAATTTGATCCAAATAATGTATA
TTTGAGAAAGTATATGGCAGACAATGATGACGATGAGAAATTATCTCTTCAAAAACTTACC
AACAATACTACCAAATAA----------

CSP1Agos

----------ATGAATATTTTAACGATTTTTTGTTATGTAACTGTGATGTGCG
ACACTCAA----------GTCAAGCCGGCTGTGAGCGCACAAAGATT
GCAGAGTGTGAACCAAAATGTGACACCAACCAAC----------

--------GATGGACGTAAAACCATCAGAGAAACGTCGTCATATCCAACTCGATACGATTAT-------

----------ATTGATATTGAAGCTGTTATGAACAATGAA
CGAATCATAAAAATTCTCTTCAACTGTGTTATGAGCCGAGGACCATGTACCAGAGAGGGTTT
GGAGCTCAAAAGG---ATTGTTCCAGATGCAATTCAAACAGAATGTGCTAAATGTAATGAAAG
ACAAAGAAAACAAGCAGGCAAAGTTTTAGCTCATTTACTACAGTACAAACCAGAGTATTGGA
AAATGTTGGTGCAAAAATTCGATCCAAATAATGTCTATTTGAGAAAGTATATGGCAGACAA
TGATGATGACGAAAAATTATCTCTTCAAAAACTTAGTAACGATACTACTAAAAAAAAGCGG
AATATTTAG----------

CSP5Apisum

----------ATGAACTGCAAAGTTTTGATCGCTCTGTGCTGCGTGGCCGTGTACGC
CGCACAGGCC----------AATCCCG
CCGGTGTGGCCACCGCCACC----------

----------GCCGCCGACGAC
GAGATCAAGGACTTCCCGGCCTACATGAAAAGGTTCGAAAAG----------
----------CTCAACGTGGAACAGGTGCTGAACAATGACCGCGTCCTGGCCAGCCA
TCTCAAGTGTTTCCTCAACGAGGGTCCGTGTGTCCAGCAGTCCAGGGATTTGAAGAGA---GT
CATCCCGGTGATCGCCAACAACAGCTGTAACGGATGCACAGAAAAGCAAAAGACCACCATCA
AGAAGACGCTGAACTTCTTGAGGACGAAGAAGCCCGACGAATGGGCTAGACTCGTCAAGATC
TACGACCCGACCGGCACCAAGTTGAACAAATTCCTCGACGCGTAA----------

CSP5Agos

----------ATGCACTGCAAGGTTTTGATCGCTCTTTGCTGCGTGGCCGTGTACGC
CGTGCAGGCT----------AGTCCTGCCG
GTACGGCCACCGCCGCCGCTGTT----------

----------TCCGCCGAC
GACGAGATCAAGGATTTCCCGGCCTACATGAAGCGGTTCGACAAG----------
----------CTCAACGTCGAACAGGTGCTGAACAACGACCGCGTCCTGGCCAG
CCATCTCAAGTGTTTCCTCAACGAGGGACCGTGCGTCCAGCAATCCAGGGACCTGAAGAGA--
-GTCATCCCAGTGATCGCTAACAACGGCTGTAACGGATGCACTGAAAGGCAAATGACCACCA
TCAAGAAGTCGCTGAACTTCCTGAGGACGAAAAAGCCAACGGAATGGGCAAGACTCGTCAAG
ATCTACGACCCATCAGGCACCAAGTTGAACAAGTTCCTCGACGCGTAA----------

CSP8Apisum

ATGACGAATAATAATATGAACTGCCCGCGGAGCCGACCAGAAATATTTTCATTGCTGGCAGT
GACGACGATCGCCGCCGTGCTCGTC----------CATCAGCCCG
CGAAAGTCTACTGTGCCGACGGTACAATTTATCCTTCGCAACAGCAGCAACAG----------

---

----------CAAACGATGATGTTCACCGCACCATCGGGCTACTACTTGTC
CACGTACGATAAC----------TTAGACGTGGGCCA
TTTACTGCGAAACAAAAAGGTTGTGTCGGGCTTCGTCAAGTGTTTCGTCAACGAGGGACCTT
GCACGCCGGACGGCAAACTAGTCAAAGCCTACCTGTTACCTGAGATAATACGTACCGTGTGT
GGAAAGTGCACGCCAAGGCAAAAGGACATGTCCCGGGCAGTGCTAAGACACCTTTACACGTA
CAGGCGAGCGGATTTCGACAAGATCATGCAAATTTACGACACGGATAACAAG---AAAAATGA
AATCATAAATTTCATGAACCAAAAATAG----------

CSP8Agos

ATGAACAATATTATAATGAACAATTCGCGGGGCCGTTATGGAATTTTTTCGCTGTTAGCCG
TTACGATTGCTGCCATCATGCTCGTC----------CATCAGCCT
GCGACCGTCCGCTGCGCCGACGGCGGAATCATAACACCGCAACAGCAGCAGCAG----------

---

----------CAGACGATGATGTTCACCGCGCCAACGGGCTACTACGTGT
CCACGTACGATCAC----------ATAGACGTCGGCC
GATTGTTGCGAAACAACAAGGTGGTGTCGGGCTACGTCAAATGTTTCGTCAACGAAGGACCC
TGCACGCCGGACGGCAAATTAGTCAAAGCCTACTTGTTGCCCGAGATCATACGTACCGTGTG
TGGAAAGTGCACGCCAAGGCAAAAAGACATGGCACGGATGGTACTCAAACACATTTACACGT
ACAGGCAAGCGGATTTCGAAAAAATCATGCAGATTTACGACACGGATGGCAAA---AGAAACG
AGATTCTAGCTTTCATGAACCATTAG----------

CSP10Apisum

----------ATGGTCTCAAAGCCGTTTGTTTTCGTGTTTGTGCTCATGTCCGTGGTCGGTG
TGTCGTTCTCCGTGACC----------GAAGAAA
ACGATGGTACAAAGGTCGTGGACAAGGAAGAGGAC----------

------CATCATCAGAACATCCAAGAGGAACTCAAGAAATTTTTGTCGACGTTAGAGAAA----------
----------ATCGACATAGACCAGATATTGAACAACCACAG
GTTGATGTCGAACAACGTCAAATGTTTTCTGAACGAAGGACCGTGTACGGCACAACTGAGGG
AAATGAAAAAA---ATGCTACCTGCGCTAGTCAAAGATAGTTGCGCGTCATGTACCAAAGAGC
AAAAGAACATTATCAAAAAATCCATGGAGGCAATTCAAGCTAGACGTCCAAATGAGTACAAA
CAAGTTAGCAAATTTTTTGACCCGGAGGGAAAATACCAGAAGAAATTCTTAGAAAACCTAAA
CACAGATTAG----------

CSP10Agos

----------ATGATCAACACTCGACCTCGGAAATTGGTCAGGTGCATAAGAGGCGTTTC
AATCTCCGTGGCT----------AAAGGAGACGA
TGCTGTAAATGCCGAAAATAAAGACGATGAC----------

-TCTCATCTGGTCAACCGAGAAGAGATTCAAAGATATATGTCAATGATGGAGAAA----------
----------ATCAACATAGACCAAATGCTGAACAACACTAGAT
TGATGTCGAACAACGTAAAATGTTTTCTGAATGAAGGACCGTGCACGGCTCATCTCAGAGAA
ATGAAAAAA---ATGGTACCTATGTTAGTCAAAGATAGTTGCTCGTCATGTACCAAAGAGCAA
AAGATTATGATGAAAAAAGCTATGGATGCGGTTAAAGCTCGTCGTCCCAATGATTACGAAA
AACTTTCCAAATTTTTCGATCCAGAAGGAAAATATGAGAAGAAATTCTTAGAAAACTTAAA
CGAATCGAAATAA----------

CSP2Apisum

----------ATGGCTCATCTTAACTTATTTGTTGTTCTGGTCGCGTCGCTTGTGTGC
TTCACGTTGGCC----------

----------GAAGAAAA
ATACACAACTAAATTCGACAAC----------TTCGA
CGTTGACAAGGTGTTGAACAACGACAGAATTTTAACCAGTTACATCAAGTGTTTGCTGGACC

AGGGAAATTGCACCAATGAAGGCCGAGAATTGAAGAAA---GTTTTACCAGACGCATTGAAAA
CTGACTGCAGCAAATGTACAGCTGTACAAAAAGACAGATCAGAAAAAGTAATCAAGTTTTTG
ATCAAAAACCGTTCTAAAGACTTTGATAATTTGACCGCCAAATACGACCCATCAGGCGAATA
CAAAAAGAAGATCGAAAAATTCGACGCGGAAAGGGCTGCGGCTGCTAAACATTAA----------
----

CSP2Agos

----------ATGGCGCATCTTAACTTATTTGTCGTCTTGATCGCATCGCTGATTTAC
TTTACTTCAGCA----------

----------GCGGAAGAAAAG
TACACAACCAAATTTGACAAC----------TTTGAC
GTCGACAAAGTGTTGAACAATAACAGAATTTTAACCAGCTACATTAAGTGTTTGCTTGACG
AAGGAAATTGTACCAACGAAGGCCGAGAATTGAAGAGA---GTTTTACCAGATGCATTAAAAA
CTGATTGCAGCAAATGTACAGATGTACAAAAAGATAGATCAGAAAAAGTGATCAAGTTTTT
GATTAAGAACCGTTCTACTGACTTTGATCGTCTGACTGCCAAATACGACCCAACCGGAGAAT
ATAAGAAGAACCTCGAAAAATTCGAAAAAGAAAGGGCTTCAGCTAAACCTCTTAAAGCTTAA

CSP3Apisum

----------ATGGTGCATCTTAACTTATTTGTTGTTGTGGTCGCGTCACTGGTGTGC
TTCACATTGGCT----------

----------CAAGAAAAGT
ACTCAACTAAATACGAAAAC----------TTTGACG
TGGACAAGGTGTTAAACAACGACAGCCTTTTAACCAGCTACATCAATTGTTTGCTCGACGAG
GGAAATTGTACCGAGGAAGGCCAAGCGTTGAAAAGA---ATTTTACCAGACGCATTAAAAACT
AACTGTGGAAAATGTACGGATGCACAAAAATTGAAAATAGAAAAAATAATGAAATTTTTGA
TCAAGAACCGTTCTATTGACTTTGATCGTTTGACCGCCAAATACGACCCATCGGGAGAATAC
AAGAAAAAGCTCGAAAAATTCTCCGCTTAA----------

CSP9Apisum

---ATGTCATCGTTTTGCCTGAACTCCGTGATCTTAATGACCGTGACCACGGTC
ATGGTGGCGCTCGTC---ACATTCACCGGGTGCACAGCCTCTGA
TGACAGACCGGGCATGAGTGACATTCGTCTGGCTAAGAGGGACGCCGAC---

---CACAAAGAAAACAACGCCGACAGCGAGGAAGGGTTTTTTTTTAGT
TTAACAAATTTTTTCGGACGCAGTAAACACGACGAAGATGACGAT---AAACCGGATTT
CATCACCACTTTCGACTTAATCCGGCTTTTGGACGAGAAATACGCTATGAAGCAATTCTAT--
-TGCGTCATAAATGAGGACCCGTGTGACTCCGTCGGAATGAGGCTCAAAGCC---ACTATACCC
GAAGAAATAAACAGAGATTGCGAACGCTGTACCGCAACAGAGACCAACAATATCCGACGAAT
TTTAAATTACGTGAAGAAACACTATCCAAAATTTTGGGAACGTGTTGAACCAATTTAT---
---AGAGATAAAATGACTGTTTTAACTGCTAATATCGTTAAAATACAATAA---

CSP9Agos

---ATGTCAGCGTTTTGCCTGAACTCCTTTATCTTAATGACCATGATCACAGTT
ATAGTGACACACGCC---ACATTCACCAGGAGCACCAAA
TTTGATGACAGAACGGGCATTGACATTCATTTGGTCAAAAGGGAT---

---ACCGACGACGTCAACGATGACGAAAATAGTGTCGAAAGCGATGAAGGTTTTTTTT
ATAGATTTACACATTTTTTCCAAGATAGCAGCGATAAAGAAGATGACGATGATGACGAAAA
AAAACCGGATTTTATTACCACTTTCGACATATTTAAACTTTTGGACGAGGAATATGCTATG
CAACAGTTCTAT---TGCGTTATAAACGAGGACCCGTGTGATGAAGTTGGAATGAGGCTCAAA
GCT---ACTATACCCGAAGAAATAAACAGGAACTGCGAACGCTGTACCTCAACAGAAAGAAAC
AATATTCGACGAATTTTGAATTACGTGAAAAAACACTATCCACAATTTTGGAAACGTGTCG
AACCAATTTAT---AAAAAAAAAATATGA---

CSP4Apisum

---ATGGATTCACGAATTGCAGTAGTCTGTGTTGTTTTGGCGGTGTTC
GCGGTGGAC---CAG

ACGGTCGGAGCGCCG----------

----------CAAAAAGATGCTGTAGC
CGCAAGTGGTACCGCCTACACTACCAAGTACGACCAT----------
----------ATTGATATTGACCAAGTTCTGGCTTCCAAAAGATTAGTCAATAGCTATGTCC
AATGTCTGTTGGACAAGAAACCGTGCACACCCGAAGGAGCTGAACTTAGAAAA---ATTTTAC
CTGATGCTTTAAAAACACAATGTGCGAAATGCAATGCCACACAAAAAAATGCTGCACTTAAA
GTAGTTGACAGACTCCAGAGAGATTATGATAAAGAGTGGAAACAGCTTCTTGACAAATGGG
ACCCTAAACGTGAACAATTCCAGAAATTCCAACAATTTTTGGCAGAAGAGAAGAAAAAAGGG
GTTGTTAAATTTTAA----------

CSP4Agos

----------ATGGATTCCAGAATTGCAGTAGTCTGTGTTGTTTTGGCTGCGTTC
GCAGTGGAC----------CAA
ACGGTCGGAGCGCCA----------

----------CAAAAAGATGCCGTAGC
CGCAAGCGGTCCCGCTTACACTACCAAATACGACCAC----------
----------ATTGATGTTGATCAAGTTCTGGCTTCCAAAAGATTAGTAAATAGCTACGTTC
AATGTCTGTTGGACAAAAAACCATGCACTCCCGAAGGAGCTGAATTGAGAAAA---ATTTTAC
CCGATGCCTTAAAAACACAATGTGCGAAATGCAATGCCACCCAAAAGAATGCTGCTCTTAAA
GTTGTTGACAGACTACAGAAAGATTATGATGCAGAGTGGAAACAGCTTCTTGACAAATGGG
ACCCAAAACGTGAACATTTCCAAAAATTCCAACAATTTTTAGCGGAAGAAAAGAAAAAGGGT
TTTACTAAATTTTAA----------

CSP6Apisum

----------ATGAACAAACTCCTTCTAGCAGTTGCTTTTTTCATCGCTACCACGATG
ACTATGGTCCAG----------

----------GCAGCACCGGCCAA
ATATACTACTAAATACGACAAC----------GTAAA
TATCGATGACATTCTGAACAACGACCGCTTGGTCGCCAGCTACTTCAAGTGTCTGATGGAAA
CAGGAAAATGTACGCCAGAAGGCGAAGAAATCAAACGT---TGGTTACCAGAAGCAGTAGAGA
ATAAATGTGAAGACTGTTCGGAAAAACAAAAAATAGGTTCCGAAAAAATCATTAAGTTTCT

ATTTGAAAAGAAAAATGATATGTGGAAACAACTTGAAGAAAAATATGACCCTAAAGGACTA
TACAGACAACGTTATTCGGAAGATGCAAAGAAATTGAACATTGACGTTTAA----------
----

CSP6Agos

----------ATGATCAAACTCATTCTAGCAATTGCTTTTTGCGTCTCTATAACTATG
ACTGTGGTCCAG----------

----------ACAGCCCCGGCT
AAATATACTACTAAATATGATAAT----------GTA
AATATCGATGAAATTCTGAACAATGACCGTTTGGTCGCCAGCTACTTCAAGTGTCTGATGGA
AACGGGAAAATGTACACCAGAAGGCGAAGAAATTAAACGG---TGGTTACCAGAAGCAATAGA
GAATAAATGTGAAGACTGTTCTGAAAAACAAAAATTGGGTTCTGAAAAAATCATTAAGTTT
TTATTTGAAAAGAAAAATGATATGTGGAAACAACTTGAAGCAAAATACGATCCTCAAGGAA
CATACAGACAACGTTATGCGGAAGAAGCAAAAAAATTGAACATTAATGTTTAA----------
----

CSP7Apisum

---------ATGGCTCGATCGTCGTCAAGTGTCACCATGAAGGTTTTCGTAATGGCCGTATGCGTG
TGCGCCGCCCTCGCCCGTCCG----------GAAGAAGTGAAAATGGAAAACAAACC
GACTGTGGTCAAATCTGAGACCTTGGCAGCACCACTTCCGACGACCATAGTGAAACGAGCT--
----

----------ACTCCTCAGGTGGTCTCTACCCAAAAAGACTCT
TCGCTTCCGAACGTCAGCGAG----------GACGTA
CTCGACAAGGCGCTCAGCGACAGGAGGTTCGTGCAAAGGCAACTCAAGTGCGCTACCGGCGA
AGGACCGTGCGACCCCATTGGCCGAAAAATTAAAGCT---CATGCACCGTTAGTGTTGAGAGG
AATGTGCGTCAAGTGTTCGCAGTCGGAAATCAAACAGATTCAACGTGTCATGTCACATATAC
AGAAGAATTATCCGAAGGAGTACACTAAGATGCTGAAACAGTAC----------CAGAGCGGT
TTCTAA----------

CSP7Agos

---------ATGTCTCGATCGTCGTCAAGTGTCACCATGAAAGTATTTGTAATCGCCATATGCGTG
TGCGCCGCTCTAGCCCGCCCG----------GAAGATGTGAAAGTGGAAAACAAACC
AGCTGTGATCAAATCTGAAACCTTAGCGGCACCACTTCCGACAAATATTGTAAAACGAGCT--
---

----------ACAGATACAATTCAGCTAGACTCATCGC
TCCCGAATGTCAGCGAA----------GACGTACTCG
ATAAAGCGCTTAGCGACAGGAGATTTGTGCAAAGGCAACTCAAGTGCGCTACAGGCGAAGGA
CCGTGTGATCCTATTGGGCGAAAAATTAAAGCT---CACGCACCGCTAGTGTTGAGAGGAATG
TGCGTCAAGTGTTCACAGTCGGAAATCAAACAGATTCAACGTGTCATGTCCCATATTCAGAA
GAATTATCCCAAGGAGTACACAAAGATGCTGAAACAGTAC----------CAGAGCGGATTTT
AA----------

Mper13164.0.v1.0.000025400.1

----------ATGAATTTGTTAGCGGTTTTTTGTTATATAACCATGATGTGTG
ACTCTCAATTATTTAAGCGACTTGAGCAGCCGGCTGCGATATCTCAAGTCAAGCGAATTGAG
CAACCAGCTATGATCGCAAATCGGATCGGACAGCCTACGGTCGCACCTAGGTTCGGACAACC
AACGATCGCACCCCGGTTCGGACTGCCTACGATCGCACCTCAGGTCGGACAGGCTGCGATCA
CACCTCAGGTCGGACAGGCTGCGATTGCATCTCGGTTTGGACTGCCTACGGTCGCACCTCAG
GTCGGACAGGCTGCGATCACACCTCAGGTCGGACAGGCTGCGATTGCATCTCGGTTTGGACT
GCCTACGGTCGCACCTCAGGTCGGACAGGCTGCGACCACACCTCAGGTCGGACAGGCTGCGA
TCGCATCTCGGATTGGACAAAATTTTCAGAATGCGAACAACAGCGTGTCACCAACCACCGAT
GGGCGTAAAACCACCAGAGAAACGTCGTCATATCCAACTCGATACGACTTC----------
----------ATTGATATTGAAGCTGTTATGAACAATGAACGAATTAT
TAAAATTCTTTTCAACTGTGTTATGAACCAAGGACCGTGTACCAGAGAAGGTTTGGAGCTT
AAAAGG---ATTGTTCCGGATGCAATACAGACAGAATGTGCTAAATGTAATGAAAGACAAAGA
AAACAAGCAGGCAAAGTTTTGGCTCATTTACTACAGTACAAACCAGAATACTGGAACATGTT
GGTGAAAAAATTTGATCCAAATAATGTATATTTGAAAAAGTATATGGCAGACAATGATGAT
GACGAGAAGGTATCTCTTCAAAAACTCACCAACGATACAACCAAATAA----------

Mper13164.0.v1.0.000099700.1

----------ATGGCTCATCTTAACTTATTTGTCGTTTTGGTCGCTTCGCTGGTGTGC
TTCACACTGGCA----------

----------GAAGAAAAGT
ACACAACTAAATTCGATAAC----------TTTGACG
TGGACAAAGTGTTGAACAATAACAGAATTTTAACCAGCTACATCAAATGCTTGCTGGACGAG
GGAAATTGCACCAACGAAGGCCGAGAATTGAGGAAA---GTTTTACCAGACGCATTAAAAACT
GACTGCAGCAAATGTACAGAAGTACAAAAAGACAGATCAGAAAAAGTGATTAAGTTTTTAA
TCAAGAACCGTTCTACTGACTTTGATCGTTTGACCGCCAAATACGACCCATCGGGCGAATAC
AAGAAGAAGATCGAAAAATTCGACTCTGAAAAGGCTGCAGCTGCTAAACATTAA----------
----

Mper13164.0.v1.0.000025410.1

----------ATGTTCAAGCTGGTCTCCATTGCGATTATTTCGGGTTTCGTATCA
ATGGTGCAG----------TGTGGTCC
AATGCCAGAACAACGAATAGAC----------

----------GGCGGACA
GAATCGCAGGCAAGAACAACAAAAATTTGCA------GTTGACCAC----------
----------TTTACGACTGGA---CTGGTGGGGAATCCTAAAATTCGTCAAAATTA
CCTCAATTGCTTTTTGGATAATGGACCGTGCAGTCCGGAAGCTAAAAATATTAAACCAGGAA
TGGTTCCCGATGCTATCCAAAATGATTGCGCACATTGTACCGAATTACAAAGAAAAGTGATT
GAAAAAATGATGTGCTACCTGAATAACCACCAACCGGATATATTAAAAGAAGTAGCTGCCAA
ATTCGATCCAAATGGAGAATATATGAAGCATTATATTAATACCATAGAGCGAAATGGAAAT
GAACAGTTTTTGAACCCAAATCTAAACCAATACCAACCACAACAAAATTCAAATCAACCACA
ACAAAATCCAAACCAACCACAACAGAACCCAAATCAACCTCAGCCACATCAAAACCAATACC
AACCACAACAAAATCAACATCAACCACAGCCTAATCCAAACCAAGCACAGCCTAATCCAAAC
CAACTACAGCCTAATCAAAACCAACCACAGCATCCAAATCAACCTCAGCAACATCAACATCA
ACACCAACCGCAACAAAATCCAAATCAGCCACAGCAACATCAACACCAAAACCAACATCAAC
CACAACAAAATCAATACCAACCACAACAACATCAACACCAACCACAACAATATCAATACCAA
CCACAACAACATCAACACGAACAACAATTAAAAGGAACGGTAGTAACAACGGCACCTATATC
TACGGCAACCCGGAAACCTTGA

Mper13164.0.v1.0.000049890.1

----------ATGAACTGCAAGGTCTTGATCGCTCTGTGCTGCGTGGCCGTGTACGC
CGCGCACGCC----------AGTCCCGCCG
GTGCGGCCACAGCCGCCGCCGCA----------

----------TCCGCCGAC

GAAGAGATCAAGGATTTCCCGGCCTACATGAAGCGGTTCGATAAG----------
----------CTCAACGTGGAACAGGTTCTGAACAACGACCGCGTCCTGGCCAG
CCATCTCAAGTGTTTCCTCAACGAGGGCCCGTGCGTCCAGCAGTCCAGGGACTTGAAGAGA--
-GTCATCCCGGTGATCGCCAACAACGGCTGCAACGGATGCACCGAAAGACAGATGACCACCAT
CAAGAAGTCGCTGAACTTCTTGAGGACGAAGAAGCCAGTCGAATGGGCGAGACTCGTCAAGA
TCTACGACCCGTCCGGCACCAAGTTGAACAAATTCCTCGACGCGTAA----------

Mper13164.0.v1.0.000025420.1

----------ATGAACACACTTCTCCTAGCAGTTGCTCTTTGCATCGCCATCACGATG
ACTGTGGTCCAG----------

----------ACAGCACCTGCTAA
ATATACTACTAAGTACGACAAC----------GTAAA
TATCGATGACATTCTGAACAACGACCGCTTGGTCGCCAGCTACTTTAAGTGTCTGATGGAAA
CCGGAAAATGTACACCAGAAGGCGAAGAAATTAAACGG---TGGTTACCAGAAGCAATAGAGA
ATAAATGTGAAAACTGTTCGGAAAAACAAAAAATTGGTTCCGAAAAAATTATTAAGTTTCT
AATTGAAAAGAAAAATGATATGTGGAAACAACTTGAACAAAAATATGACCCCCAAGGACTCT
ACAAACAACGTTATTCGGAAGAGGCAAAGAAATTGAACCTTGATGTTTAA----------

Mper13164.0.v1.0.000067300.1

---------ATGGATCGATCGTCGTCAAGTGTCACCATGAAAGTATTCGTAATCGCCGTGTGCGTG
TGCGCCGCACTCGCCCGTCCG----------GAAGACTCGAAAGTGGAAAACAAACC
GGCTGCGGTCAAATCGGAGACCTTGGCCGCACCACTTCCGACGACCATAGTGAAACGGGCT--
----

----------ACTCCTCAGGTCGTCTCAACCCAGCAAGGCGCA
TCGCTTCCGAACGTCAGCGAG----------GACGTA
CTTGATAAGGCGCTCAGTGACAGGAGGTTCGTGCTAAGGCAACTCAAGTGTGCTACGGGCGA
AGGACCGTGCGACCCCATTGGTCGAAAAATTAAAGCT---CATGCACCGCTAGTGTTGAGAGG
AATGTGCGTCAAGTGCTCGCAGTCGGAAATCAAACAGATTCAACGTGTCATGTCACATATAC
AGAAGAATTATCCCAAGGAGTACACTATGATGCTCAAACAGTAC----------CAGAGCGGA
TTCTAA----------

Mper13164.0.v1.0.000049900.1

ATGACGAATAATAATATGAACAGTCCGCGGTGCCGTCCCGAAATCTTCTCGCTGCTGGCCGT
CGCGGCGATCGCCACCGTGCTCGTC----------CATCAGCCAT
CGACCGTCCACTGCGCCGACGCCGGAGTTTATCCTCCGCAGCAACAGCAACAG----------

---

----------GAGGCGACGATGTTCACCGCTCCGTCGGGCTACTACGTGTC
CACGTACGACCAC----------ATGGACGTGGGCCG
ATTGCTGAGAAACAACAAGGTGGTTGCGGGCTTCGTCAAGTGTTTCACCAACGAAGGACCTT
GCACGCCGGAAGGCAGACTAGCAAAAGCCTACCTGTTGCCCGAGATCATACGTACCGTGTGC
GGAAAGTGCACCCCGAGGCAAAAGGACATGGCGCGGTTAGTGATAAGACACATTTACACGTA
CAGGCGAGGGGATTTCGATAAGATCATGCAGATTTACGACACGGATGGCAAG---AAAAACGA
GATCATCGATTTCATGAACCAAAAATAG----------

Mper13164.0.v1.0.000099690.1

----------ATGACGTCGTTTTGTCTGAACTCTGTGATCTTGATGACCATTACCACGGTT
ATAGTGGCGCACGCC----------GCATCCACCGGGATGACCGCC
TTCAATAACAGATCGGGCAGTGATATACATATGGCCCAAAGAGAC----------

----------TACAACGAAAACAAAGCTGATAAAGCTGAAGGTTTTTTTTTTACTATC
ACAAATTTTTTCAGTCGCAGAAAACACGATGACGATAAACCGGATTTCATTACCACT----------
--------TTCGACATAATCAGGCTTTTGGACGAGAAATACGCTATGAAACAATTCTAT---TGCGT
CATCAACAAGGAGCCTTGTGACGCCACCGGATTGAGGCTCAAAGCT---ACTATACCCGAAGA
AATAAACAATGATTGTGAGCGCTGTACAGCAACAGAGACCAGCAACATCCGACGAATTTTGA
ATTACGTGAAAAAACACTATCCAGAATTTTGGGATCGTGTCGAACCAATTTAT----------
-AGAAATAATATGACAGCTTAA----------

Mper13164.0.v1.0.000067280.1

----------ATGGTCTCAAAACTGTTTGTTTCCGTGTTCGTGCTCATGTCCGTGGTGGGTG
TGTCGTACTCCGTGACC----------GAAGGA
GATGATGATGCCGCAAAGGTGGCTGACAAGGAC----------

----CTTCATCCTGTCAACCAGGAGGAGCTCAAAAAATTTTTGTCGATGATGGAGAAA----------
----------GTCGACATCGACCAAATACTGAACAACAACAGG
TTGATGTCGAACAACGTCAAATGCTTTCTGAATGAAGGACCGTGCACGGGCCAACTCAGGGA
AATGAAAAAA---ATGGTACCTATGCTAGTCAAAGATAGTTGTTCATCGTGTAATAAAGAACA
AAAGAACATGATGAAAAAAGCTATGGACGCGATGAAAGCTCGACGTCCAAATGAGTACGAAC
AAATTTCCAAATTTTTTGATCCAGAGGGAAAATACGAAAAGAAATTTTTAGAGAACTTAAA
CGAATCGAAATAA----------

Mper13164.0.v1.0.000025390.1

---------ATGTTCAGTACACAATCGCAAGCCATGGATTCACGAATTGCAGTAGTCTGTGTTGTT
TTGGCAGTGTTCGCGGTGGAC----------
----------CAGACGGTCGGAGCGCCG----------

----------CAAAA
AGATGCTGTGGCAGCAAGCGGTCCCGCCTACACCACAAAATACGACCAT----------
----------ATTGACATCGACCAAGTTTTGGGTTCCAAAAGATTAGTAA
ACAGCTACGTTCAATGTCTGCTGGACAAGAAACCGTGCACTCCCGAAGGAGCTGAACTTAGA
AAA---ATTTTACCTGATGCCTTAAAAACCCAATGTGTGAAATGCAATGCTACGCAAAAAAAT
GCTGCTCTTAAAGTAGTTGACCGACTCCAGAGAGATTATGATAAAGAATGGAAACAGCTTCT
TGACAAATGGGACCCTAAGCGTGAATATTTCCAGAAATTCCAACAATTTTTGGCAGAAGAGA
AGAAAAAAGGTGTTGTCAAATTTTAA----------

Appendix E

All NBS-LRR-aphid effector and effector-effector interactions found in Michelmore yeast two-hybrid screens

	Ap1	Ap2	Mp1	Mp10	Mp11	Mp14	Mp15	Mp17	Mp23	Mp32	Mp36	Mp41	Mp43.1	Mp44.1	Mp44.3	Mp47	Mp51	Mp53	Mp6
Mp3																			
Mp5																			
Mp10									x										
Mp15									x										
Mp17	x		x		x	x		y	x				x	x	x			x	
Mp19	x	x						x	x	x		x						x	
Mp21								x	x										
Mp22								x											
Mp23																			
Mp29											x						x		
Mp35	x									x									
Mp39		x						x											
Mp43.6				x				x	x			x					x		
Mp44.1								x						y					
Mp44.3								y	y					y	x				
Mp46																z			
Mp47									x			x	x						
Mp50									x				x						
Mp54								x											

Table showing all effector-effector interactions identified in the Michelmore screen.
Light blue (x) indicates an interaction whereas dark blue (y) indicates strong interaction. Only clones that were found to have an interaction are shown. Red (z) is an inconsistent interaction.
Candidate effectors along the top were tested as bait (pLAW10) and effectors along the side were tested as prey (pLAW11).

Gene	RPS5 (At1g12220)	RPS5 (At1g12220)	RPS5 (At1g12220)	SUMM2 (At1g12280)	SUMM2 (At1g12280)	At1g56520	At1g59218	At1g61190	At1g63350	At1g63360	At1g63860	At1g63880	At2g16870	At3g14470	At3g15700	At4g19060	At5g18360	At5g40100	At5g43740	RPS4 (At5g45250)	At5g45440	At5g45490	At5g46270	At5g46490	At5g46510	At5g46520	
Type	CC	CC	CC	CC	CC	TIR	CC	CC	CC	CC	TIR	TIR	TIR	NB-ARC	NB-ARC	NB-ARC	TIR	TIR	CC	TIR	NB-ARC	NB-ARC	TIR	TIR	TIR	TIR	
Domain interacted	CC	TIR + NBS	LRR	CC	LRR	TIR	CC	CC	TIR + NBS	LRR	TIR	TIR+NBS	TIR	NB-ARC	NB-ARC	NB-ARC	TIR	TIR	CC	TIR	NB-ARC	NB-ARC	TIR	TIR	TIR	TIR	
Mp2																										x	Mp2
Mp3						x							x	x													Mp3
Mp6.6						x							x				x							x	x	x	Mp6.6
Mp11																								x			Mp11
Mp14						x											x							x		x	Mp14
Mp19	x	x	x		y	x			y	x	x	x	x	x						y	x	x	x	x	x	x	Mp19
Mp20						x								x											x	x	Mp20
Mp21	x					x					x		x	x					x	y	x			x	x	x	Mp21
Mp22																											Mp22
Mp23																											Mp23
Mp24											x		x											x			Mp24
Mp28						x																					Mp28
Mp29						x					x		x										x	x	x	x	Mp29
Mp33.3																										x	Mp33.3
Mp33.2						x							x													x	Mp33.2
Mp35.9																											Mp35.9
Mp39			x			x								y		x			x	x	x						Mp39
Mp42						x					x		x	x								x		x	x	x	Mp42
Mp43.6											x		x	x			x						x	x	x	x	Mp43.6
Mp44.1						x					x	y	x	x			x			y			x	x	x	x	Mp44.1
Mp44.3						x						y	x							y							Mp44.3
Mp46						x			x																		Mp46
Mp47	x	x	x	x	x		x	x	x	x	x	x	y	y	x	x	x	x	x	x	x	x		x		y	Mp47
Mp49				x		x					x		x	x								x				x	Mp49
Mp50																											Mp50
Mp53														x											x	x	Mp53
Mp54					x	x					x		x	x						x			x	x	x	x	Mp54
Gene	RPS5 (At1g12220)	RPS5 (At1g12220)	RPS5 (At1g12220)	SUMM2 (At1g12280)	SUMM2 (At1g12280)	At1g56520	At1g59218	At1g61190	At1g63350	At1g63360	At1g63860	At1g63880	At2g16870	At3g14470	At3g15700	At4g19060	At5g18360	At5g40100	At5g43740	RPS4 (At5g45250)	At5g45440	At5g45490	At5g46270	At5g46490	At5g46510	At5g46520	

Table showing all effector-R-protein interactions identified in the Michelmore screen.
Light blue (x) indicates an interaction whereas dark blue (y) indicates strong interaction. Only clones that were found to have an interaction are shown.
Candidate effectors were tested as prey (pLAW11) and NBS-LRR fragments as bait (pLAW10)

Bibliography

[1] C. Dedryver, A. Le Ralec, and F. Fabre, "The conflicting relationships between aphids and men: A review of aphid damage and control strategies," *Comptes Rendus Biologies*, vol. 333, no. 6, pp. 539–553, 2010.

[2] A. Dixon, P. Kindlmann, J. Leps, and J. Holman, "Why there are so few species of aphids, especially in the tropics," *American Naturalist*, pp. 580–592, 1987.

[3] CABI, "*Myzus persicae*," *Invasive Species Compendium*, 2015.

[4] F. Legeai, S. Shigenobu, J.-P. Gauthier, J. Colbourne, C. Rispe, O. Collin, S. Richards, A. C. Wilson, T. Murphy, and D. Tagu, "AphidBase: a centralized bioinformatic resource for annotation of the pea aphid genome," *Insect Molecular Biology*, vol. 19, no. s2, pp. 5–12, 2010.

[5] I. A. G. Consortium, "Genome sequence of the pea aphid *Acyrthosiphon pisum*," *PLoS Biology*, vol. 8, no. 2, p. e1000313, 2010.

[6] R. L. Blackman and V. F. Eastop, *Aphids on the world's crops. An identification and information guide*. John Wiley & Sons, 2000.

[7] F. L. Goggin, "Plant–aphid interactions: molecular and ecological perspectives," *Current Opinion in Plant Biology*, vol. 10, no. 4, pp. 399–408, 2007.

[8] A. Dixon, "Parthenogenetic reproduction and the rate of increase in aphids," *Aphids: Their Biology, Natural Enemies, and Control*, pp. 269–287, 1987.

[9] L. Taylor, "Synoptic dynamics, migration and the Rothamsted insect survey: Presidential address to the British Ecological Society, December 1984," *The Journal of Animal Ecology*, pp. 1–38, 1986.

[10] A. Shingleton, G. Sisk, and D. Stern, "Diapause in the pea aphid (*Acyrthosiphon pisum*) is a slowing but not a cessation of development," *BMC Developmental Biology*, vol. 3, no. 1, p. 7, 2003.

[11] B. Fenton, J. Woodford, and G. Malloch, "Analysis of clonal diversity of the peach–potato aphid, *Myzus persicae* (Sulzer), in Scotland, UK and evidence for the existence of a predominant clone," *Molecular Ecology*, vol. 7, no. 11, pp. 1475–1487, 1998.

[12] C. Taylor and W. Robertson, "Electron microscopy evidence for the association of Tobacco Severe Etch Virus with the maxillae in *Myzus persicae* (Sulzer)," *Journal of Phytopathology*, vol. 80, no. 3, pp. 257–266, 1974.

[13] A. Forbes, "The stylets of the green peach aphid, *Myzus persicae* (Homoptera: Aphididae)," *The Canadian Entomologist*, vol. 101, no. 1, pp. 31–41, 1969.

[14] M. Uzest, D. Gargani, A. Dombrovsky, C. Cazevieille, D. Cot, and S. Blanc, "The acrostyle: a newly described anatomical structure in aphid stylets," *Arthropod Structure & Development*, vol. 39, no. 4, pp. 221–229, 2010.

[15] M. Uzest, D. Gargani, M. Drucker, E. Hébrard, E. Garzo, T. Candresse, A. Fereres, and S. Blanc, "A protein key to plant virus transmission at the tip of the insect vector stylet," *Proceedings of the National Academy of Sciences*, vol. 104, no. 46, pp. 17959–17964, 2007.

[16] A. Douglas, "Phloem-sap feeding by animals: problems and solutions," *Journal of Experimental Botany*, vol. 57, no. 4, pp. 747–754, 2006.

[17] A. Douglas, "Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*," *Annual Review of Entomology*, vol. 43, no. 1, pp. 17–37, 1998.

[18] T. Tsuchida, R. Koga, A. Fujiwara, and T. Fukatsu, "Phenotypic effect of *Candidatus Rickettsiella viridis*, a facultative symbiont of the pea aphid (*Acyrthosiphon pisum*), and its interaction with a coexisting symbiont," *Applied and Environmental Microbiology*, vol. 80, no. 2, pp. 525–533, 2014.

[19] K. M. Oliver, P. H. Degnan, G. R. Burke, and N. A. Moran, "Facultative symbionts in aphids and the horizontal transfer of ecologically important traits," *Annual Review of Entomology*, vol. 55, pp. 247–266, 2010.

[20] J. Ferrari, C. L. Scarborough, and H. C. J. Godfray, "Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids," *Oecologia*, vol. 153, no. 2, pp. 323–329, 2007.

[21] W. Tjallingii, "Salivary secretions by aphids interacting with proteins of phloem wound responses," *Journal of Experimental Botany*, vol. 57, no. 4, pp. 739–745, 2006.

[22] W. Tjallingii and T. Esch, "Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals," *Physiological Entomology*, vol. 18, no. 3, pp. 317–328, 1993.

[23] A. Hewer, T. Will, and A. J. van Bel, "Plant cues for aphid navigation in vascular tissues," *The Journal of Experimental Biology*, vol. 213, no. 23, pp. 4030–4042, 2010.

[24] S. Hogenhout and J. Bos, "Effector proteins that modulate plant-insect interactions," *Current Opinion in Plant Biology*, vol. 14, no. 4, pp. 422–428, 2011.

[25] W. Tjallingii, "Regulation of phloem sap feeding by aphids," in *Regulatory mechanisms in insect feeding*, pp. 190–209, Springer, 1995.

[26] J. K. Morgan, G. A. Luzio, E.-D. Ammar, W. B. Hunter, D. G. Hall, and R. G. Shatters Jr, "Formation of stylet sheaths in āere (in air) from eight species of phytophagous hemipterans from six families (Suborders: Auchenorrhyncha and Sternorrhyncha)," *PloS One*, vol. 8, no. 4, p. e62444, 2013.

[27] M. Knoblauch, W. S. Peters, K. Ehlers, and A. J. van Bel, "Reversible calcium-regulated stopcocks in legume sieve tubes," *The Plant Cell*, vol. 13, no. 5, pp. 1221–1230, 2001.

[28] E. Prado and W. Tjallingii, "Aphid activities during sieve element punctures," *Entomologia Experimentalis et Applicata*, vol. 72, no. 2, pp. 157–165, 1994.

[29] T. Will, W. Tjallingii, A. Thönnessen, and A. van Bel, "Molecular sabotage of plant defense by aphid saliva," *Proceedings of the National Academy of Sciences*, vol. 104, no. 25, pp. 10536–10541, 2007.

[30] A. Cherqui and W. F. Tjallingii, "Salivary proteins of aphids, a pilot study on identification, separation and immunolocalisation," *Journal of Insect Physiology*, vol. 46, no. 8, pp. 1177–1186, 2000.

[31] P. W. Miles, "Aphid saliva," *Biological Reviews of the Cambridge Philosophical Society*, vol. 74, no. 1, pp. 41–85, 1999.

[32] J. C. Carolan, C. I. Fitzroy, P. D. Ashton, A. E. Douglas, and T. L. Wilkinson, "The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry," *Proteomics*, vol. 9, no. 9, pp. 2457–2467, 2009.

[33] T. Will and A. Vilcinskas, "The structural sheath protein of aphids is required for phloem feeding," *Insect Biochemistry and Molecular Biology*, vol. 57, pp. 34–40, 2015.

[34] N. Harmel, E. Létocart, A. Cherqui, P. Giordanengo, G. Mazzucchelli, F. Guillonneau, E. De Pauw, E. Haubruge, and F. Francis, "Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*," *Insect Molecular Biology*, vol. 17, no. 2, pp. 165–174, 2008.

[35] S. J. Nicholson, S. D. Hartson, and G. J. Puterka, "Proteomic analysis of secreted saliva from Russian wheat aphid (*Diuraphis noxia* kurd.) biotypes that differ in virulence to wheat," *Journal of Proteomics*, vol. 75, no. 7, pp. 2252–2268, 2012.

[36] R. Ma, J. C. Reese, W. C. Black, and P. Bramel-Cox, "Detection of pectinesterase and polygalacturonase from salivary secretions of living greenbugs, *Schizaphis graminum* (Homoptera: Aphididae)," *Journal of Insect Physiology*, vol. 36, no. 7, pp. 507–512, 1990.

[37] P. Miles and Z. Peng, "Studies on the salivary physiology of plant bugs: detoxification of phytochemicals by the salivary peroxidase of aphids," *Journal of Insect Physiology*, vol. 35, no. 11, pp. 865–872, 1989.

[38] A. Urbanska, W. F. Tjallingii, A. F. Dixon, and B. Leszczynski, "Phenol oxidising enzymes in the grain aphid's saliva," *Entomologia Experimentalis et Applicata*, vol. 86, no. 2, pp. 197–203, 1998.

[39] J. Carolan, D. Caragea, K. Reardon, N. Mutti, N. Dittmer, K. Pappan, F. Cui, M. Castaneto, J. Poulain, C. Dossat, D. Tagu, J. Reese, G. Reeck, T. Wilkinson, and O. Edwards, "Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*)-a dual transcriptomic-proteomic approach.," *Journal of Proteome Research*, vol. 10, no. 4, pp. 1505–1518, 2011.

[40] T. Schultz, L. van Eck, and A.-M. Botha, "Phi-class glutathione-S-transferase is involved in Dn1-mediated resistance," *Physiologia Plantarum*, vol. 154, no. 1, pp. 1–12, 2015.

[41] N. Mutti, J. Louis, L. Pappan, K. Pappan, K. Begum, M. Chen, Y. Park, N. Dittmer, J. Marshall, J. Reese, and G. Reeck, "A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant," *Proceedings of the National Academy of Sciences*, vol. 105, no. 29, pp. 9965–9969, 2008.

[42] N. Mutti, Y. Park, J. Reese, and G. Reeck, "RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*," *Journal of Insect Science*, vol. 6, 2006.

[43] M. Pitino, A. Coleman, M. Maffei, C. Ridout, and S. Hogenhout, "Silencing of aphid genes by dsRNA feeding from plants," *PLoS One*, vol. 6, no. 10, p. e25709, 2011.

[44] M. Pitino and S. A. Hogenhout, "Aphid protein effectors promote aphid colonization in a plant species-specific manner," *Molecular Plant-Microbe Interactions*, vol. 26, no. 1, pp. 130–139, 2013.

[45] A. Coleman, R. Wouters, S. Mugford, and S. Hogenhout, "Persistence and transgenerational effect of plant-mediated RNAi in aphids," *Journal of Experimental Botany*, vol. 66, no. 2, pp. 541–548, 2015.

[46] J. Bos, D. Prince, M. Pitino, M. Maffei, J. Win, and S. Hogenhout, "A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid)," *PLoS Genetics*, vol. 6, no. 11, p. e1001216, 2010.

[47] M. De Vos and G. Jander, "*Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*," *Plant, Cell & Environment*, vol. 32, no. 11, pp. 1548–1560, 2009.

[48] C. Hawkins, M. Whitecross, and M. Aston, "The effect of short-term aphid feeding on the partitioning of 14CO2 photoassimilate in three legume species," *Canadian Journal of Botany*, vol. 65, no. 4, pp. 666–672, 1987.

[49] S. Quisenberry and X. Ni, "13 Feeding Injury," *Aphids as Crop Pests*, p. 331, 2007.

[50] P. D. Nabity, M. J. Haus, M. R. Berenbaum, and E. H. DeLucia, "Leaf-galling phylloxera on grapes reprograms host metabolism and morphology," *Proceedings of the National Academy of Sciences*, vol. 110, no. 41, pp. 16663–16668, 2013.

[51] B. J. Rehill and J. C. Schultz, "*Hormaphis hamamelidis* fundatrices benefit by manipulating phenolic metabolism of their host," *Journal of Chemical Ecology*, vol. 38, no. 5, pp. 496–498, 2012.

[52] M. S. Zinkgraf, N. Meneses, T. G. Whitham, and G. J. Allan, "Genetic variation in NIN1 and C/VIF1 genes is significantly associated with *Populus angustifolia* resistance to a galling herbivore, *Pemphigus betae*," *Journal of Insect Physiology*, vol. 84, pp. 50–59, 2016.

[53] K. Goethals, D. Vereecke, M. Jaziri, M. Van Montagu, and M. Holsters, "Leafy gall formation by *Rhodococcus fascians*," *Annual Review of Phytopathology*, vol. 39, no. 1, pp. 27–52, 2001.

[54] A. Robert-Seilaniantz, L. Navarro, R. Bari, and J. Jones, "Pathological hormone imbalances," *Current Opinion in Plant Biology*, vol. 10, no. 4, pp. 372–379, 2007.

[55] M. Brown, D. Glenn, and M. Wisniewski, "Functional and anatomical disruption of apple roots by the woolly apple aphid (Homoptera: Aphididae)," *Journal of Economic Entomology*, vol. 84, no. 6, pp. 1823–1826, 1991.

[56] W. Tedders and J. Smith, "Shading effect on pecan by sooty mold growth," *Journal of Economic Entomology*, vol. 69, no. 4, pp. 551–553, 1976.

[57] L. Nault, "Arthropod transmission of plant viruses: a new synthesis," *Annals of the Entomological Society of America*, vol. 90, no. 5, pp. 521–541, 1997.

[58] N. Katis, J. Tsitsipis, M. Stevens, and G. Powell, "14 transmission of plant viruses," *Aphids as Crop Pests*, p. 353, 2007.

[59] J. Ng and K. Perry, "Transmission of plant viruses by aphid vectors," *Molecular Plant Pathology*, vol. 5, no. 5, pp. 505–511, 2004.

[60] S. Hogenhout, E. Ammar, A. Whitfield, and M. Redinbaugh, "Insect-vector interactions with persistently transmitted viruses," *Annual Review of Phytopathology*, vol. 46, pp. 327–359, 2008.

[61] G. Tatchell, "An estimate of the potential economic losses to some crops due to aphids in Britain," *Crop Protection*, vol. 8, no. 1, pp. 25–29, 1989.

[62] X. Wang, C. Fang, X. Zheng, Z. Lin, L. Zhang, and H. Wang, "A study on the damage and economic threshold of the soyabean aphid at the seedling stage.," *Plant Protection*, vol. 20, no. 4, pp. 12–13, 1994.

[63] Y. Li, C. B. Hill, S. R. Carlson, B. W. Diers, and G. L. Hartman, "Soybean aphid resistance genes in the soybean cultivars Dowling and Jackson map to linkage group M," *Molecular Breeding*, vol. 19, no. 1, pp. 25–34, 2007.

[64] V. Bhatia, P. Uniyal, and R. Bhattacharya, "Aphid resistance in *Brassica* crops: challenges, biotechnological progress and emerging possibilities," *Biotechnology Advances*, vol. 29, no. 6, pp. 879–888, 2011.

[65] D. Goulson, "Review: An overview of the environmental risks posed by neonicotinoid insecticides," *Journal of Applied Ecology*, vol. 50, no. 4, pp. 977–987, 2013.

[66] K. M. Theiling and B. Croft, "Pesticide side-effects on arthropod natural enemies: a database summary," *Agriculture, Ecosystems & Environment*, vol. 21, no. 3, pp. 191–218, 1988.

[67] T. Blacquiere, G. Smagghe, C. A. Van Gestel, and V. Mommaerts, "Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment," *Ecotoxicology*, vol. 21, no. 4, pp. 973–992, 2012.

[68] H. C. J. Godfray, T. Blacquière, L. M. Field, R. S. Hails, G. Petrokofsky, S. G. Potts, N. E. Raine, A. J. Vanbergen, and A. R. McLean, "A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators," *Proceedings of the Royal Society B: Biological Sciences*, vol. 281, no. 1786, p. 20140558, 2014.

[69] A. Devonshire and G. Moores, "A carboxylesterase with broad substrate specificity causes organophosphorus, carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*)," *Pesticide Biochemistry and Physiology*, vol. 18, no. 2, pp. 235–246, 1982.

[70] G. Moores, G. Devine, and A. Devonshire, "Insecticide-insensitive acetylcholinesterase can enhance esterase-based resistance in *Myzus persicae* and *Myzus nicotianae*," *Pesticide Biochemistry and Physiology*, vol. 49, no. 2, pp. 114–120, 1994.

[71] D. Martinez-Torres, S. Foster, L. Field, A. Devonshire, and M. Williamson, "A sodium channel point mutation is associated with resistance to DDT and pyrethroid insecticides in the peach-potato aphid, *Myzus persicae* (Sulzer)(Hemiptera: Aphididae)," *Insect Molecular Biology*, vol. 8, no. 3, pp. 339–346, 1999.

[72] S. Foster, I. Denholm, and A. Devonshire, "The ups and downs of insecticide resistance in peach-potato aphids (*Myzus persicae*) in the uk," *Crop Protection*, vol. 19, no. 8-10, pp. 873–879, 2000.

[73] C. Bass, A. M. Puinean, C. T. Zimmer, I. Denholm, L. M. Field, S. P. Foster, O. Gutbrod, R. Nauen, R. Slater, and M. S. Williamson, "The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*," *Insect Biochemistry and Molecular Biology*, vol. 51, pp. 41–51, 2014.

[74] P. Shaw, J. Walker, and M. O'-Callaghan, "Biological control of woolly apple aphid by *Aphelinus mali* in an integrated fruit production programme in Nelson," in *Proceedings of the New Zealand Plant Protection Conference*, pp. 59–63, New Zealand Plant Protection Society Inc., 1996.

[75] A. Levie, M. Legrand, P. Dogot, C. Pels, P. Baret, and T. Hance, "Mass releases of *Aphidius rhopalosiphi* (Hymenoptera: Aphidiinae), and strip management to control of wheat aphids," *Agriculture, Ecosystems & Environment*, vol. 105, no. 1, pp. 17–21, 2005.

[76] S. Li, P. Falabella, S. Giannantonio, P. Fanti, D. Battaglia, M. Digilio, W. Völkl, J. Sloggett, W. Weisser, and F. Pennacchio, "Pea aphid clonal resistance to the endophagous parasitoid *Aphidius ervi*," *Journal of Insect Physiology*, vol. 48, no. 10, pp. 971–980, 2002.

[77] K. M. Oliver, J. A. Russell, N. A. Moran, and M. S. Hunter, "Facultative bacterial symbionts in aphids confer resistance to parasitic wasps," *Proceedings of the National Academy of Sciences*, vol. 100, no. 4, pp. 1803–1807, 2003.

[78] J. S. Bale, G. J. Masters, I. D. Hodkinson, C. Awmack, T. M. Bezemer, V. K. Brown, J. Butterfield, A. Buse, J. C. Coulson, J. Farrar, J. E. Good, R. Harrington, S. Hartley, H. Jones, R. L. Lindroth, M. C. Press, I. Symrnioudis, A. D. Watt, and J. B. Whittaker, "Herbivory in global climate change research: direct effects of rising temperature on insect herbivores," *Global Change Biology*, vol. 8, no. 1, pp. 1–16, 2002.

[79] J. Dunn, "The biology of lettuce root aphid," *Annals of Applied Biology*, vol. 47, no. 3, pp. 475–491, 1959.

[80] Z. Khan, C. Midega, J. Pittchar, J. Pickett, and T. Bruce, "Push-pull technology: a conservation agriculture approach for integrated management of insect pests, weeds and soil health in Africa," *International Journal of Agricultural Sustainability*, vol. 9, no. 1, pp. 162–170, 2011.

[81] S. Milligan, J. Bodeau, J. Yaghoobi, I. Kaloshian, P. Zabel, and V. Williamson, "The root knot nematode resistance gene Mi from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes," *The Plant Cell*, vol. 10, no. 8, pp. 1307–1320, 1998.

[82] G. Zhang, C. Gu, and D. Wang, "A novel locus for soybean aphid resistance," *TAG Theoretical and Applied Genetics*, vol. 120, no. 6, pp. 1183–1191, 2010.

[83] J. Klingler, I. Kovalski, L. Silberstein, G. Thompson, and R. Perl-Treves, "Mapping of cotton-melon aphid resistance in melon," *Journal of the American Society for Horticultural Science*, vol. 126, no. 1, pp. 56–63, 2001.

[84] J. Klingler, R. Creasy, L. Gao, R. Nair, A. Calix, H. Jacob, O. Edwards, and K. Singh, "Aphid resistance in *Medicago truncatula* involves antixenosis and phloem-specific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogs," *Plant Physiology*, vol. 137, no. 4, pp. 1445–1455, 2005.

[85] S. Guo, L. Kamphuis, L. Gao, J. Klingler, J. Lichtenzveig, O. Edwards, and K. Singh, "Identification of distinct quantitative trait loci associated with defence against the closely related aphids *Acyrthosiphon pisum* and *A. kondoi* in *Medicago truncatula*," *Journal of Experimental Botany*, 2012.

[86] J. Pauquet, E. Burget, L. Hagen, V. Chovelon, A. Menn, N. Valot, S. Desloire, M. Caboche, P. Rousselle, M. Pitrat, A. Bendahmane, and C. Dogimont, "Map-based cloning of the Vat gene from melon conferring resistance to both aphid colonization and aphid transmission of several viruses.," in *Proceedings of Cucurbitaceae 2004, the 8th EUCARPIA Meeting on Cucurbit Genetics and Breeding, Olomouc, Czech Republic*, pp. 325–329, 2004.

[87] E. Lombaert, J. Carletto, C. Piotte, X. Fauvergue, H. Lecoq, F. Vanlerberghe-Masutti, and L. Lapchin, "Response of the melon aphid, *Aphis gossypii*, to host-plant resistance: evidence for high adaptive potential despite low genetic variability," *Entomologia Experimentalis et Applicata*, vol. 133, no. 1, pp. 46–56, 2009.

[88] A. van der Arendt, A. Ester, and J. Van Schijndel, "Developing an aphid resistant butterhead lettuce Dynamite," in *Eucarpia Leafy Vegetables' 99, Olomouc (Czech Republic)*, Palacky University, 1999.

[89] E. Keep, "Breeding red raspberry for resistance to diseases and pests," *Plant Breeding Reviews, Volume 6*, pp. 245–321, 1989.

[90] B. Du, W. Zhang, B. Liu, J. Hu, Z. Wei, Z. Shi, R. He, L. Zhu, R. Chen, B. Han, *et al.*, "Identification and characterization of Bph14, a gene conferring resistance to brown planthopper in rice," *Proceedings of the National Academy of Sciences*, vol. 106, no. 52, pp. 22163–22168, 2009.

[91] Y. Tamura, M. Hattori, H. Yoshioka, M. Yoshioka, A. Takahashi, J. Wu, N. Sentoku, and H. Yasui, "Map-based cloning and characterization of a brown planthopper resistance gene BPH26 from *Oryza sativa* l. ssp. indica cultivar ADR52," *Scientific Reports*, vol. 4, 2014.

[92] S. Kumar, C. Atri, M. Sangha, and S. Banga, "Screening of wild crucifers for resistance to mustard aphid, *Lipaphis erysimi* (Kaltenbach) and attempt at introgression of resistance gene (s) from *Brassica fruticulosa* to *Brassica juncea*," *Euphytica*, vol. 179, no. 3, pp. 461–470, 2011.

[93] V. Hilder and D. Boulter, "Genetic engineering of crop plants for insect resistance–a critical review," *Crop Protection*, vol. 18, no. 3, pp. 177–191, 1999.

[94] N. P. Chougule, H. Li, S. Liu, L. B. Linz, K. E. Narva, T. Meade, and B. C. Bonning, "Retargeting of the *Bacillus thuringiensis* toxin Cyt2Aa against hemipteran insect pests," *Proceedings of the National Academy of Sciences*, vol. 110, no. 21, pp. 8465–8470, 2013.

[95] B. C. Bonning, N. Pal, S. Liu, Z. Wang, S. Sivakumar, P. M. Dixon, G. F. King, and W. A. Miller, "Toxin delivery by the coat protein of an aphid-vectored plant virus provides plant resistance to aphids," *Nature Biotechnology*, vol. 32, no. 1, pp. 102–105, 2014.

[96] B. Tabashnik, A. Gassmann, D. Crowder, and Y. Carrière, "Insect resistance to Bt crops: evidence versus theory," *Nature Biotechnology*, vol. 26, no. 2, pp. 199–202, 2008.

[97] R. Roush, "Bt-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management?," *Pesticide Science*, vol. 51, no. 3, pp. 328–334, 1997.

[98] V. Hilder, A. Gatehouse, S. Sheerman, R. Barker, and D. Boulter, "A novel mechanism of insect resistance engineered into tobacco," *Nature*, vol. 330, no. 6144, pp. 160–163, 1987.

[99] P. Tran, T. Cheesbrough, and R. Keickhefer, "Plant proteinase inhibitors are potential anticereal aphid compounds," *Journal of Economic Entomology*, vol. 90, no. 6, pp. 1672–1677, 1997.

[100] J. Pickett, L. Wadhams, C. Woodcock, and J. Hardie, "The chemical ecology of aphids," *Annual Review of Entomology*, vol. 37, no. 1, pp. 67–90, 1992.

[101] L. E. Vet and M. Dicke, "Ecology of infochemical use by natural enemies in a tritrophic context," *Annual Review of Entomology*, vol. 37, no. 1, pp. 141–172, 1992.

[102] S. Vandermoten, M. C. Mescher, F. Francis, E. Haubruge, and F. J. Verheggen, "Aphid alarm pheromone: an overview of current knowledge on biosynthesis and functions," *Insect Biochemistry and Molecular Biology*, vol. 42, no. 3, pp. 155–163, 2012.

[103] R. Gibson and J. Pickett, "Wild potato repels aphids by release of aphid alarm pheromone," *Nature*, vol. 302, pp. 608–609, 1983.

[104] W. Tingey, R. Plaisted, J. Laubengayer, and S. Mehlenbacher, "Green peach aphid resistance by glandular trichomes in *Solanum tuberosum x S. berthaultii* hybrids," *American Journal of Potato Research*, vol. 59, no. 6, pp. 241–251, 1982.

[105] J. Crock, M. Wildung, and R. Croteau, "Isolation and bacterial expression of a sesquiterpene synthase cDNA clone from peppermint (*Mentha x piperita*, l.) that produces the aphid alarm pheromone (E)-β-farnesene," *Proceedings of the National Academy of Sciences*, vol. 94, no. 24, pp. 12833–12838, 1997.

[106] V. Gosset, N. Harmel, C. Göbel, F. Francis, E. Haubruge, J.-P. Wathelet, P. Du Jardin, I. Feussner, and M.-L. Fauconnier, "Attacks by a piercing-sucking insect (*Myzus persicae* sultzer) or a chewing insect (*Leptinotarsa decemlineata* say) on potato plants (*Solanum tuberosum* l.) induce differential changes in volatile compound release and oxylipin synthesis," *Journal of Experimental Botany*, vol. 60, no. 4, pp. 1231–1240, 2009.

[107] M. Beale, M. Birkett, T. Bruce, K. Chamberlain, L. Field, A. Huttly, J. Martin, R. Parker, A. Phillips, J. Pickett, I. Prosser, P. Shewry, L. Smart, L. Wadhams, C. Woodcock, and Y. Zhang, "Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior," *Proceedings of the National Academy of Sciences*, vol. 103, no. 27, pp. 10509–10513, 2006.

[108] M. de Vos, W. Y. Cheng, H. E. Summers, R. A. Raguso, and G. Jander, "Alarm pheromone habituation in *Myzus persicae* has fitness consequences and causes extensive gene expression changes," *Proceedings of the National Academy of Sciences*, vol. 107, no. 33, pp. 14673–14678, 2010.

[109] T. J. Bruce, G. I. Aradottir, L. E. Smart, J. L. Martin, J. C. Caulfield, A. Doherty, C. A. Sparks, C. M. Woodcock, M. A. Birkett, J. A. Napier, H. D. Jones, and J. A. Pickett, "The first crop plant genetically engineered to release an insect pheromone for defence," *Scientific Reports*, vol. 5, no. 11183, 2015.

[110] D. Yang, C. Hettenhausen, I. Baldwin, and J. Wu, "BAK1 regulates the accumulation of jasmonic acid and the levels of trypsin proteinase inhibitors in *Nicotiana attenuata*'s responses to herbivory," *Journal of Experimental Botany*, vol. 62, no. 2, p. 641, 2011.

[111] I. Kaloshian and L. Walling, "Hemipterans as plant pathogens," *Annual Review of Phytopathology*, vol. 43, pp. 491–521, 2005.

[112] C. Smith and S. Clement, "Molecular bases of plant resistance to Arthropods," *Annual Review of Entomology*, vol. 57, no. 1, pp. 309–328, 2012.

[113] D. Walters and M. Heil, "Costs and trade-offs associated with induced resistance," *Physiological and Molecular Plant Pathology*, vol. 71, no. 1, pp. 3–17, 2007.

[114] T. Boller and G. Felix, "A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors," *Annual Review of Plant Biology*, vol. 60, pp. 379–406, 2009.

[115] J. Wu and I. Baldwin, "New insights into plant responses to the attack from insect herbivores," *Annual Review of Genetics*, vol. 44, pp. 1–24, 2010.

[116] P. Paré, H. Alborn, and J. Tumlinson, "Concerted biosynthesis of an insect elicitor of plant volatiles," *Proceedings of the National Academy of Sciences*, vol. 95, no. 23, pp. 13971–13975, 1998.

[117] C. Hettenhausen, M. Heinrich, I. T. Baldwin, and J. Wu, "Fatty acid-amino acid conjugates are essential for systemic activation of salicylic acid-induced protein kinase and accumulation of jasmonic acid in *Nicotiana attenuata*," *BMC Plant Biology*, vol. 14, no. 1, p. 326, 2014.

[118] J. Wu, C. Hettenhausen, S. Meldau, and I. T. Baldwin, "Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*," *The Plant Cell*, vol. 19, no. 3, pp. 1096–1122, 2007.

[119] J. A. Zavala, A. G. Patankar, K. Gase, D. Hui, and I. T. Baldwin, "Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as antiherbivore defenses," *Plant Physiology*, vol. 134, no. 3, pp. 1181–1190, 2004.

[120] N. Yoshinaga, T. Aboshi, C. Ishikawa, M. Fukui, M. Shimoda, R. Nishida, C. G. Lait, J. H. Tumlinson, and N. Mori, "Fatty acid amides, previously identified in caterpillars, found in the cricket *Teleogryllus taiwanemma* and fruit fly *Drosophila melanogaster* larvae," *Journal of Chemical Ecology*, vol. 33, no. 7, pp. 1376–1381, 2007.

[121] N. Yoshinaga, T. Aboshi, H. Abe, R. Nishida, H. T. Alborn, J. H. Tumlinson, and N. Mori, "Active role of fatty acid amino acid conjugates in nitrogen metabolism in *Spodoptera litura* larvae," *Proceedings of the National Academy of Sciences*, vol. 105, no. 46, pp. 18058–18063, 2008.

[122] H. T. Alborn, T. V. Hansen, T. H. Jones, D. C. Bennett, J. H. Tumlinson, E. A. Schmelz, and P. E. Teal, "Disulfooxy fatty acids from the American bird grasshopper *Schistocerca americana*, elicitors of plant volatiles," *Proceedings of the National Academy of Sciences*, vol. 104, no. 32, pp. 12976–12981, 2007.

[123] E. A. Schmelz, M. J. Carroll, S. LeClere, S. M. Phipps, J. Meredith, P. S. Chourey, H. T. Alborn, and P. E. Teal, "Fragments of ATP synthase mediate plant perception of insect attack," *Proceedings of the National Academy of Sciences*, vol. 103, no. 23, pp. 8894–8899, 2006.

[124] J. Louis, M. Peiffer, S. Ray, D. S. Luthe, and G. W. Felton, "Host-specific salivary elicitor(s) of European corn borer induce defenses in tomato and maize," *New Phytologist*, vol. 199, no. 1, pp. 66–73, 2013.

[125] D. Tian, M. Peiffer, E. Shoemaker, J. Tooker, E. Haubruge, F. Francis, D. S. Luthe, and G. W. Felton, "Salivary glucose oxidase from caterpillars mediates the induction of rapid and delayed-induced defenses in the tomato plant," *PLoS One*, vol. 7, no. 4, p. e36168, 2012.

[126] E. A. Schmelz, S. LeClere, M. J. Carroll, H. T. Alborn, and P. E. Teal, "Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory," *Plant Physiology*, vol. 144, no. 2, pp. 793–805, 2007.

[127] N. Lapitan, Y. Li, J. Peng, and A. Botha, "Fractionated extracts of Russian wheat aphid eliciting defense responses in wheat," *Journal of Economic Entomology*, vol. 100, no. 3, pp. 990–999, 2007.

[128] C. A. Ryan and G. Pearce, "Systemins: a functionally defined family of peptide signals that regulate defensive genes in Solanaceae species," *Proceedings of the National Academy of Sciences*, vol. 100, no. 2, pp. 14577–14580, 2003.

[129] B. McGurl, G. Pearce, M. Orozco-Cardenas, and C. A. Ryan, "Structure, expression, and antisense inhibition of the systemin precursor gene," *Science*, vol. 255, no. 5051, pp. 1570–1573, 1992.

[130] G. Corrado, R. Sasso, M. Pasquariello, L. Iodice, A. Carretta, P. Cascone, L. Ariati, M. Digilio, E. Guerrieri, and R. Rao, "Systemin regulates both systemic and volatile signaling in tomato plants," *Journal of Chemical Ecology*, vol. 33, no. 4, pp. 669–681, 2007.

[131] M. Lintle and A. Van Der Westhuizen, "Glycoproteins from Russian wheat aphid infested wheat induce defence responses.," *Zeitschrift für Naturforschung. C, Journal of Biosciences*, vol. 57, no. 9-10, p. 867, 2002.

[132] A. Huffaker, G. Pearce, and C. Ryan, "An endogenous peptide signal in *Arabidopsis* activates components of the innate immune response," *Proceedings of the National Academy of Sciences*, vol. 103, no. 26, pp. 10098–10103, 2006.

[133] S.-H. Shiu and A. B. Bleecker, "Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases," *Proceedings of the National Academy of Sciences*, vol. 98, no. 19, pp. 10763–10768, 2001.

[134] J. M. Scheer and C. A. Ryan, "The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family," *Proceedings of the National Academy of Sciences*, vol. 99, no. 14, pp. 9585–9590, 2002.

[135] F. C. Lanfermeijer, M. Staal, R. Malinowski, J. W. Stratmann, and J. T. M. Elzenga, "Micro-electrode flux estimation confirms that the *Solanum pimpinellifolium* cu3 mutant still responds to systemin," *Plant Physiology*, vol. 146, no. 1, pp. 129–139, 2008.

[136] Y. Liu, H. Wu, H. Chen, Y. Liu, J. He, H. Kang, Z. Sun, G. Pan, Q. Wang, J. Hu, F. Zhou, K. Zhou, X. Zheng, Y. Ren, L. Chen, Y. Wang, Z. Zhao, Q. Lin, F. Wu, X. Zhang, X. Guo, X. Cheng, L. Jiang, C. Wu, H. Wang, and J. Wan, "A gene cluster encoding lectin receptor kinases confers broad-spectrum and durable insect resistance in rice," *Nature Biotechnology*, 2014.

[137] C. Zipfel, G. Kunze, D. Chinchilla, A. Caniard, J. Jones, T. Boller, and G. Felix, "Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation," *Cell*, vol. 125, no. 4, pp. 749–760, 2006.

[138] D. Chinchilla, Z. Bauer, M. Regenass, T. Boller, and G. Felix, "The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception," *The Plant Cell*, vol. 18, no. 2, pp. 465–476, 2006.

[139] G. Felix, J. Duran, S. Volko, and T. Boller, "Plants have a sensitive perception system for the most conserved domain of bacterial flagellin," *The Plant Journal*, vol. 18, no. 3, pp. 265–276, 1999.

[140] C. Zipfel, S. Robatzek, L. Navarro, E. Oakeley, J. Jones, G. Felix, and T. Boller, "Bacterial disease resistance in *Arabidopsis* through flagellin perception," *Nature*, vol. 428, no. 6984, pp. 764–767, 2004.

[141] G. Kunze, C. Zipfel, S. Robatzek, K. Niehaus, T. Boller, and G. Felix, "The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants," *The Plant Cell*, vol. 16, no. 12, pp. 3496–3507, 2004.

[142] E. Krol, T. Mentzel, D. Chinchilla, T. Boller, G. Felix, B. Kemmerling, S. Postel, M. Arents, E. Jeworutzki, K. Al-Rasheid, D. Becker, and R. Hedrich, "Perception of the *Arabidopsis* danger signal peptide 1 involves the pattern recognition receptor AtPEPR1 and its close homologue AtPEPR2," *Journal of Biological Chemistry*, vol. 285, no. 18, pp. 13471–13479, 2010.

[143] A. Heese, D. Hann, S. Gimenez-Ibanez, A. Jones, K. He, J. Li, J. Schroeder, S. Peck, and J. Rathjen, "The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants," *Proceedings of the National Academy of Sciences*, vol. 104, no. 29, p. 12217, 2007.

[144] A. Chaparro-Garcia, R. C. Wilkinson, S. Gimenez-Ibanez, K. Findlay, M. D. Coffey, C. Zipfel, J. P. Rathjen, S. Kamoun, and S. Schornack, "The receptor-like kinase SERK3/BAK1 is required for basal resistance against the late blight pathogen *Phytophthora infestans* in *Nicotiana benthamiana*," *PLoS One*, vol. 6, no. 1, p. e16608, 2011.

[145] J. Li, J. Wen, K. Lease, J. Doke, F. Tax, and J. Walker, "BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling," *Cell*, vol. 110, no. 2, pp. 213–222, 2002.

[146] D. Chinchilla, C. Zipfel, S. Robatzek, B. Kemmerling, T. Nürnberger, J. Jones, G. Felix, and T. Boller, "A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence," *Nature*, vol. 448, no. 7152, pp. 497–500, 2007.

[147] B. Schwessinger, M. Roux, Y. Kadota, V. Ntoukakis, J. Sklenar, A. Jones, and C. Zipfel, "Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1," *PLoS Genetics*, vol. 7, no. 4, p. e1002046, 2011.

[148] D. Chinchilla, L. Shan, P. He, S. de Vries, and B. Kemmerling, "One for all: the receptor-associated kinase BAK1," *Trends in Plant Science*, vol. 14, no. 10, pp. 535–541, 2009.

[149] Y. Sun, L. Li, A. P. Macho, Z. Han, Z. Hu, C. Zipfel, J.-M. Zhou, and J. Chai, "Structural basis for flg22-induced activation of the *Arabidopsis* FLS2-BAK1 immune complex," *Science*, vol. 342, no. 6158, pp. 624–628, 2013.

[150] B. Schulze, T. Mentzel, A. K. Jehle, K. Mueller, S. Beeler, T. Boller, G. Felix, and D. Chinchilla, "Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1," *Journal of Biological Chemistry*, vol. 285, no. 13, pp. 9444–9451, 2010.

[151] D. Lu, S. Wu, X. Gao, Y. Zhang, L. Shan, and P. He, "A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity," *Proceedings of the National Academy of Sciences*, vol. 107, no. 1, pp. 496–501, 2010.

[152] J. Lei, S. A. Finlayson, R. A. Salzman, L. Shan, and K. Zhu-Salzman, "BOTRYTIS-INDUCED KINASE1 modulates *Arabidopsis* resistance to green peach aphids via PHYTOALEXIN DEFICIENT4," *Plant Physiology*, vol. 165, no. 4, pp. 1657–1670, 2014.

[153] F. Ikeda and I. Dikic, "Atypical ubiquitin chains: new molecular signals," *EMBO Reports*, vol. 9, no. 6, pp. 536–542, 2008.

[154] R. D. Vierstra, "The ubiquitin–26S proteasome system at the nexus of plant biology," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 6, pp. 385–397, 2009.

[155] D. Lu, W. Lin, X. Gao, S. Wu, C. Cheng, J. Avila, A. Heese, T. P. Devarenne, P. He, and L. Shan, "Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity," *Science*, vol. 332, no. 6036, pp. 1439–1442, 2011.

[156] S. Robatzek, D. Chinchilla, and T. Boller, "Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*," *Genes & Development*, vol. 20, no. 5, pp. 537–542, 2006.

[157] M. Beck, J. Zhou, C. Faulkner, D. MacLean, and S. Robatzek, "Spatio-temporal cellular dynamics of the *Arabidopsis* flagellin receptor reveal activation status-dependent endosomal sorting," *The Plant Cell*, vol. 24, no. 10, pp. 4205–4219, 2012.

[158] M. E. Maffei, A. Mithöfer, G.-I. Arimura, H. Uchtenhagen, S. Bossi, C. M. Bertea, L. S. Cucuzza, M. Novero, V. Volpe, S. Quadro, and W. Boland, "Effects of feeding *Spodoptera littoralis* on lima bean leaves.III. Membrane depolarization and involvement of hydrogen peroxide," *Plant Physiology*, vol. 140, no. 3, pp. 1022–1035, 2006.

[159] G.-i. Arimura and M. E. Maffei, "Calcium and secondary CPK signaling in plants in response to herbivore attack," *Biochemical and Biophysical Research Communications*, vol. 400, no. 4, pp. 455–460, 2010.

[160] I. Bricchi, M. Leitner, M. Foti, A. Mithöfer, W. Boland, and M. E. Maffei, "Robotic mechanical wounding (MecWorm) versus herbivore-induced responses: early signaling and volatile emission in Lima bean (*Phaseolus lunatus* L.)," *Planta*, vol. 232, no. 3, pp. 719–729, 2010.

[161] M. Maffei, S. Bossi, D. Spiteller, A. Mithöfer, and W. Boland, "Effects of feeding *Spodoptera littoralis* on lima bean leaves. I. Membrane potentials, intracellular calcium variations, oral secretions, and regurgitate components," *Plant Physiology*, vol. 134, no. 4, pp. 1752–1762, 2004.

[162] C. Smith and E. Boyko, "The molecular bases of plant resistance and defense responses to aphid feeding: current status," *Entomologia Experimentalis et Applicata*, vol. 122, no. 1, pp. 1–16, 2007.

[163] D. Lecourieux, C. Mazars, N. Pauly, R. Ranjeva, and A. Pugin, "Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells," *The Plant Cell*, vol. 14, no. 10, pp. 2627–2641, 2002.

[164] C. Cheval, D. Aldon, J.-P. Galaud, and B. Ranty, "Calcium/calmodulin-mediated regulation of plant immunity," *Biochimica et Biophysica Acta Molecular Cell Research*, vol. 1833, no. 7, pp. 1766–1771, 2013.

[165] D. Lecourieux, R. Ranjeva, and A. Pugin, "Calcium in plant defence-signalling pathways," *New Phytologist*, vol. 171, no. 2, pp. 249–269, 2006.

[166] W. Ma and G. A. Berkowitz, "Ca2+ conduction by plant cyclic nucleotide gated channels and associated signaling components in pathogen defense signal transduction cascades," *New Phytologist*, vol. 190, no. 3, pp. 566–572, 2011.

[167] L. Steinhorst and J. Kudla, "Calcium and ROS rule the waves of signaling," *Plant Physiology*, vol. 163, no. 2, pp. 471–485, 2013.

[168] B. G. Forde and M. R. Roberts, "Glutamate receptor-like channels in plants: a role as amino acid sensors in plant defence?," *F1000 Prime Reports*, vol. 6, no. 37, 2014.

[169] K. Mark, H. Rik, M. Jens, R. Anja, and P. Ralph, "Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*," *Biochemical Journal*, vol. 440, no. 3, pp. 355–365, 2011.

[170] Y. Ma, R. K. Walker, Y. Zhao, and G. A. Berkowitz, "Linking ligand perception by PEPR pattern recognition receptors to cytosolic Ca2+ elevation and downstream immune signaling in plants," *Proceedings of the National Academy of Sciences*, vol. 109, no. 48, pp. 19852–19857, 2012.

[171] L. Li, M. Li, L. Yu, Z. Zhou, X. Liang, Z. Liu, G. Cai, L. Gao, X. Zhang, Y. Wang, S. Chen, and J.-M. Zhou, "The FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RbohD to control plant immunity," *Cell Host & Microbe*, vol. 15, no. 3, pp. 329–338, 2014.

[172] S. A. Mousavi, A. Chauvin, F. Pascaud, S. Kellenberger, and E. E. Farmer, "GLUTAMATE RECEPTOR-LIKE genes mediate leaf-to-leaf wound signalling," *Nature*, vol. 500, no. 7463, pp. 422–426, 2013.

[173] Z. Qi, N. R. Stephens, and E. P. Spalding, "Calcium entry mediated by GLR3.3, an *Arabidopsis* glutamate receptor with a broad agonist profile," *Plant Physiology*, vol. 142, no. 3, pp. 963–971, 2006.

[174] M. Torres, "ROS in biotic interactions," *Physiologia Plantarum*, vol. 138, no. 4, pp. 414–429, 2010.

[175] A. Kuśnierczyk, P. Winge, T. Jørstad, J. Troczyńska, J. Rossiter, and A. Bones, "Towards global understanding of plant defence against aphids–timing and dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack," *Plant, Cell & Environment*, vol. 31, no. 8, pp. 1097–1115, 2008.

[176] M. Maffei, A. Mithöfer, and W. Boland, "Insects feeding on plants: rapid signals and responses preceding the induction of phytochemical release," *Phytochemistry*, vol. 68, no. 22-24, pp. 2946–2959, 2007.

[177] M. Jaouannet, J. A. Morris, P. E. Hedley, and J. I. Bos, "Characterization of *Arabidopsis* transcriptional responses to different aphid species reveals genes that contribute to host susceptibility and non-host resistance," *PLoS Pathogens*, vol. 11, no. 5, p. e1004918, 2015.

[178] M. A. Torres, J. L. Dangl, and J. D. Jones, "*Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response," *Proceedings of the National Academy of Sciences*, vol. 99, no. 1, pp. 517–522, 2002.

[179] G. Miller, K. Schlauch, R. Tam, D. Cortes, M. A. Torres, V. Shulaev, J. L. Dangl, and R. Mittler, "The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli," *Science Signaling*, vol. 2, no. 84, pp. 45–45, 2009.

[180] Y. Kadota, J. Sklenar, P. Derbyshire, L. Stransfeld, S. Asai, V. Ntoukakis, J. D. Jones, K. Shirasu, F. Menke, A. Jones, and C. Zipfel, "Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity," *Molecular Cell*, vol. 54, no. 1, pp. 43–55, 2014.

[181] U. Dubiella, H. Seybold, G. Durian, E. Komander, R. Lassig, C.-P. Witte, W. X. Schulze, and T. Romeis, "Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation," *Proceedings of the National Academy of Sciences*, vol. 110, no. 21, pp. 8744–8749, 2013.

[182] D. Marino, C. Dunand, A. Puppo, and N. Pauly, "A burst of plant NADPH oxidases," *Trends in Plant Science*, vol. 17, no. 1, pp. 9–15, 2012.

[183] S. Kimura, H. Kaya, T. Kawarazaki, G. Hiraoka, E. Senzaki, M. Michikawa, and K. Kuchitsu, "Protein phosphorylation is a prerequisite for the calcium-dependent activation of *Arabidopsis* NADPH oxidases and may function as a trigger for the positive feedback regulation of calcium and reactive oxygen species," *Biochimica et Biophysica Acta-Molecular Cell Research*, vol. 1823, no. 2, pp. 398–405, 2012.

[184] L. Steinhorst and J. Kudla, "Signaling in cells and organisms - calcium holds the line," *Current Opinion in Plant Biology*, vol. 22, pp. 14–21, 2014.

[185] C. Segonzac, D. Feike, S. Gimenez-Ibanez, D. Hann, C. Zipfel, and J. Rathjen, "Hierarchy and roles of pathogen-associated molecular pattern-induced responses in *Nicotiana benthamiana*," *Plant Physiology*, vol. 156, no. 2, pp. 687–699, 2011.

[186] T. Asai, G. Tena, J. Plotnikova, M. Willmann, W. Chiu, L. Gomez-Gomez, T. Boller, F. Ausubel, and J. Sheen, "MAP kinase signalling cascade in *Arabidopsis* innate immunity," *Nature*, vol. 415, no. 6875, pp. 977–983, 2002.

[187] A. Lebrun-Garcia, F. Ouaked, A. Chiltz, and A. Pugin, "Activation of MAPK homologues by elicitors in tobacco cells," *The Plant Journal*, vol. 15, no. 6, pp. 773–781, 1998.

[188] J. Dahan, C. Pichereaux, M. Rossignol, S. Blanc, D. Wendehenne, A. Pugin, and S. Bourque, "Activation of a nuclear-localized SIPK in tobacco cells challenged by cryptogein, an elicitor of plant defence reactions.," *Biochemical Journal*, vol. 418, no. 1, pp. 191–200, 2009.

[189] M. Kallenbach, F. Alagna, I. Baldwin, and G. Bonaventure, "Nicotiana attenuata SIPK, WIPK, NPR1, and fatty acid-amino acid conjugates participate in the induction of jasmonic acid biosynthesis by affecting early enzymatic steps in the pathway," *Plant Physiology*, vol. 152, no. 1, pp. 96–106, 2010.

[190] M. Schäfer, C. Fischer, S. Meldau, E. Seebald, R. Oelmüller, and I. T. Baldwin, "Lipase activity in insect oral secretions mediates defense responses in *Arabidopsis*," *Plant Physiology*, vol. 156, no. 3, pp. 1520–1534, 2011.

[191] A. Pitzschke and H. Hirt, "Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signaling," *Plant Physiology*, vol. 149, no. 2, pp. 606–615, 2009.

[192] S. Asai, K. Ohta, and H. Yoshioka, "MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*," *The Plant Cell*, vol. 20, no. 5, pp. 1390–1406, 2008.

[193] Z. Cheng, J.-F. Li, Y. Niu, X.-C. Zhang, O. Z. Woody, Y. Xiong, S. Djonović, Y. Millet, J. Bush, B. J. McConkey, J. Sheen, and F. M. Ausubel, "Pathogen-secreted proteases activate a novel plant immune pathway," *Nature*, 2015.

[194] A. Ishikawa, "The *Arabidopsis* G-protein β-subunit is required for defense response against *Agrobacterium tumefaciens*," *Bioscience, Biotechnology and Biochemistry*, vol. 73, no. 1, pp. 47–52, 2009.

[195] M. A. Torres, J. Morales, C. Sánchez-Rodríguez, A. Molina, and J. L. Dangl, "Functional interplay between *Arabidopsis* NADPH oxidases and heterotrimeric G-protein," *Molecular Plant-Microbe Interactions*, vol. 26, no. 6, pp. 686–694, 2013.

[196] D. P. S. Verma and Z. Hong, "Plant callose synthase complexes," *Plant Molecular Biology*, vol. 47, no. 6, pp. 693–701, 2001.

[197] T. A. Richmond and C. R. Somerville, "The cellulose synthase superfamily," *Plant Physiology*, vol. 124, no. 2, pp. 495–498, 2000.

[198] A. K. Jacobs, V. Lipka, R. A. Burton, R. Panstruga, N. Strizhov, P. Schulze-Lefert, and G. B. Fincher, "An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation," *The Plant Cell*, vol. 15, no. 11, pp. 2503–2513, 2003.

[199] M. Nishimura, M. Stein, B. Hou, J. Vogel, H. Edwards, and S. Somerville, "Loss of a callose synthase results in salicylic acid-dependent disease resistance," *Science*, vol. 301, no. 5635, pp. 969–972, 2003.

[200] S. Saheed, I. Cierlik, K. Larsson, G. Delp, G. Bradley, L. Jonsson, and C. Botha, "Stronger induction of callose deposition in barley by Russian wheat aphid than bird cherry-oat aphid is not associated with differences in callose synthase or β-1, 3-glucanase transcript abundance," *Physiologia Plantarum*, vol. 135, no. 2, pp. 150–161, 2009.

[201] P. Hao, C. Liu, Y. Wang, R. Chen, M. Tang, B. Du, L. Zhu, and G. He, "Herbivore-induced callose deposition on the sieve plates of rice: an important mechanism for host resistance," *Plant Physiology*, vol. 146, no. 4, pp. 1810–1820, 2008.

[202] W. Lv, B. Du, X. Shangguan, Y. Zhao, Y. Pan, L. Zhu, Y. He, and G. He, "BAC and RNA sequencing reveal the brown planthopper resistance gene BPH15 in a recombination cold spot that mediates a unique defense mechanism," *BMC Genomics*, vol. 15, no. 1, p. 674, 2014.

[203] R. Galletti, C. Denoux, S. Gambetta, J. Dewdney, F. Ausubel, G. De Lorenzo, and S. Ferrari, "The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in *Arabidopsis* is dispensable for the activation of defense responses effective against *Botrytis cinerea*," *Plant Physiology*, vol. 148, no. 3, pp. 1695–1706, 2008.

[204] J. Ton and B. Mauch-Mani, "β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose," *The Plant Journal*, vol. 38, no. 1, pp. 119–130, 2004.

[205] N. Clay, A. Adio, C. Denoux, G. Jander, and F. Ausubel, "Glucosinolate metabolites required for an *Arabidopsis* innate immune response," *Science*, vol. 323, no. 5910, pp. 95–101, 2009.

[206] E. Luna, V. Pastor, J. Robert, V. Flors, B. Mauch-Mani, and J. Ton, "Callose deposition: a multifaceted plant defense response," *Molecular Plant-Microbe Interactions*, vol. 24, no. 2, pp. 183–193, 2011.

[207] A. Robert-Seilaniantz, M. Grant, and J. Jones, "Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism," *Annual Review of Phytopathology*, vol. 49, pp. 317–343, 2011.

[208] G. A. Howe and G. Jander, "Plant immunity to insect herbivores," *Annual Review of Plant Biology*, vol. 59, pp. 41–66, 2008.

[209] M. De Vos, V. Van Oosten, R. Van Poecke, J. Van Pelt, M. Pozo, M. Mueller, A. Buchala, J. Métraux, L. Van Loon, M. Dicke, and C. M. J. Pieterse, "Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack," *Molecular Plant-Microbe Interactions*, vol. 18, no. 9, pp. 923–937, 2005.

[210] J. Glazebrook, "Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens," *Annual Review of Phytopathology*, vol. 43, pp. 205–227, 2005.

[211] C. Diezel, C. von Dahl, E. Gaquerel, and I. Baldwin, "Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling," *Plant Physiology*, vol. 150, no. 3, pp. 1576–1586, 2009.

[212] J. Louis, S. Basu, S. Varsani, L. Castano-Duque, V. Jiang, W. P. Williams, G. W. Felton, and D. S. Luthe, "Ethylene contributes to maize insect resistance1-mediated maize defense against the phloem sap-sucking corn leaf aphid," *Plant Physiology*, vol. 169, no. 1, pp. 313–324, 2015.

[213] G. Thompson and F. Goggin, "Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects," *Journal of Experimental Botany*, vol. 57, no. 4, pp. 755–766, 2006.

[214] C. Ellis, I. Karafyllidis, and J. G. Turner, "Constitutive activation of jasmonate signaling in an *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*," *Molecular Plant-Microbe Interactions*, vol. 15, no. 10, pp. 1025–1030, 2002.

[215] M. de Vos, J. Kim, and G. Jander, "Biochemistry and molecular biology of *Arabidopsis*–aphid interactions," *Bioessays*, vol. 29, no. 9, pp. 871–883, 2007.

[216] C. M. Pieterse, A. Leon-Reyes, S. Van der Ent, and S. C. Van Wees, "Networking by small-molecule hormones in plant immunity," *Nature Chemical Biology*, vol. 5, no. 5, pp. 308–316, 2009.

[217] D. M. Brooks, C. L. Bender, and B. N. Kunkel, "The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*," *Molecular Plant Pathology*, vol. 6, no. 6, pp. 629–639, 2005.

[218] S. R. Uppalapati, Y. Ishiga, T. Wangdi, B. N. Kunkel, A. Anand, K. S. Mysore, and C. L. Bender, "The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic

acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. tomato DC3000," *Molecular Plant-Microbe Interactions*, vol. 20, no. 8, pp. 955–965, 2007.

[219] J. X. Becerra, "Macroevolutionary and geographical intensification of chemical defense in plants driven by insect herbivore selection pressure," *Current Opinion in Insect Science*, vol. 8, pp. 15–21, 2015.

[220] D. Kliebenstein, J. Kroymann, P. Brown, A. Figuth, D. Pedersen, J. Gershenzon, and T. Mitchell-Olds, "Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation," *Plant Physiology*, vol. 126, no. 2, pp. 811–825, 2001.

[221] M. Reichelt, P. Brown, B. Schneider, N. Oldham, E. Stauber, J. Tokuhisa, D. Kliebenstein, T. Mitchell-Olds, and J. Gershenzon, "Benzoic acid glucosinolate esters and other glucosinolates from *Arabidopsis thaliana*," *Phytochemistry*, vol. 59, no. 6, pp. 663–671, 2002.

[222] J. W. Fahey, A. T. Zalcmann, and P. Talalay, "The chemical diversity and distribution of glucosinolates and isothiocyanates among plants," *Phytochemistry*, vol. 56, no. 1, pp. 5–51, 2001.

[223] B. A. Halkier and J. Gershenzon, "Biology and biochemistry of glucosinolates," *Annual Review of Plant Biology*, vol. 57, pp. 303–333, 2006.

[224] R. J. Hopkins, N. M. van Dam, and J. J. van Loon, "Role of glucosinolates in insect-plant relationships and multitrophic interactions," *Annual Review of Entomology*, vol. 54, pp. 57–83, 2009.

[225] I. Mewis, J. G. Tokuhisa, J. C. Schultz, H. M. Appel, C. Ulrichs, and J. Gershenzon, "Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways," *Phytochemistry*, vol. 67, no. 22, pp. 2450–2462, 2006.

[226] J. H. Kim and G. Jander, "*Myzus persicae* (green peach aphid) feeding on *Arabidopsis* induces the formation of a deterrent indole glucosinolate," *The Plant Journal*, vol. 49, no. 6, pp. 1008–1019, 2007.

[227] P. Bednarek, M. Piślewska-Bednarek, A. Svatoš, B. Schneider, J. Doubskỳ, M. Mansurova, M. Humphry, C. Consonni, R. Panstruga, A. Sanchez-Vallet, A. Molina, and P. Schulze-Lefert, "A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense," *Science*, vol. 323, no. 5910, pp. 101–106, 2009.

[228] V. Lipka, J. Dittgen, P. Bednarek, R. Bhat, M. Wiermer, M. Stein, J. Landtag, W. Brandt, S. Rosahl, D. Scheel, F. Llorente, A. Molina, J. Parker, S. Somerville, and P. Schulze-Lefert, "Pre-and postinvasion defenses both contribute to nonhost resistance in *Arabidopsis*," *Science*, vol. 310, no. 5751, pp. 1180–1183, 2005.

[229] M. Levy, Q. Wang, R. Kaspi, M. Parrella, and S. Abel, "*Arabidopsis* IQD1, a novel calmodulin-binding nuclear protein, stimulates glucosinolate accumulation and plant defense," *The Plant Journal*, vol. 43, no. 1, pp. 79–96, 2005.

[230] J. H. Kim, B. W. Lee, F. C. Schroeder, and G. Jander, "Identification of indole glucosinolate breakdown products with antifeedant effects on *Myzus persicae* (green peach aphid)," *The Plant Journal*, vol. 54, no. 6, pp. 1015–1026, 2008.

[231] I. Mewis, H. Appel, A. Hom, R. Raina, and J. Schultz, "Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects," *Plant Physiology*, vol. 138, no. 2, p. 1149, 2005.

[232] B. Pontoppidan, B. Ekbom, S. Eriksson, and J. Meijer, "Purification and characterization of myrosinase from the cabbage aphid (*Brevicoryne brassicae*), a brassica herbivore," *European Journal of Biochemistry*, vol. 268, no. 4, pp. 1041–1048, 2001.

[233] F. Francis, G. Lognay, J. Wathelet, and E. Haubruge, "Effects of allelochemicals from first (*Brassicaceae*) and second (*Myzus persicae* and *Brevicoryne brassicae*) trophic levels on *Adalia bipunctata*," *Journal of Chemical Ecology*, vol. 27, no. 2, pp. 243–256, 2001.

[234] F. Francis, G. Lognay, J. Wathelet, and E. Haubruge, "Characterisation of aphid myrosinase and degradation studies of glucosinolates," *Archives of Insect Biochemistry and Physiology*, vol. 50, no. 4, pp. 173–182, 2002.

[235] N. Zhou, T. Tootle, and J. Glazebrook, "*Arabidopsis* PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase," *The Plant Cell*, vol. 11, no. 12, pp. 2419–2428, 1999.

[236] R. Schuhegger, M. Nafisi, M. Mansourova, B. L. Petersen, C. E. Olsen, A. Svatoš, B. A. Halkier, and E. Glawischnig, "CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis," *Plant Physiology*, vol. 141, no. 4, pp. 1248–1254, 2006.

[237] C. Böttcher, L. Westphal, C. Schmotz, E. Prade, D. Scheel, and E. Glawischnig, "The multifunctional enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) converts cysteine-indole-3-acetonitrile to camalexin in the indole-3-acetonitrile metabolic network of *Arabidopsis thaliana*," *The Plant Cell*, vol. 21, no. 6, pp. 1830–1845, 2009.

[238] G. J. Kettles, C. Drurey, H.-j. Schoonbeek, A. J. Maule, and S. A. Hogenhout, "Resistance of *Arabidopsis thaliana* to the green peach aphid, *Myzus persicae*, involves camalexin and is regulated by microRNAs," *New Phytologist*, vol. 198, pp. 1178–1190, June 2013.

[239] B. P. Thomma, I. Nelissen, K. Eggermont, and W. F. Broekaert, "Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*," *The Plant Journal*, vol. 19, no. 2, pp. 163–171, 1999.

[240] S. Ferrari, J. M. Plotnikova, G. De Lorenzo, and F. M. Ausubel, "*Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4," *The Plant Journal*, vol. 35, no. 2, pp. 193–205, 2003.

[241] I. Ahuja, R. Kissen, and A. M. Bones, "Phytoalexins in defense against pathogens," *Trends in plant science*, vol. 17, no. 2, pp. 73–90, 2012.

[242] M. Dicke, J. Van Loon, and R. Soler, "Chemical complexity of volatiles from plants induced by multiple attack," *Nature Chemical Biology*, vol. 5, no. 5, pp. 317–324, 2009.

[243] M. Dicke and I. Baldwin, "The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'," *Trends in Plant Science*, vol. 15, no. 3, pp. 167–175, 2010.

[244] J. Zhu and K. Park, "Methyl salicylate, a soybean aphid-induced plant volatile attractive to the predator *Coccinella septempunctata*," *Journal of Chemical Ecology*, vol. 31, no. 8, pp. 1733–1746, 2005.

[245] F. Verheggen, L. Arnaud, S. Bartram, M. Gohy, and E. Haubruge, "Aphid and plant volatiles induce oviposition in an aphidophagous hoverfly," *Journal of Chemical Ecology*, vol. 34, no. 3, pp. 301–307, 2008.

[246] G. Imbiscuso, A. Trotta, M. Maffei, and S. Bossi, "Herbivory induces a ROS burst and the release of volatile organic compounds in the fern *Pteris vittata* l.," *Journal of Plant Interactions*, vol. 4, no. 1, pp. 15–22, 2009.

[247] K. Shiojiri, K. Kishimoto, R. Ozawa, S. Kugimiya, S. Urashimo, G. Arimura, J. Horiuchi, T. Nishioka, K. Matsui, and J. Takabayashi, "Changing green leaf volatile biosynthesis in plants: an approach for improving plant resistance against both herbivores and pathogens," *Proceedings of the National Academy of Sciences*, vol. 103, no. 45, pp. 16672–16676, 2006.

[248] K. Kishimoto, K. Matsui, R. Ozawa, and J. Takabayashi, "Components of C6-aldehyde-induced resistance in *Arabidopsis thaliana* against a necrotrophic fungal pathogen, *Botrytis cinerea*," *Plant Science*, vol. 170, no. 4, pp. 715–723, 2006.

[249] K. Matsui, A. Minami, E. Hornung, H. Shibata, K. Kishimoto, V. Ahnert, H. Kindl, T. Kajiwara, and I. Feussner, "Biosynthesis of fatty acid derived aldehydes is induced upon mechanical wounding and its products show fungicidal activities in cucumber," *Phytochemistry*, vol. 67, no. 7, pp. 649–657, 2006.

[250] E. Schwartzberg, K. Böröczky, and J. Tumlinson, "Pea aphids, *Acyrthosiphon pisum*, suppress induced plant volatiles in broad bean, *Vicia faba*," *Journal of Chemical Ecology*, pp. 1–8, 2011.

[251] J. E. Galán and A. Collmer, "Type III secretion machines: bacterial devices for protein delivery into host cells," *Science*, vol. 284, no. 5418, pp. 1322–1328, 1999.

[252] D. P. Garnica, A. Nemri, N. M. Upadhyaya, J. P. Rathjen, and P. N. Dodds, "The ins and outs of rust haustoria," *PLoS Pathogens*, vol. 10, no. 9, p. e1004329, 2014.

[253] T. O. Bozkurt, S. Schornack, M. J. Banfield, and S. Kamoun, "Oomycetes, effectors, and all that jazz," *Current Opinion in Plant Biology*, vol. 15, no. 4, pp. 483–492, 2012.

[254] E. Davis, R. Hussey, M. Mitchum, and T. Baum, "Parasitism proteins in nematode–plant interactions," *Current Opinion in Plant Biology*, vol. 11, no. 4, pp. 360–366, 2008.

[255] R. Sarmento, F. Lemos, P. Bleeker, R. Schuurink, A. Pallini, M. Oliveira, E. Lima, M. Kant, M. Sabelis, and A. Janssen, "A herbivore that manipulates plant defence," *Ecology Letters*, vol. 14, pp. 229–236, 2011.

[256] R. O. Musser, S. M. Hum-Musser, H. Eichenseer, M. Peiffer, G. Ervin, J. B. Murphy, and G. W. Felton, "Herbivory: caterpillar saliva beats plant defences," *Nature*, vol. 416, no. 6881, pp. 599–600, 2002.

[257] R. O. Musser, D. F. Cipollini, S. M. Hum-Musser, S. A. Williams, J. K. Brown, and G. W. Felton, "Evidence that the caterpillar salivary enzyme glucose oxidase provides herbivore offense in Solanaceous plants," *Archives of Insect Biochemistry and Physiology*, vol. 58, no. 2, pp. 128–137, 2005.

[258] H. Eichenseer, M. Mathews, J. S. Powell, and G. W. Felton, "Survey of a salivary effector in caterpillars: glucose oxidase variation and correlation with host range," *Journal of Chemical Ecology*, vol. 36, no. 8, pp. 885–897, 2010.

[259] E. Naessens, G. Dubreuil, P. Giordanengo, O. L. Baron, N. Minet-Kebdani, H. Keller, and C. Coustau, "A secreted MIF cytokine enables aphid feeding and represses plant immune responses," *Current Biology*, vol. 25, no. 14, pp. 1898–1903, 2015.

[260] V. Göhre and S. Robatzek, "Breaking the barriers: microbial effector molecules subvert plant immunity," *Annual Review of Phytopathology*, vol. 46, pp. 189–215, 2008.

[261] L. Shan, P. He, J. Li, A. Heese, S. Peck, T. Nürnberger, G. Martin, and J. Sheen, "Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity," *Cell Host & Microbe*, vol. 4, no. 1, pp. 17–27, 2008.

[262] M. De Torres, J. Mansfield, N. Grabov, I. Brown, H. Ammouneh, G. Tsiamis, A. Forsyth, S. Robatzek, M. Grant, and J. Boch, "*Pseudomonas syringae* effector AvrPtoB suppresses basal defence in *Arabidopsis*," *The Plant Journal*, vol. 47, no. 3, pp. 368–382, 2006.

[263] J. Zhou, S. Wu, X. Chen, C. Liu, J. Sheen, L. Shan, and P. He, "The *Pseudomonas syringae* effector HopF2 suppresses *Arabidopsis* immunity by targeting BAK1," *The Plant Journal*, vol. 77, no. 2, pp. 235–245, 2014.

[264] S. Gimenez-Ibanez, D. R. Hann, V. Ntoukakis, E. Petutschnig, V. Lipka, and J. P. Rathjen, "AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants," *Current Biology*, vol. 19, no. 5, pp. 423–429, 2009.

[265] V. Göhre, T. Spallek, H. Häweker, S. Mersmann, T. Mentzel, T. Boller, M. de Torres, J. W. Mansfield, and S. Robatzek, "Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB," *Current Biology*, vol. 18, no. 23, pp. 1824–1832, 2008.

[266] J. Zhang, W. Li, T. Xiang, Z. Liu, K. Laluk, X. Ding, Y. Zou, M. Gao, X. Zhang, S. Chen, T. Mengiste, Y. Zhang, and J.-M. Zhou, "Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector," *Cell Host & Microbe*, vol. 7, no. 4, pp. 290–301, 2010.

[267] C. Hemetsberger, C. Herrberger, B. Zechmann, M. Hillmer, and G. Doehlemann, "The *Ustilago maydis* effector Pep1 suppresses plant immunity by inhibition of host peroxidase activity," *PLoS Pathogens*, vol. 8, no. 5, p. e1002684, 2012.

[268] J. Zhang, F. Shao, Y. Li, H. Cui, L. Chen, H. Li, Y. Zou, C. Long, L. Lan, J. Chai, S. Chen, X. Tang, and J.-M. Zhou, "A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants," *Cell Host & Microbe*, vol. 1, no. 3, pp. 175–185, 2007.

[269] P. Bednarek, C. Kwon, and P. Schulze-Lefert, "Not a peripheral issue: secretion in plant–microbe interactions," *Current Opinion in Plant Biology*, vol. 13, no. 4, pp. 378–387, 2010.

[270] K. Nomura, S. DebRoy, Y. H. Lee, N. Pumplin, J. Jones, and S. Y. He, "A bacterial virulence protein suppresses host innate immunity to cause plant disease," *Science*, vol. 313, no. 5784, pp. 220–223, 2006.

[271] S. M. Schmidt, H. Kuhn, C. Micali, C. Liller, M. Kwaaitaal, and R. Panstruga, "Interaction of a *Blumeria graminis f. sp. hordei* effector candidate with a barley ARF-GAP suggests that host vesicle trafficking is a fungal pathogenicity target," *Molecular Plant Pathology*, vol. 15, no. 6, pp. 535–549, 2014.

[272] E. Nielsen, A. Y. Cheung, and T. Ueda, "The regulatory RAB and ARF GTPases for vesicular trafficking," *Plant Physiology*, vol. 147, no. 4, pp. 1516–1526, 2008.

[273] J. Jones and J. Dangl, "The plant immune system," *Nature*, vol. 444, no. 7117, pp. 323–329, 2006.

[274] W. Gassmann and S. Bhattacharjee, "Effector-triggered immunity signaling: from gene-for-gene pathways to protein-protein interaction networks," *Molecular Plant-Microbe Interactions*, vol. 25, no. 7, pp. 862–868, 2012.

[275] K. Tsuda and F. Katagiri, "Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity," *Current opinion in Plant Biology*, vol. 13, no. 4, pp. 459–465, 2010.

[276] K. Naito, Y. Ishiga, K. Toyoda, T. Shiraishi, and Y. Ichinose, "N-terminal domain including conserved flg22 is required for flagellin-induced hypersensitive cell death in *Arabidopsis thaliana*," *Journal of General Plant Pathology*, vol. 73, no. 4, pp. 281–285, 2007.

[277] L. Wirthmueller, Y. Zhang, J. Jones, and J. Parker, "Nuclear accumulation of the *Arabidopsis* immune receptor RPS4 is necessary for triggering EDS1-dependent defense," *Current Biology*, vol. 17, no. 23, pp. 2023–2029, 2007.

[278] B. Thomma, T. Nürnberger, and M. Joosten, "Of PAMPs and effectors: the blurred PTI-ETI dichotomy," *The Plant Cell*, vol. 23, no. 1, pp. 4–15, 2011.

[279] T. K. Eitas and J. L. Dangl, "NB-LRR proteins: pairs, pieces, perception, partners, and pathways," *Current Opinion in Plant Biology*, vol. 13, no. 4, pp. 472–477, 2010.

[280] M. Rossi, F. L. Goggin, S. B. Milligan, I. Kaloshian, D. E. Ullman, and V. M. Williamson, "The nematode resistance gene Mi of tomato confers resistance against the potato aphid," *Proceedings of the National Academy of Sciences*, vol. 95, no. 17, pp. 9750–9754, 1998.

[281] G. Nombela, F. Beitia, and M. Muniz, "A differential interaction study of *Bemisia tabaci* Q-biotype on commercial tomato varieties with or without the Mi resistance gene, and comparative host responses with the B-biotype," *Entomologia Experimentalis et Applicata*, vol. 98, no. 3, pp. 339–344, 2001.

[282] C. L. Casteel, L. L. Walling, and T. D. Paine, "Behavior and biology of the tomato psyllid, *Bactericerca cockerelli*, in response to the Mi-1.2 gene," *Entomologia Experimentalis et Applicata*, vol. 121, no. 1, pp. 67–72, 2006.

[283] C. Hwang, A. Bhakta, G. Truesdell, W. Pudlo, and V. Williamson, "Evidence for a role of the N terminus and leucine-rich repeat region of the Mi gene product in regulation of localized cell death," *The Plant Cell*, vol. 12, no. 8, pp. 1319–1330, 2000.

[284] I. Kaloshian, M. Kinsey, D. Ullman, and V. Williamson, "The impact of Meu1-mediated resistance in tomato on longevity, fecundity and behavior of the potato aphid, *Macrosiphum euphorbiae*," *Entomologia Experimentalis et Applicata*, vol. 83, no. 2, pp. 181–187, 1997.

[285] Q. Li, Q. Xie, J. Smith-Becker, D. Navarre, and I. Kaloshian, "Mi-1-mediated aphid resistance involves salicylic acid and mitogen-activated protein kinase signaling cascades," *Molecular Plant-Microbe Interactions*, vol. 19, no. 6, pp. 655–664, 2006.

[286] K. Bhattarai, H. Atamian, I. Kaloshian, and T. Eulgem, "WRKY72-type transcription factors contribute to basal immunity in tomato and *Arabidopsis* as well as gene-for-gene resistance mediated by the tomato R-gene Mi-1," *The Plant Journal*, vol. 63, no. 2, pp. 229–240, 2010.

[287] L. Deslandes, J. Olivier, F. Theulières, J. Hirsch, D. X. Feng, P. Bittner-Eddy, J. Beynon, and Y. Marco, "Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes," *Proceedings of the National Academy of Sciences*, vol. 99, no. 4, pp. 2404–2409, 2002.

[288] L. Deslandes, J. Olivier, N. Peeters, D. X. Feng, M. Khounlotham, C. Boucher, I. Somssich, S. Genin, and Y. Marco, "Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus," *Proceedings of the National Academy of Sciences*, vol. 100, no. 13, pp. 8024–8029, 2003.

[289] E. A. Van Der Biezen and J. D. Jones, "Plant disease-resistance proteins and the gene-for-gene concept," *Trends in Biochemical Sciences*, vol. 23, no. 12, pp. 454–456, 1998.

[290] J. L. Dangl and J. D. Jones, "Plant pathogens and integrated defence responses to infection," *Nature*, vol. 411, no. 6839, pp. 826–833, 2001.

[291] D. Mackey, B. F. Holt, A. Wiig, and J. L. Dangl, "RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*," *Cell*, vol. 108, no. 6, pp. 743–754, 2002.

[292] D. Selote and A. Kachroo, "RIN4-like proteins mediate resistance protein-derived soybean defense against *Pseudomonas syringae*," *Plant Signaling & Behavior*, vol. 5, no. 11, pp. 1453–1456, 2010.

[293] M. G. Kim, L. da Cunha, A. J. McFall, Y. Belkhadir, S. DebRoy, J. L. Dangl, and D. Mackey, "Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in *Arabidopsis*," *Cell*, vol. 121, no. 5, pp. 749–759, 2005.

[294] J. Liu, J. Elmore, A. Fuglsang, M. Palmgren, B. Staskawicz, and G. Coaker, "RIN4 functions with plasma membrane H+-ATPases to regulate stomatal apertures during pathogen attack," *PLoS Biology*, vol. 7, no. 6, p. e1000139, 2009.

[295] D. Mackey, Y. Belkhadir, J. M. Alonso, J. R. Ecker, and J. L. Dangl, "Arabidopsis RIN4 is a target of the type iii virulence effector AvrRpt2 and modulates RPS2-mediated resistance," *Cell*, vol. 112, no. 3, pp. 379–389, 2003.

[296] M. Axtell, S. Chisholm, D. Dahlbeck, and B. Staskawicz, "Genetic and molecular evidence that the *Pseudomonas syringae* type III effector protein AvrRpt2 is a cysteine protease," *Molecular Microbiology*, vol. 49, no. 6, pp. 1537–1546, 2003.

[297] A. J. Afzal, L. da Cunha, and D. Mackey, "Separable fragments and membrane tethering of *Arabidopsis* RIN4 regulate its suppression of PAMP-triggered immunity," *The Plant Cell*, vol. 23, no. 10, pp. 3798–3811, 2011.

[298] B. Day, D. Dahlbeck, J. Huang, S. Chisholm, D. Li, and B. Staskawicz, "Molecular basis for the RIN4 negative regulation of RPS2 disease resistance," *The Plant Cell*, vol. 17, no. 4, pp. 1292–1305, 2005.

[299] E.-H. Chung, L. da Cunha, A.-J. Wu, Z. Gao, K. Cherkis, A. J. Afzal, D. Mackey, and J. L. Dangl, "Specific threonine phosphorylation of a host target by two unrelated type III effectors activates a host innate immune receptor in plants," *Cell Host & Microbe*, vol. 9, no. 2, pp. 125–136, 2011.

[300] J. Liu, J. M. Elmore, Z.-J. D. Lin, and G. Coaker, "A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor," *Cell Host & Microbe*, vol. 9, no. 2, pp. 137–146, 2011.

[301] M. S. Mukhtar, A.-R. Carvunis, M. Dreze, P. Epple, J. Steinbrenner, J. Moore, M. Tasan, M. Galli, T. Hao, M. T. Nishimura, S. J. Pevzner, S. E. Donovan, L. Ghamsari, B. Santhanam, V. Romero, M. M. Poulin, F. Gebreab, B. J. Gutierrez, S. Tam, D. Monachello, M. Boxem, C. J. Harbort, N. McDonald, L. Gal, H. Chen, Y. He, E. U. E. Consortium, J. Vandenhaute, F. P. Roth, D. E. Hill, J. R. Ecker, M. Vidal, J. Beynon, P. Braun, and J. L. Dangl, "Independently evolved virulence effectors converge onto hubs in a plant immune system network," *Science*, vol. 333, no. 6042, pp. 596–601, 2011.

[302] R. A. van der Hoorn and S. Kamoun, "From guard to decoy: a new model for perception of plant pathogen effectors," *The Plant Cell*, vol. 20, no. 8, pp. 2009–2017, 2008.

[303] A. P. Macho and C. Zipfel, "Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors," *Current Opinion in Microbiology*, vol. 23, pp. 14–22, 2015.

[304] T. Mucyn, A. Clemente, V. Andriotis, A. Balmuth, G. Oldroyd, B. Staskawicz, and J. Rathjen, "The tomato NBARC-LRR protein Prf interacts with Pto kinase in vivo to regulate specific plant immunity," *The Plant Cell*, vol. 18, no. 10, pp. 2792–2806, 2006.

[305] V. Ntoukakis, I. M. Saur, B. Conlan, and J. P. Rathjen, "The changing of the guard: the Pto/Prf receptor complex of tomato and pathogen recognition," *Current Opinion in Plant Biology*, vol. 20, pp. 69–74, 2014.

[306] Y. Zhao, A. K. Hull, N. R. Gupta, K. A. Goss, J. Alonso, J. R. Ecker, J. Normanly, J. Chory, and J. L. Celenza, "Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3," *Genes & Development*, vol. 16, no. 23, pp. 3100–3112, 2002.

[307] J. Glazebrook and F. Ausubel, "Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens," *Proceedings of the National Academy of Sciences*, vol. 91, no. 19, pp. 8955–8959, 1994.

[308] S. E. Jacobsen, M. P. Running, and E. M. Meyerowitz, "Disruption of an RNA helicase/RNAse iii gene in *Arabidopsis* causes unregulated cell division in floral meristems," *Development*, vol. 126, no. 23, pp. 5231–5243, 1999.

[309] H. Ullah, J.-G. Chen, B. Temple, D. C. Boyes, J. M. Alonso, K. R. Davis, J. R. Ecker, and A. M. Jones, "The β-subunit of the *Arabidopsis* G protein negatively regulates auxin-induced cell division and affects multiple developmental processes," *The Plant Cell*, vol. 15, no. 2, pp. 393–409, 2003.

[310] M. Gao, X. Wang, D. Wang, F. Xu, X. Ding, Z. Zhang, D. Bi, Y. T. Cheng, S. Chen, X. Li, and Y. Zhang, "Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*," *Cell Host & Microbe*, vol. 6, no. 1, pp. 34–44, 2009.

[311] S. J. Clough and A. F. Bent, "Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*," *The Plant Journal*, vol. 16, no. 6, pp. 735–743, 1998.

[312] G. J. Kettles, *Analysis of Arabidopsis thaliana small RNA-mediated defence responses to infestation by the green peach aphid Myzus persicae*. PhD thesis, University of East Anglia, September 2011.

[313] N. Kleinboelting, G. Huep, A. Kloetgen, P. Viehoever, and B. Weisshaar, "GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database," *Nucleic Acids Research*, vol. 40, pp. 1211–1215, 2011.

[314] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman, "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes," *Genome Biology*, vol. 3, no. 7, p. 34, 2002.

[315] T. Czechowski, M. Stitt, T. Altmann, M. K. Udvardi, and W.-R. Scheible, "Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*," *Plant Physiology*, vol. 139, no. 1, pp. 5–17, 2005.

[316] D. C. Prince, *Dissecting the role of plant immunity in plant-aphid interactions*. PhD thesis, University of East Anglia, September 2012.

[317] A. Coleman, *Control of Turnip Yellows Virus: Assessing impact on oilseed rape quality traits and dissecting circulative transmission by aphids*. PhD thesis, University of East Anglia, September 2013.

[318] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT–PCR," *Nucleic Acids Research*, vol. 29, no. 9, p. 45, 2001.

[319] C. Segonzac, Z. L. Nimchuk, M. Beck, P. T. Tarr, S. Robatzek, E. M. Meyerowitz, and C. Zipfel, "The shoot apical meristem regulatory peptide CLV3 does not activate innate immunity," *The Plant Cell*, vol. 24, no. 8, pp. 3186–3192, 2012.

[320] C. Chassot, A. Buchala, H.-j. Schoonbeek, J.-P. Métraux, and O. Lamotte, "Wounding of *Arabidopsis* leaves causes a powerful but transient protection against *Botrytis* infection," *The Plant Journal*, vol. 55, no. 4, pp. 555–567, 2008.

[321] D. C. Prince, S. T. Mugford, T. R. Vincent, and S. A. Hogenhout, "Pea aphid survival assays on *Arabidopsis thaliana*," *Bio-protocol*, vol. 4, no. 19, p. e1251, 2014.

[322] Y. Nishinaka, Y. Aramaki, H. Yoshida, H. Masuya, T. Sugawara, and Y. Ichimori, "A new sensitive chemiluminescence probe, L-012, for measuring the production of superoxide anion by cells," *Biochemical and Biophysical Research Communications*, vol. 193, no. 2, pp. 554–559, 1993.

[323] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[324] F. He, "Laemmli-SDS-PAGE." Bio Protocol, 2011.

[325] M. Fromont-Racine, J.-C. Rain, and P. Legrain, "Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens," *Nature Genetics*, vol. 16, no. 3, pp. 277–282, 1997.

[326] J. A. Gatehouse, "Plant resistance towards insect herbivores: a dynamic interaction," *New Phytologist*, vol. 156, no. 2, pp. 145–169, 2002.

[327] M. Skibbe, N. Qu, I. Galis, and I. T. Baldwin, "Induced plant defenses in the natural environment: *Nicotiana attenuata* WRKY3 and WRKY6 coordinate responses to herbivory," *The Plant Cell*, vol. 20, no. 7, pp. 1984–2000, 2008.

[328] V. Nicaise, M. Roux, and C. Zipfel, "Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm," *Plant Physiology*, vol. 150, no. 4, pp. 1638–1647, 2009.

[329] C. Zipfel, "Plant pattern-recognition receptors," *Trends in Immunology*, vol. 35, no. 7, pp. 345–351, 2014.

[330] T. Boller, "Chemoperception of microbial signals in plant cells," *Annual Review of Plant Biology*, vol. 46, no. 1, pp. 189–214, 1995.

[331] N. Shibuya and E. Minami, "Oligosaccharide signalling for defence responses in plant," *Physiological and Molecular Plant Pathology*, vol. 59, no. 5, pp. 223–233, 2001.

[332] S. Ferrari, D. V. Savatin, F. Sicilia, G. Gramegna, F. Cervone, and G. De Lorenzo, "Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development," *Frontiers in Plant Science*, vol. 4, 2013.

[333] H. Alborn, T. Turlings, T. Jones, G. Stenhagen, J. Loughrin, and J. Tumlinson, "An elicitor of plant volatiles from beet armyworm oral secretion," *Science*, vol. 276, no. 5314, pp. 945–949, 1997.

[334] Y. Liu, W.-L. Wang, G.-X. Guo, and X.-L. Ji, "Volatile emission in wheat and parasitism by *Aphidius avenae* after exogenous application of salivary enzymes of *Sitobion avenae*," *Entomologia Experimentalis et Applicata*, vol. 130, no. 3, pp. 215–221, 2009.

[335] R. Ma, J.-L. Chen, D.-F. Cheng, and J.-R. Sun, "Activation of defense mechanism in wheat by polyphenol oxidase from aphid saliva," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 4, pp. 2410–2418, 2010.

[336] S. R. Lloyd, H.-j. Schoonbeek, M. Trick, C. Zipfel, and C. J. Ridout, "Methods to study PAMP-triggered immunity in Brassica species," *Molecular Plant-Microbe Interactions*, vol. 27, no. 3, pp. 286–295, 2014.

[337] X. Chen and J. Kim, "Callose synthesis in higher plants," *Plant Signaling & Behavior*, vol. 4, no. 6, p. 489, 2009.

[338] E. S. Villada, E. G. González, A. I. López-Sesé, A. F. Castiel, and M. L. Gómez-Guillamón, "Hypersensitive response to *Aphis gossypii* glover in melon genotypes carrying the Vat gene," *Journal of Experimental Botany*, vol. 60, no. 11, pp. 3269–3277, 2009.

[339] P. J. Moran and G. A. Thompson, "Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways," *Plant Physiology*, vol. 125, no. 2, pp. 1074–1085, 2001.

[340] D. C. Prince, C. Drurey, C. Zipfel, and S. A. Hogenhout, "The leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 and the cytochrome P450 PHYTOALEXIN DEFICIENT3 contribute to innate immunity to aphids in *Arabidopsis*," *Plant Physiology*, vol. 164, no. 4, pp. 2207–2219, 2014.

[341] M. Pfalz, H. Vogel, and J. Kroymann, "The gene controlling the indole glucosinolate modifier1 quantitative trait locus alters indole glucosinolate structures and aphid resistance in *Arabidopsis*," *The Plant Cell*, vol. 21, no. 3, pp. 985–999, 2009.

[342] A. Gust, R. Biswas, H. Lenz, T. Rauhut, S. Ranf, B. Kemmerling, F. Götz, E. Glawischnig, J. Lee, G. Felix, and T. Nurnberger, "Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*," *Journal of Biological Chemistry*, vol. 282, no. 44, pp. 32338–32348, 2007.

[343] C. Denoux, R. Galletti, N. Mammarella, S. Gopalan, D. Werck, G. De Lorenzo, S. Ferrari, F. M. Ausubel, and J. Dewdney, "Activation of defense response pathways by OGs and flg22 elicitors in *Arabidopsis* seedlings," *Molecular Plant*, vol. 1, no. 3, pp. 423–445, 2008.

[344] L. Gómez-Gómez, G. Felix, and T. Boller, "A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*," *The Plant Journal*, vol. 18, no. 3, pp. 277–284, 1999.

[345] R. Hammerschmidt, "Induced disease resistance: how do induced plants stop pathogens?," *Physiological and Molecular Plant Pathology*, vol. 55, no. 2, pp. 77–84, 1999.

[346] L. D. Keppler, C. J. Baker, and M. M. Atkinson, "Active oxygen production during a bacteria-induced hypersensitive reaction in tobacco suspension cells.," *Phytopathology*, vol. 79, no. 9, pp. 974–978, 1989.

[347] R. Medzhitov and C. A. Janeway, "Innate immunity: the virtues of a nonclonal system of recognition," *Cell*, vol. 91, no. 3, pp. 295–298, 1997.

[348] Y. Kadota, K. Shirasu, and C. Zipfel, "Regulation of the NADPH oxidase RBOHD during plant immunity," *Plant and Cell Physiology*, vol. 56, no. 8, pp. 1472–1480, 2015.

[349] J. Monaghan and C. Zipfel, "Plant pattern recognition receptor complexes at the plasma membrane," *Current Opinion in Plant Biology*, vol. 15, no. 4, pp. 349–357, 2012.

[350] B. Kemmerling, A. Schwedt, P. Rodriguez, S. Mazzotta, M. Frank, S. A. Qamar, T. Mengiste, S. Betsuyaku, J. E. Parker, C. Müssig, B. Thomma, C. Albrecht, S. C. de Vries, H. Hirt, and T. Nurnberger, "The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control," *Current Biology*, vol. 17, no. 13, pp. 1116–1122, 2007.

[351] W. Park, J. Li, R. Song, J. Messing, and X. Chen, "CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*," *Current Biology*, vol. 12, no. 17, pp. 1484–1495, 2002.

[352] Y. Kurihara and Y. Watanabe, "*Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions," *Proceedings of the National Academy of Sciences*, vol. 101, no. 34, pp. 12753–12758, 2004.

[353] I. R. Henderson, X. Zhang, C. Lu, L. Johnson, B. C. Meyers, P. J. Green, and S. E. Jacobsen, "Dissecting *Arabidopsis thaliana* DICER function in small RNA processing, gene silencing and DNA methylation patterning," *Nature Genetics*, vol. 38, no. 6, pp. 721–725, 2006.

[354] Y. Yamaguchi and A. Huffaker, "Endogenous peptide elicitors in higher plants," *Current Opinion in Plant Biology*, vol. 14, no. 4, pp. 351–357, 2011.

[355] S. Ranf, L. Eschen-Lippold, P. Pecher, J. Lee, and D. Scheel, "Interplay between calcium signalling and early signalling elements during defence responses to microbe-or damage-associated molecular patterns," *The Plant Journal*, vol. 68, no. 1, pp. 100–113, 2011.

[356] W. R. Cooper, J. W. Dillwith, and G. J. Puterka, "Salivary proteins of Russian wheat aphid (Hemiptera: Aphididae)," *Environmental Entomology*, vol. 39, no. 1, pp. 223–231, 2010.

[357] W. Wang, L. Luo, H. Lu, S. Chen, L. Kang, and F. Cui, "Angiotensin-converting enzymes modulate aphid-plant interactions," *Scientific Reports*, vol. 5, no. 8885, 2015.

[358] T. Will, K. Steckbauer, M. Hardt, and A. van Bel, "Aphid gel saliva: Sheath structure, protein composition and secretory dependence on stylet-tip milieu," *PloS One*, vol. 7, no. 10, p. e46903, 2012.

[359] D. A. Elzinga, M. De Vos, and G. Jander, "Suppression of plant defenses by a *Myzus persicae* (green peach aphid) salivary effector protein," *Molecular Plant-Microbe Interactions*, vol. 27, no. 7, pp. 747–756, 2014.

[360] S. A. Rao, J. C. Carolan, and T. L. Wilkinson, "Proteomic profiling of cereal aphid saliva reveals both ubiquitous and adaptive secreted proteins," *PLoS One*, vol. 8, no. 2, p. e57413, 2013.

[361] S. Gunderson and R. Schiavone, "The insect exoskeleton: a natural structural composite," *The Journal of The Minerals, Metals & Materials Society*, vol. 41, no. 11, pp. 60–63, 1989.

[362] A. Miya, P. Albert, T. Shinya, Y. Desaki, K. Ichimura, K. Shirasu, Y. Narusaka, N. Kawakami, H. Kaku, and N. Shibuya, "CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*," *Proceedings of the National Academy of Sciences*, vol. 104, no. 49, pp. 19613–19618, 2007.

[363] R. Chaudhary, H. S. Atamian, Z. Shen, S. P. Briggs, and I. Kaloshian, "GroEL from the endosymbiont *Buchnera aphidicola* betrays the aphid by triggering plant defense," *Proceedings of the National Academy of Sciences*, p. 201407687, 2014.

[364] S. Bouvaine, N. Boonham, and A. E. Douglas, "Interactions between a luteovirus and the GroEL chaperonin protein of the symbiotic bacterium *Buchnera aphidicola* of aphids," *Journal of General Virology*, vol. 92, no. 6, pp. 1467–1474, 2011.

[365] S. Vandermoten, N. Harmel, G. Mazzucchelli, E. De Pauw, E. Haubruge, and F. Francis, "Comparative analyses of salivary proteins from three aphid species," *Insect Molecular Biology*, vol. 23, no. 1, pp. 67–77, 2014.

[366] J. Song, J. Win, M. Tian, S. Schornack, F. Kaschani, M. Ilyas, R. A. van der Hoorn, and S. Kamoun, "Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3," *Proceedings of the National Academy of Sciences*, vol. 106, no. 5, pp. 1654–1659, 2009.

[367] Y. Yamaguchi, G. Pearce, and C. A. Ryan, "The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in *Arabidopsis*, is functional in transgenic tobacco cells," *Proceedings of the National Academy of Sciences*, vol. 103, no. 26, pp. 10104–10109, 2006.

[368] Y. Yamaguchi, A. Huffaker, A. C. Bryan, F. E. Tax, and C. A. Ryan, "PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in *Arabidopsis*," *The Plant Cell*, vol. 22, no. 2, pp. 508–522, 2010.

[369] M. Kutsukake, H. Shibao, N. Nikoh, M. Morioka, T. Tamura, T. Hoshino, S. Ohgiya, and T. Fukatsu, "Venomous protease of aphid soldier for colony defense," *Proceedings of the National Academy of Sciences*, vol. 101, no. 31, pp. 11338–11343, 2004.

[370] L. Mattiacci, M. Dicke, and M. A. Posthumus, "beta-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps," *Proceedings of the National Academy of Sciences*, vol. 92, no. 6, pp. 2036–2040, 1995.

[371] J. Glazebrook, M. Zook, F. Mert, I. Kagan, E. E. Rogers, I. R. Crute, E. B. Holub, R. Hammerschmidt, and F. M. Ausubel, "Phytoalexin-deficient mutants of *Arabidopsis* reveal that PAD4 encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance," *Genetics*, vol. 146, no. 1, pp. 381–392, 1997.

[372] V. Pegadaraju, C. Knepper, J. Reese, and J. Shah, "Premature leaf senescence modulated by the *Arabidopsis* PHYTOALEXIN DEFICIENT4 gene is associated with defense against the phloem-feeding green peach aphid," *Plant Physiology*, vol. 139, no. 4, pp. 1927–1934, 2005.

[373] E. E. Rogers, J. Glazebrook, and F. M. Ausubel, "Mode of action of the *Arabidopsis thaliana* phytoalexin camalexin and its role in *Arabidopsis*-pathogen interactions," *Molecular Plant Microbe Interactions*, vol. 9, pp. 748–757, 1996.

[374] R. Mezencev, T. Updegrove, P. Kutschy, M. Repovská, and J. F. McDonald, "Camalexin induces apoptosis in T-leukemia Jurkat cells by increased concentration of reactive oxygen species and activation of caspase-8 and caspase-9," *Journal of Natural Medicines*, vol. 65, no. 3-4, pp. 488–499, 2011.

[375] B. A. Smith, C. L. Neal, M. Chetram, B. Vo, R. Mezencev, C. Hinton, and V. A. Odero-Marah, "The phytoalexin camalexin mediates cytotoxicity towards aggressive prostate cancer cells via reactive oxygen species," *Journal of Natural Medicines*, vol. 67, no. 3, pp. 607–618, 2013.

[376] H. Saga, T. Ogawa, K. Kai, H. Suzuki, Y. Ogata, N. Sakurai, D. Shibata, and D. Ohta, "Identification and characterization of ANAC042, a transcription factor family gene involved in the regulation of

camalexin biosynthesis in *Arabidopsis*," *Molecular Plant-Microbe Interactions*, vol. 25, no. 5, pp. 684–696, 2012.

[377] R. P. Birkenbihl, C. Diezel, and I. E. Somssich, "*Arabidopsis* WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection," *Plant Physiology*, vol. 159, no. 1, pp. 266–285, 2012.

[378] G. Li, X. Meng, R. Wang, G. Mao, L. Han, Y. Liu, and S. Zhang, "Dual-level regulation of ACC synthase activity by MPK3/MPK6 cascade and its downstream WRKY transcription factor during ethylene induction in *Arabidopsis*," *PLoS Genetics*, vol. 8, no. 6, p. e1002767, 2012.

[379] R. Datta, D. Kumar, A. Sultana, S. Hazra, D. Bhattacharyya, and S. Chattopadhyay, "Glutathione regulates 1-aminocyclopropane-1-carboxylate synthase transcription via WRKY33 and 1-aminocyclopropane-1-carboxylate oxidase by modulating messenger RNA stability to induce ethylene synthesis during stress," *Plant Physiology*, vol. 169, no. 4, pp. 2963–2981, 2015.

[380] G. Mao, X. Meng, Y. Liu, Z. Zheng, Z. Chen, and S. Zhang, "Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in *Arabidopsis*," *The Plant Cell*, vol. 23, no. 4, pp. 1639–1653, 2011.

[381] J.-L. Qiu, B. K. Fiil, K. Petersen, H. B. Nielsen, C. J. Botanga, S. Thorgrimsen, K. Palma, M. C. Suarez-Rodriguez, S. Sandbech-Clausen, J. Lichota, P. Broderson, K. D. Grasser, O. Mattsson, J. Glazebrook, J. Mundy, and M. Petersen, "*Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus," *The EMBO Journal*, vol. 27, no. 16, pp. 2214–2221, 2008.

[382] G. Tena, M. Boudsocq, and J. Sheen, "Protein kinase signaling networks in plant innate immunity," *Current Opinion in Plant Biology*, vol. 14, no. 5, pp. 519–529, 2011.

[383] H. Adachi, T. Nakano, N. Miyagawa, N. Ishihama, M. Yoshioka, Y. Katou, T. Yaeno, K. Shirasu, and H. Yoshioka, "WRKY transcription factors phosphorylated by MAPK regulate a plant immune NADPH oxidase in *Nicotiana benthamiana*," *The Plant Cell*, vol. 27, no. 9, pp. 2645–2663, 2015.

[384] L. Navarro, P. Dunoyer, F. Jay, B. Arnold, N. Dharmasiri, M. Estelle, O. Voinnet, and J. Jones, "A plant miRNA contributes to antibacterial resistance by repressing auxin signaling," *Science*, vol. 312, no. 5772, pp. 436–439, 2006.

[385] W. Zhang, S. Gao, X. Zhou, P. Chellappan, Z. Chen, X. Zhou, X. Zhang, N. Fromuth, G. Coutino, M. Coffey, and H. Jin, "Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks," *Plant Molecular Biology*, vol. 75, no. 1-2, pp. 93–105, 2011.

[386] A. Robert-Seilaniantz, D. MacLean, Y. Jikumaru, L. Hill, S. Yamaguchi, Y. Kamiya, and J. D. Jones, "The microRNA miR393 re-directs secondary metabolite biosynthesis away from camalexin and towards glucosinolates," *The Plant Journal*, vol. 67, no. 2, pp. 218–231, 2011.

[387] S. Sattar, Y. Song, J. A. Anstead, R. Sunkar, and G. A. Thompson, "*Cucumis melo* microRNA expression profile during aphid herbivory in a resistant and susceptible interaction," *Molecular Plant-Microbe Interactions*, vol. 25, no. 6, pp. 839–848, 2012.

[388] S. P. Pandey, P. Shahi, K. Gase, and I. T. Baldwin, "Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in *Nicotiana attenuata*," *Proceedings of the National Academy of Sciences*, vol. 105, no. 12, pp. 4559–4564, 2008.

[389] C. Botha, B. Matsiliza, and C. Bornman, "Reduction in transport in wheat (*Triticum aestivum*) is caused by sustained phloem feeding by the Russian wheat aphid (*Diuraphis noxia*)," *South African Journal of Botany*, vol. 70, no. 2, pp. 249–254, 2004.

[390] M. Leitner, W. Boland, and A. Mithöfer, "Direct and indirect defences induced by piercing-sucking and chewing herbivores in *Medicago truncatula*," *New Phytologist*, vol. 167, no. 2, pp. 597–606, 2005.

[391] X. Liu, C. E. Williams, J. A. Nemacheck, H. Wang, S. Subramanyam, C. Zheng, and M.-S. Chen, "Reactive oxygen species are involved in plant defense against a gall midge," *Plant Physiology*, vol. 152, no. 2, pp. 985–999, 2010.

[392] I. Albert, H. Böhm, M. Albert, C. E. Feiler, J. Imkampe, N. Wallmeroth, C. Brancato, T. M. Raaymakers, S. Oome, H. Zhang, *et al.*, "An RLP23–SOBIR1–BAK1 complex mediates NLP-triggered immunity," *Nature Plants*, vol. 1, p. 15140, 2015.

[393] C. J. Kørner, D. Klauser, A. Niehl, A. Domínguez-Ferreras, D. Chinchilla, T. Boller, M. Heinlein, and D. Hann, "The immunity regulator BAK1 contributes to resistance against diverse RNA viruses," *Molecular Plant-Microbe Interactions*, vol. 26, no. 11, pp. 1271–1280, 2013.

[394] D. Yang, C. Hettenhausen, I. Baldwin, and J. Wu, "The multifaceted function of BAK1/SERK3: Plant immunity to pathogens and responses to insect herbivores.," *Plant Signaling & Behavior*, vol. 6, no. 9, 2011.

[395] S. Mantelin, H. Peng, B. Li, H. Atamian, F. Takken, and I. Kaloshian, "The receptor-like kinase SlSERK1 is required for Mi-1-mediated resistance to potato aphids in tomato," *The Plant Journal*, vol. 67, pp. 459–471, 2011.

[396] S.-H. Shiu and A. B. Bleecker, "Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis*," *Plant Physiology*, vol. 132, no. 2, pp. 530–543, 2003.

[397] G. Wang, U. Ellendorff, B. Kemp, J. W. Mansfield, A. Forsyth, K. Mitchell, K. Bastas, C.-M. Liu, A. Woods-Tör, C. Zipfel, P. de Wit, J. Jones, M. Tor, and B. Thomma, "A genome-wide functional investigation into the roles of receptor-like proteins in *Arabidopsis*," *Plant Physiology*, vol. 147, no. 2, pp. 503–517, 2008.

[398] T. Liu, Z. Liu, C. Song, Y. Hu, Z. Han, J. She, F. Fan, J. Wang, C. Jin, J. Chang, J.-M. Zhou, and J. Chai, "Chitin-induced dimerization activates a plant immune receptor," *Science*, vol. 336, no. 6085, pp. 1160–1164, 2012.

[399] R. Willmann, H. M. Lajunen, G. Erbs, M.-A. Newman, D. Kolb, K. Tsuda, F. Katagiri, J. Fliegmann, J.-J. Bono, J. V. Cullimore, A. K. Jehle, F. Gotz, A. Kulik, A. Molinaro, V. Lipka, A. A. Gust, and T. Nurnberger, "*Arabidopsis* lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection," *Proceedings of the National Academy of Sciences*, vol. 108, no. 49, pp. 19824–19829, 2011.

[400] H. Cho and J. H. Kehrl, "Regulation of immune function by G-protein-coupled receptors, trimeric G-proteins, and RGS proteins," *Progress in Molecular Biology and Translational Science*, vol. 86, pp. 249–298, 2009.

[401] H. Zhang, Z. Gao, X. Zheng, and Z. Zhang, "The role of G-proteins in plant immunity," *Plant Signaling & Behaviour*, vol. 7, pp. 1284–1288, 2012.

[402] Y. Trusov, N. Sewelam, J. E. Rookes, M. Kunkel, E. Nowak, P. M. Schenk, and J. R. Botella, "Heterotrimeric G proteins-mediated resistance to necrotrophic pathogens includes mechanisms independent of salicylic acid-, jasmonic acid/ethylene-and abscisic acid-mediated defense signaling," *The Plant Journal*, vol. 58, no. 1, pp. 69–81, 2009.

[403] K. Maeda, Y. Houjyou, T. Komatsu, H. Hori, T. Kodaira, and A. Ishikawa, "AGB1 and PMR5 contribute to PEN2-mediated preinvasion resistance to *Magnaporthe oryzae* in *Arabidopsis thaliana*," *Molecular Plant-Microbe Interactions*, vol. 22, no. 11, pp. 1331–1340, 2009.

[404] D. Urano, J.-G. Chen, J. R. Botella, and A. M. Jones, "Heterotrimeric G protein signalling in the plant kingdom," *Open Biology*, vol. 3, no. 3, pp. 2046–2441, 2013.

[405] Y. Trusov, J. E. Rookes, D. Chakravorty, D. Armour, P. M. Schenk, and J. R. Botella, "Heterotrimeric G proteins facilitate *Arabidopsis* resistance to necrotrophic pathogens and are involved in jasmonate signaling," *Plant Physiology*, vol. 140, no. 1, pp. 210–220, 2006.

[406] N. Idänheimo, A. Gauthier, J. Salojärvi, R. Siligato, M. Brosché, H. Kollist, A. P. Mähönen, J. Kangasjärvi, and M. Wrzaczek, "The *Arabidopsis thaliana* cysteine-rich receptor-like kinases CRK6 and CRK7 protect against apoplastic oxidative stress," *Biochemical and Biophysical Research Communications*, vol. 445, no. 2, pp. 457–462, 2014.

[407] J.-H. Mu, H.-S. Lee, and T. Kao, "Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase.," *The Plant Cell*, vol. 6, no. 5, pp. 709–721, 1994.

[408] H.-S. Lee, B. Karunanandaa, A. McCubbin, S. Gilroy, and T.-h. Kao, "PRK1, a receptor-like kinase of *Petunia inflata*, is essential for postmeiotic development of pollen," *The Plant Journal*, vol. 9, no. 5, pp. 613–624, 1996.

[409] B. Kobe and A. V. Kajava, "The leucine-rich repeat as a protein recognition motif," *Current Opinion in Structural Biology*, vol. 11, no. 6, pp. 725–732, 2001.

[410] Z. Chen, "A superfamily of proteins with novel cysteine-rich repeats," *Plant Physiology*, vol. 126, no. 2, pp. 473–476, 2001.

[411] G. Bourdais, P. Burdiak, A. Gauthier, L. Nitsch, J. Salojärvi, C. Rayapuram, N. Idänheimo, K. Hunter, S. Kimura, E. Merilo, *et al.*, "Large-scale phenomics identifies primary and fine-tuning roles for CRKs in responses related to oxidative stress," *PLoS Genetics*, vol. 11, no. 7, p. e1005373, 2015.

[412] S. Postel, I. Küfner, C. Beuter, S. Mazzotta, A. Schwedt, A. Borlotti, T. Halter, B. Kemmerling, and T. Nürnberger, "The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in *Arabidopsis* development and immunity," *European Journal of Cell Biology*, vol. 89, no. 2, pp. 169–174, 2010.

[413] E. Iizasa, M. Mitsutomi, and Y. Nagano, "Direct binding of a plant LysM receptor-like kinase, LysM RLK1/CERK1, to chitin in vitro," *Journal of Biological Chemistry*, vol. 285, no. 5, pp. 2996–3004, 2010.

[414] N. Moriwaki, K. Matsushita, M. Nishina, and Y. Kono, "High concentrations of trehalose in aphid hemolymph," *Applied Entomology and Zoology*, vol. 38, no. 2, pp. 241–248, 2003.

[415] O. Fernandez, L. Béthencourt, A. Quero, R. S. Sangwan, and C. Clément, "Trehalose and plant stress responses: friend or foe?," *Trends in Plant Science*, vol. 15, no. 7, pp. 409–417, 2010.

[416] S. Hodge, J. L. Ward, M. H. Beale, M. Bennett, J. W. Mansfield, and G. Powell, "Aphid-induced accumulation of trehalose in *Arabidopsis thaliana* is systemic and dependent upon aphid density," *Planta*, vol. 237, no. 4, pp. 1057–1064, 2013.

[417] V. Singh, J. Louis, B. G. Ayre, J. C. Reese, and J. Shah, "TREHALOSE PHOSPHATE SYNTHASE11-dependent trehalose metabolism promotes *Arabidopsis thaliana* defense against the phloem-feeding insect *Myzus persicae*," *The Plant Journal*, vol. 67, no. 1, pp. 94–104, 2011.

[418] J. Louis and J. Shah, "*Arabidopsis thaliana-Myzus persicae* interaction: shaping the understanding of plant defense against phloem-feeding aphids," *Frontiers in Plant Science*, vol. 4, p. 213, 2013.

[419] T. Halter, J. Imkampe, S. Mazzotta, M. Wierzba, S. Postel, C. Bücherl, C. Kiefer, M. Stahl, D. Chinchilla, X. Wang, T. Nurnberger, C. Zipfel, S. Clouse, J. W. Borst, S. Boeren, S. C. de Vries, F. Tax, and B. Kemmerling, "The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity," *Current Biology*, vol. 24, no. 2, pp. 134–143, 2014.

[420] A. M. Jones and S. M. Assmann, "Plants: the latest model system for G-protein research," *EMBO Reports*, vol. 5, no. 6, p. 572, 2004.

[421] J. Zhao, C. C. Williams, and R. L. Last, "Induction of *Arabidopsis* tryptophan pathway enzymes and camalexin by amino acid starvation, oxidative stress, and an abiotic elicitor," *The Plant Cell*, vol. 10, no. 3, pp. 359–370, 1998.

[422] K. F.-J. Tierens, B. P. Thomma, R. P. Bari, M. Garmier, K. Eggermont, M. Brouwer, I. A. Penninckx, W. F. Broekaert, and B. Cammue, "Esa1, an *Arabidopsis* mutant with enhanced susceptibility to a range of necrotrophic fungal pathogens, shows a distorted induction of defense responses by reactive oxygen generating compounds," *The Plant Journal*, vol. 29, no. 2, pp. 131–140, 2002.

[423] Y. Narusaka, M. Narusaka, M. Seki, J. Ishida, M. Nakashima, A. Kamiya, A. Enju, T. Sakurai, M. Satoh, M. Kobayashi, Y. Tosa, P. Park, and K. Shinozaki, "The cDNA microarray analysis using an *Arabidopsis pad3* mutant reveals the expression profiles and classification of genes induced by *Alternaria brassicicola* attack," *Plant and Cell Physiology*, vol. 44, no. 4, pp. 377–387, 2003.

[424] H. Hao, L. Fan, T. Chen, R. Li, X. Li, Q. He, M. A. Botella, and J. Lin, "Clathrin and membrane microdomains cooperatively regulate RbohD dynamics and activity in *Arabidopsis*," *The Plant Cell*, vol. 26, no. 4, pp. 1729–1745, 2014.

[425] S. Hogenhout, R. Van der Hoorn, R. Terauchi, and S. Kamoun, "Emerging concepts in effector biology of plant-associated organisms," *Molecular Plant-Microbe Interactions*, vol. 22, no. 2, pp. 115–122, 2009.

[426] C. Azevedo, S. Betsuyaku, J. Peart, A. Takahashi, L. Noel, A. Sadanandom, C. Casais, J. Parker, and K. Shirasu, "Role of SGT1 in resistance protein accumulation in plant immunity," *The EMBO Journal*, vol. 25, no. 9, pp. 2007–2016, 2006.

[427] S. Meldau, I. T. Baldwin, and J. Wu, "SGT1 regulates wounding-and herbivory-induced jasmonic acid accumulation and *Nicotiana attenuata*s resistance to the specialist lepidopteran herbivore *Manduca sexta*," *New Phytologist*, vol. 189, no. 4, pp. 1143–1156, 2011.

[428] Y. Xu, P. He, L. Zhang, S. Fang, S. Dong, Y. Zhang, and F. Li, "Large-scale identification of odorant-binding proteins and chemosensory proteins from expressed sequence tags in insects," *BMC Genomics*, vol. 10, no. 1, pp. 632–645, 2009.

[429] P. Pelosi, M. Calvello, and L. Ban, "Diversity of odorant-binding proteins and chemosensory proteins in insects," *Chemical Senses*, vol. 30, no. 1, pp. 291–292, 2005.

[430] A. Lartigue, V. Campanacci, A. Roussel, A. M. Larsson, T. A. Jones, M. Tegoni, and C. Cambillau, "X-ray structure and ligand binding study of a moth chemosensory protein," *Journal of Biological Chemistry*, vol. 277, no. 35, pp. 32094–32098, 2002.

[431] A. Mosbah, V. Campanacci, A. Lartigue, M. Tegoni, C. Cambillau, and H. Darbon, "Solution structure of a chemosensory protein from the moth *Mamestra brassicae*.," *Biochemical Journal*, vol. 369, pp. 39–44, 2003.

[432] K. W. Wanner, L. G. Willis, D. A. Theilmann, M. B. Isman, Q. Feng, and E. Plettner, "Analysis of the insect OS-D-like gene family," *Journal of Chemical Ecology*, vol. 30, no. 5, pp. 889–911, 2004.

[433] A. Sanchez-Gracia, F. Vieira, and J. Rozas, "Molecular evolution of the major chemosensory gene families in insects," *Heredity*, vol. 103, no. 3, pp. 208–216, 2009.

[434] M. Calvello, N. Guerra, A. Brandazza, C. D'ambrosio, A. Scaloni, F. Dani, S. Turillazzi, and P. Pelosi, "Soluble proteins of chemical communication in the social wasp *Polistes dominulus*," *Cellular and Molecular Life Sciences*, vol. 60, no. 9, pp. 1933–1943, 2003.

[435] Y. Ishida, V. Chiang, and W. S. Leal, "Protein that makes sense in the Argentine ant," *Naturwissenschaften*, vol. 89, no. 11, pp. 505–507, 2002.

[436] M. Ozaki, A. Wada-Katsumata, K. Fujikawa, M. Iwasaki, F. Yokohari, Y. Satoji, T. Nisimura, and R. Yamaoka, "Ant nestmate and non-nestmate discrimination by a chemosensory sensillum," *Science*, vol. 309, no. 5732, pp. 311–314, 2005.

[437] A. N. Kitabayashi, T. Arai, T. Kubo, and S. Natori, "Molecular cloning of cDNA for p10, a novel protein that increases in the regenerating legs of *Periplaneta americana* (American cockroach)," *Insect Biochemistry and Molecular Biology*, vol. 28, no. 10, pp. 785–790, 1998.

[438] J. Maleszka, S. Forêt, R. Saint, and R. Maleszka, "RNAi-induced phenotypes suggest a novel role for a chemosensory protein CSP5 in the development of embryonic integument in the honeybee (*Apis mellifera*)," *Development Genes and Evolution*, vol. 217, no. 3, pp. 189–196, 2007.

[439] W. Guo, X. Wang, Z. Ma, L. Xue, J. Han, D. Yu, and L. Kang, "CSP and takeout genes modulate the switch between attraction and repulsion during behavioral phase change in the migratory locust," *PLoS Genetics*, vol. 7, no. 2, p. e1001291, 2011.

[440] R. J. Beynon and J. L. Hurst, "Urinary proteins and the modulation of chemical scents in mice and rats," *Peptides*, vol. 25, no. 9, pp. 1553–1563, 2004.

[441] S. Jacobs, A. Liggins, J. Zhou, J. Pickett, X. Jin, and L. Field, "OS-D-like genes and their expression in aphids (Hemiptera: Aphididae)," *Insect Molecular Biology*, vol. 14, no. 4, pp. 423–432, 2005.

[442] R. C. Edgar, "MUSCLE: multiple sequence alignment with high accuracy and high throughput," *Nucleic Acids Research*, vol. 32, no. 5, pp. 1792–1797, 2004.

[443] K. Tamura, J. Dudley, M. Nei, and S. Kumar, "MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0," *Molecular Biology and Evolution*, vol. 24, no. 8, pp. 1596–1599, 2007.

[444] K. L. Morrison and G. A. Weiss, "Combinatorial alanine-scanning," *Current Opinion in Chemical Biology*, vol. 5, no. 3, pp. 302–307, 2001.

[445] F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. Thompson, and D. Higgins, "Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega," *Molecular Systems Biology*, vol. 7, no. 1, p. 539, 2011.

[446] S. Tomaselli, O. Crescenzi, D. Sanfelice, E. Ab, R. Wechselberger, S. Angeli, A. Scaloni, R. Boelens, T. Tancredi, P. Pelosi, *et al.*, "Solution structure of a chemosensory protein from the desert locust *Schistocerca gregaria*," *Biochemistry*, vol. 45, no. 35, pp. 10606–10613, 2006.

[447] L. A. Kelley, S. Mezulis, C. M. Yates, M. N. Wass, and M. J. Sternberg, "The Phyre2 web portal for protein modeling, prediction and analysis," *Nature Protocols*, vol. 10, no. 6, pp. 845–858, 2015.

[448] V. Campanacci, A. Lartigue, B. M. Hällberg, T. A. Jones, M.-T. Giudici-Orticoni, M. Tegoni, and C. Cambillau, "Moth chemosensory protein exhibits drastic conformational changes and cooperativity on ligand binding," *Proceedings of the National Academy of Sciences*, vol. 100, no. 9, pp. 5069–5074, 2003.

[449] E. F. Pettersen, T. D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, and T. E. Ferrin, "UCSF Chimera - a visualization system for exploratory research and analysis," *Journal of Computational Chemistry*, vol. 25, no. 13, pp. 1605–1612, 2004.

[450] A. A. Gust and T. Nürnberger, "Plant immunology: A life or death switch," *Nature*, vol. 486, no. 7402, pp. 198–199, 2012.

[451] S.-K. Oh, H. Kim, and D. Choi, "*Rpi-blb2*-mediated late blight resistance in *Nicotiana benthamiana* requires SGT1 and salicylic acid-mediated signaling but not RAR1 or HSP90," *FEBS Letters*, vol. 588, no. 7, pp. 1109–1115, 2014.

[452] W. Cheng, K. R. Munkvold, H. Gao, J. Mathieu, S. Schwizer, S. Wang, Y.-b. Yan, J. Wang, G. B. Martin, and J. Chai, "Structural analysis of *Pseudomonas syringae* AvrPtob bound to host BAK1 reveals two similar kinase-interacting domains in a type III effector," *Cell Host & Microbe*, vol. 10, no. 6, pp. 616–626, 2011.

[453] R. B. Abramovitch, R. Janjusevic, C. E. Stebbins, and G. B. Martin, "Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity," *Proceedings of the National Academy of Sciences*, vol. 103, no. 8, pp. 2851–2856, 2006.

[454] M. Ghanim, A. Dombrovsky, B. Raccah, and A. Sherman, "A microarray approach identifies ANT, OS-D and takeout-like genes as differentially regulated in alate and apterous morphs of the green peach aphid *Myzus persicae* (sulzer)," *Insect Biochemistry and Molecular Biology*, vol. 36, no. 11, pp. 857–868, 2006.

[455] S.-H. Gu, K.-M. Wu, Y.-Y. Guo, L. M. Field, J. A. Pickett, Y.-J. Zhang, and J.-J. Zhou, "Identification and expression profiling of odorant binding proteins and chemosensory proteins between two wingless morphs and a winged morph of the cotton aphid *Aphis gossypii* glover," *PLoS One*, vol. 8, no. 9, p. e73524, 2013.

[456] H. S. Atamian, R. Chaudhary, V. D. Cin, E. Bao, T. Girke, and I. Kaloshian, "*In planta* expression or delivery of potato aphid *Macrosiphum euphorbiae* effectors Me10 and Me23 enhances aphid fecundity," *Molecular Plant-Microbe Interactions*, vol. 26, no. 1, pp. 67–74, 2013.

[457] S.-Y. Seong and P. Matzinger, "Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses," *Nature Reviews Immunology*, vol. 4, no. 6, pp. 469–478, 2004.

[458] R. de Jonge, H. P. van Esse, A. Kombrink, T. Shinya, Y. Desaki, R. Bours, S. van der Krol, N. Shibuya, M. H. Joosten, and B. P. Thomma, "Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants," *Science*, vol. 329, no. 5994, pp. 953–955, 2010.

[459] E. Blée, "Impact of phyto-oxylipins in plant defense," *Trends in Plant Science*, vol. 7, no. 7, pp. 315–322, 2002.

[460] A. Mosblech, I. Feussner, and I. Heilmann, "Oxylipins: structurally diverse metabolites from fatty acid oxidation," *Plant Physiology and Biochemistry*, vol. 47, no. 6, pp. 511–517, 2009.

[461] V. Nalam, J. Keeretaweep, S. Sarowar, and J. Shah, "Root-derived oxylipins promote green peach aphid performance on *Arabidopsis* foliage," *The Plant Cell*, vol. 24, no. 4, pp. 1643–1653, 2012.

[462] C. M. Smith, X. Liu, L. J. Wang, X. Liu, M.-S. Chen, S. Starkey, and J. Bai, "Aphid feeding activates expression of a transcriptome of oxylipin-based defense signals in wheat involved in resistance to herbivory," *Journal of Chemical Ecology*, vol. 36, no. 3, pp. 260–276, 2010.

[463] M. Hegde, J. N. Oliveira, J. G. da Costa, E. Loza-Reyes, E. Bleicher, A. E. Santana, J. C. Caulfield, P. Mayon, S. Y. Dewhirst, T. J. Bruce, J. A. Pickett, and M. A. Birkett, "Aphid antixenosis in cotton is activated by the natural plant defence elicitor cis-jasmone," *Phytochemistry*, vol. 78, pp. 81–88, 2012.

[464] S. T. Schenk and A. Schikora, "AHL-priming functions via oxylipin and salicylic acid," *Frontiers in Plant Science*, vol. 5, 2014.

[465] J.-L. Montillet, N. Leonhardt, S. Mondy, S. Tranchimand, D. Rumeau, M. Boudsocq, A. V. Garcia, T. Douki, J. Bigeard, C. Laurière, A. Chevalier, C. Castresana, and H. Hirt, "An abscisic acid-independent oxylipin pathway controls stomatal closure and immune defense in *Arabidopsis*," *PLoS Biology*, vol. 11, no. 3, p. e1001513, 2013.

[466] K. W. Dorschner, J. Ryan, R. C. Johnson, and R. Eikenbary, "Modification of host nitrogen levels by the greenbug (Homoptera: Aphididae): its role in resistance of winter wheat to aphids," *Environmental Entomology*, vol. 16, no. 4, pp. 1007–1011, 1987.

[467] J. Sandström, A. Telang, and N. Moran, "Nutritional enhancement of host plants by aphids - a comparison of three aphid species on grasses," *Journal of Insect Physiology*, vol. 46, no. 1, pp. 33–40, 2000.

[468] G. Loake, "Plant cell death: Unmasking the gatekeepers," *Current Biology*, vol. 11, no. 24, pp. 1028–1031, 2001.

[469] J. L. Boatwright and K. Pajerowska-Mukhtar, "Salicylic acid: an old hormone up to new tricks," *Molecular Plant Pathology*, vol. 14, no. 6, pp. 623–634, 2013.

[470] K. Tsuda, M. Sato, T. Stoddard, J. Glazebrook, and F. Katagiri, "Network properties of robust immunity in plants," *PLoS Genetics*, vol. 5, no. 12, p. e1000772, 2009.

[471] K. Bhattarai, Q. Li, Y. Liu, S. Dinesh-Kumar, and I. Kaloshian, "The Mi-1-mediated pest resistance requires *Hsp90* and *Sgt1*," *Plant Physiology*, vol. 144, no. 1, pp. 312–323, 2007.

[472] J.-J. Zhou, F. G. Vieira, X.-L. He, C. Smadja, R. Liu, J. Rozas, and L. M. Field, "Genome annotation and comparative analyses of the odorant-binding proteins and chemosensory proteins in the pea aphid *Acyrthosiphon pisum*," *Insect Molecular Biology*, vol. 19, no. s2, pp. 113–122, 2010.

[473] F. G. Vieira and J. Rozas, "Comparative genomics of the odorant-binding and chemosensory protein gene families across the Arthropoda: origin and evolutionary history of the chemosensory system," *Genome Biology and Evolution*, vol. 3, pp. 476–490, 2011.

[474] M. de la Paz Celorio-Mancera, S. M. Sundmalm, H. Vogel, D. Rutishauser, A. J. Ytterberg, R. A. Zubarev, and N. Janz, "Chemosensory proteins, major salivary factors in caterpillar mandibular glands," *Insect Biochemistry and Molecular Biology*, vol. 42, no. 10, pp. 796–805, 2012.

[475] S. Sim, J. L. Ramirez, and G. Dimopoulos, "Dengue virus infection of the *Aedes aegypti* salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior," *PLoS Pathology*, vol. 8, no. 3, p. e1002631, 2012.

[476] Z. Liu, Y. Wu, F. Yang, Y. Zhang, S. Chen, Q. Xie, X. Tian, and J.-M. Zhou, "BIK1 interacts with PEPRs to mediate ethylene-induced immunity," *Proceedings of the National Academy of Sciences*, vol. 110, no. 15, pp. 6205–6210, 2013.

[477] W. Lin, B. Li, D. Lu, S. Chen, N. Zhu, P. He, and L. Shan, "Tyrosine phosphorylation of protein kinase complex BAK1/BIK1 mediates *Arabidopsis* innate immunity," *Proceedings of the National Academy of Sciences*, vol. 111, no. 9, pp. 3632–3637, 2014.

[478] C.-Y. Hung, P. Aspesi Jr, M. R. Hunter, A. W. Lomax, and I. Y. Perera, "Phosphoinositide-signaling is one component of a robust plant defense response," *Frontiers in Plant Science*, vol. 5, 2014.

[479] Y. Wang, J. Li, S. Hou, X. Wang, Y. Li, D. Ren, S. Chen, X. Tang, and J.-M. Zhou, "A *Pseudomonas syringae* ADP-ribosyltransferase inhibits *Arabidopsis* mitogen-activated protein kinase kinases," *The Plant Cell*, vol. 22, no. 6, pp. 2033–2044, 2010.

[480] M. Wilton, R. Subramaniam, J. Elmore, C. Felsensteiner, G. Coaker, and D. Desveaux, "The type III effector HopF2 Pto targets *Arabidopsis* RIN4 protein to promote *Pseudomonas syringae* virulence," *Proceedings of the National Academy of Sciences*, vol. 107, no. 5, pp. 2349–2354, 2010.

[481] S. N. Aslam, M.-A. Newman, G. Erbs, K. L. Morrissey, D. Chinchilla, T. Boller, T. T. Jensen, C. De Castro, T. Ierano, A. Molinaro, *et al.*, "Bacterial polysaccharides suppress induced innate immunity by calcium chelation," *Current Biology*, vol. 18, no. 14, pp. 1078–1083, 2008.

[482] M. Jaouannet, M. Magliano, M. Arguel, M. Gourgues, E. Evangelisti, P. Abad, and M. Rosso, "The root-knot nematode calreticulin Mi-CRT is a key effector in plant defense suppression," *Molecular Plant-Microbe Interactions*, vol. 26, no. 1, pp. 97–105, 2013.

[483] M. Hattori, M. Nakamura, S. Komatsu, K. Tsuchihara, Y. Tamura, and T. Hasegawa, "Molecular cloning of a novel calcium-binding protein in the secreted saliva of the green rice leafhopper *Nephotettix cincticeps*," *Insect Biochemistry and Molecular Biology*, vol. 42, no. 1, pp. 1–9, 2012.

[484] J. T. Greenberg and B. A. Vinatzer, "Identifying type III effectors of plant pathogens and analyzing their interaction with plant cells," *Current Opinion in Microbiology*, vol. 6, no. 1, pp. 20–28, 2003.

[485] M. Beth Mudgett, "New insights to the function of phytopathogenic bacterial type III effectors in plants," *Annual Review of Plant Biology*, vol. 56, pp. 509–531, 2005.

[486] P. A. Rodriguez, R. Stam, T. Warbroek, and J. I. Bos, "Mp10 and Mp42 from the aphid species *Myzus persicae* trigger plant defenses in *Nicotiana benthamiana* through different activities," *Molecular Plant-Microbe Interactions*, vol. 27, no. 1, pp. 30–39, 2014.

[487] A. Kuśnierczyk, P. Winge, H. Midelfart, W. S. Armbruster, J. T. Rossiter, and A. M. Bones, "Transcriptional responses of *Arabidopsis thaliana* ecotypes with different glucosinolate profiles after attack by polyphagous *Myzus persicae* and oligophagous *Brevicoryne brassicae*," *Journal of Experimental Botany*, vol. 58, no. 10, pp. 2537–2552, 2007.

[488] S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Research*, vol. 25, no. 17, pp. 3389–3402, 1997.

[489] P. Lamesch, T. Z. Berardini, D. Li, D. Swarbreck, C. Wilks, R. Sasidharan, R. Muller, K. Dreher, D. L. Alexander, M. Garcia-Hernandez, A. S. Karthikeyan, C. Lee, W. D. Nelson, L. Ploetz, S. Singh, A. Wensel, and E. Huala, "The *Arabidopsis* Information Resource (TAIR): improved gene annotation and new tools," *Nucleic Acids Research*, vol. 40, no. D1, pp. 1202–1210, 2012.

[490] A. Katsiarimpa, K. Kalinowska, F. Anzenberger, C. Weis, M. Ostertag, C. Tsutsumi, C. Schwechheimer, F. Brunner, R. Hückelhoven, and E. Isono, "The deubiquitinating enzyme AMSH1 and the ESCRT-III subunit VPS2.1 are required for autophagic degradation in *Arabidopsis*," *The Plant Cell*, vol. 25, no. 6, pp. 2236–2252, 2013.

[491] A. Mitchell, H.-Y. Chang, L. Daugherty, M. Fraser, S. Hunter, R. Lopez, C. McAnulla, C. McMenamin, G. Nuka, S. Pesseat, A. Sangrador-Vegas, M. Scheremetjew, C. Rato, S.-Y. Yong, A. Bateman, M. Punta, T. K. Attwood, C. J. Sigrist, N. Redaschi, C. Rivoire, I. Xenarios, D. Kahn, D. Guyot, P. Bork, I. Letunic, J. Gough, M. Oates, D. Haft, H. Huang, D. A. Natale, C. H. Wu, C. Orengo,

I. Sillitoe, H. Mi, P. D. Thomas, and R. D. Finn, "The InterPro protein families database: the classification resource after 15 years," *Nucleic Acids Research*, vol. 43, pp. 213–221, 2015.

[492] A. Katsiarimpa, F. Anzenberger, N. Schlager, S. Neubert, M.-T. Hauser, C. Schwechheimer, and E. Isono, "The *Arabidopsis* deubiquitinating enzyme AMSH3 interacts with ESCRT-III subunits and regulates their localization," *The Plant Cell*, vol. 23, no. 8, pp. 3026–3040, 2011.

[493] A. Katsiarimpa, A. Muñoz, K. Kalinowska, T. Uemura, E. Rojo, and E. Isono, "The ESCRT-III interacting deubiquitinating enzyme AMSH3 is essential for degradation of ubiquitinated membrane proteins in *Arabidopsis thaliana*," *Plant and Cell Physiology*, vol. 55, no. 4, pp. 727–736, 2014.

[494] K. Kalinowska, M.-K. Nagel, K. Goodman, L. Cuyas, F. Anzenberger, A. Alkofer, J. Paz-Ares, P. Braun, V. Rubio, M. S. Otegui, and E. Isono, "Arabidopsis ALIX is required for the endosomal localization of the deubiquitinating enzyme AMSH3," *Proceedings of the National Academy of Sciences*, vol. 112, no. 40, pp. 5543–5551, 2015.

[495] K. Ebine, M. Fujimoto, Y. Okatani, T. Nishiyama, T. Goh, E. Ito, T. Dainobu, A. Nishitani, T. Uemura, M. H. Sato, *et al.*, "A membrane trafficking pathway regulated by the plant-specific RAB GTPase ARA6," *Nature Cell Biology*, vol. 13, no. 7, pp. 853–859, 2011.

[496] E. M. Cooper, C. Cutcliffe, T. Z. Kristiansen, A. Pandey, C. M. Pickart, and R. E. Cohen, "K63-specific deubiquitination by two JAMM/MPN+ complexes: BRISC-associated Brcc36 and proteasomal Poh1," *The EMBO Journal*, vol. 28, no. 6, pp. 621–631, 2009.

[497] C. W. Davies, L. N. Paul, and C. Das, "Mechanism of recruitment and activation of the endosome-associated deubiquitinase AMSH," *Biochemistry*, vol. 52, no. 44, pp. 7818–7829, 2013.

[498] F. Huang, X. Zeng, W. Kim, M. Balasubramani, A. Fortian, S. P. Gygi, N. A. Yates, and A. Sorkin, "Lysine 63-linked polyubiquitination is required for EGF receptor degradation," *Proceedings of the National Academy of Sciences*, vol. 110, no. 39, pp. 15722–15727, 2013.

[499] F. Herrera-Vigenor, R. Hernández-García, M. Valadez-Sánchez, J. Vázquez-Prado, and G. Reyes-Cruz, "AMSH regulates calcium-sensing receptor signaling through direct interactions," *Biochemical and Biophysical Research Communications*, vol. 347, no. 4, pp. 924–930, 2006.

[500] A. P. Reyes-Ibarra, A. Garcia-Regalado, I. Ramírez-Rangel, A. L. Esparza-Silva, M. Valadez-Sanchez, J. Vazquez-Prado, and G. Reyes-Cruz, "Calcium-sensing receptor endocytosis links extracellular calcium signaling to parathyroid hormone-related peptide secretion via a Rab11a-dependent and AMSH-sensitive mechanism," *Molecular Endocrinology*, vol. 21, no. 6, pp. 1394–1407, 2007.

[501] W. Zhang, B. W. Jeon, and S. M. Assmann, "Heterotrimeric G-protein regulation of ROS signalling and calcium currents in *Arabidopsis* guard cells," *Journal of Experimental Botany*, vol. 62, no. 7, pp. 2371–2379, 2011.

[502] S. B. Khaled, J. Postma, and S. Robatzek, "A moving view: Subcellular transport processes in pattern recognition receptor-triggered plant immunity," *Annual Review of Phytopathology*, vol. 53, no. 1, pp. 379–402, 2015.

[503] M. Trujillo, K. Ichimura, C. Casais, and K. Shirasu, "Negative regulation of PAMP-triggered immunity by an E3 ubiquitin ligase triplet in *Arabidopsis*," *Current Biology*, vol. 18, no. 18, pp. 1396–1401, 2008.

[504] M. Stegmann, R. G. Anderson, K. Ichimura, T. Pecenkova, P. Reuter, V. Žárskỳ, J. M. McDowell, K. Shirasu, and M. Trujillo, "The ubiquitin ligase PUB22 targets a subunit of the exocyst complex required for PAMP-triggered responses in *Arabidopsis*," *The Plant Cell*, vol. 24, no. 11, pp. 4703–4716, 2012.

[505] T. Pečenková, M. Hála, I. Kulich, D. Kocourková, E. Drdová, M. Fendrych, T. Hana, and V. Žárskỳ, "The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant–pathogen interaction," *Journal of Experimental Botany*, vol. 62, no. 6, pp. 2107–2116, 2011.

[506] G. Furlan, J. Klinkenberg, and M. Trujillo, "Regulation of plant immune receptors by ubiquitination," *Frontiers in Plant Science*, vol. 3, no. 238, 2012.

[507] N. Geldner and S. Robatzek, "Plant receptors go endosomal: a moving view on signal transduction," *Plant Physiology*, vol. 147, no. 4, pp. 1565–1574, 2008.

[508] A. Małolepszy, D. F. Urbański, E. K. James, N. Sandal, E. Isono, J. Stougaard, and S. U. Andersen, "The deubiquitinating enzyme AMSH1 is required for rhizobial infection and nodule organogenesis in *Lotus japonicus*," *The Plant Journal*, vol. 83, no. 4, pp. 719–731, 2015.

[509] M. J. Banfield, "Perturbation of host ubiquitin systems by plant pathogen/pest effector proteins," *Cellular Microbiology*, vol. 17, no. 1, pp. 18–25, 2015.

[510] A.-S. Dielen, S. Badaoui, T. Candresse, and S. German-Retana, "The ubiquitin/26S proteasome system in plant–pathogen interactions: A never-ending hide-and-seek game," *Molecular Plant Pathology*, vol. 11, no. 2, pp. 293–308, 2010.

[511] A. Gangadharan, M.-V. Sreerekha, J. Whitehill, J. H. Ham, and D. Mackey, "The *Pseudomonas syringae* pv. *tomato* type III effector HopM1 suppresses *Arabidopsis* defenses independent of suppressing salicylic acid signaling and of targeting AtMIN7," *PLoS One*, 2013.

[512] R. Lozano-Durán, G. Bourdais, S. Y. He, and S. Robatzek, "The bacterial effector HopM1 suppresses PAMP-triggered oxidative burst and stomatal immunity," *New Phytologist*, vol. 202, no. 1, pp. 259–269, 2014.

[513] C.-H. Park, S. Chen, G. Shirsekar, B. Zhou, C. H. Khang, P. Songkumarn, A. J. Afzal, Y. Ning, R. Wang, M. Bellizzi, B. Valent, and G.-L. Wang, "The *Magnaporthe oryzae* effector AvrPiz-t targets the RING E3 ubiquitin ligase APIP6 to suppress pathogen-associated molecular pattern–triggered immunity in rice," *The Plant Cell*, vol. 24, no. 11, pp. 4748–4762, 2012.

[514] C. Dogimont, A. Bendahmane, V. Chovelon, and N. Boissot, "Host plant resistance to aphids in cultivated crops: genetic and molecular bases, and interactions with aphid populations," *Comptes Rendus Biologies*, vol. 333, no. 6, pp. 566–573, 2010.

[515] G. R. Pallipparambil, R. J. Sayler, J. P. Shapiro, J. M. Thomas, T. J. Kring, and F. L. Goggin, "Mi-1.2, an R gene for aphid resistance in tomato, has direct negative effects on a zoophytophagous biocontrol agent, *Orius insidiosus*," *Journal of Experimental Botany*, vol. 66, no. 2, pp. 549–557, 2015.

[516] J. P. Klingler, R. M. Nair, O. R. Edwards, and K. B. Singh, "A single gene, AIN, in *Medicago truncatula* mediates a hypersensitive response to both bluegreen aphid and pea aphid, but confers resistance only to bluegreen aphid," *Journal of Experimental Botany*, p. erp244, 2009.

[517] J. Hatchett and R. L. Gallun, "Genetics of the ability of the Hessian fly, *Mayetiola destructor*, to survive on wheats having different genes for resistance," *Annals of the Entomological Society of America*, vol. 63, no. 5, pp. 1400–1407, 1970.

[518] M.-S. Chen, J. Fellers, J. Stuart, J. Reese, and X. Liu, "A group of related cDNAs encoding secreted proteins from Hessian fly [*Mayetiola destructor* (Say)] salivary glands," *Insect Molecular Biology*, vol. 13, no. 1, pp. 101–108, 2004.

[519] K. Shirasu, "The HSP90-SGT1 chaperone complex for NLR immune sensors," *Annual Review of Plant Biology*, vol. 60, pp. 139–164, 2009.

[520] D. Jirage, T. L. Tootle, T. L. Reuber, L. N. Frost, B. J. Feys, J. E. Parker, F. M. Ausubel, and J. Glazebrook, "*Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling," *Proceedings of the National Academy of Sciences*, vol. 96, no. 23, pp. 13583–13588, 1999.

[521] V. Pegadaraju, J. Louis, V. Singh, J. C. Reese, J. Bautor, B. J. Feys, G. Cook, J. E. Parker, and J. Shah, "Phloem-based resistance to green peach aphid is controlled by *Arabidopsis* PHYTOALEXIN DEFICIENT4 without its signaling partner ENHANCED DISEASE SUSCEPTIBILITY1," *The Plant Journal*, vol. 52, no. 2, pp. 332–341, 2007.

[522] J. Louis, E. Gobbato, H. A. Mondal, B. J. Feys, J. E. Parker, and J. Shah, "Discrimination of *Arabidopsis* PAD4 activities in defense against green peach aphid and pathogens," *Plant Physiology*, vol. 158, no. 4, pp. 1860–1872, 2012.

[523] Y. J. Kim, N.-C. Lin, and G. B. Martin, "Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity," *Cell*, vol. 109, no. 5, pp. 589–598, 2002.

[524] E. Richly, J. Kurth, and D. Leister, "Mode of amplification and reorganization of resistance genes during recent *Arabidopsis thaliana* evolution," *Molecular Biology and Evolution*, vol. 19, no. 1, pp. 76–84, 2002.

[525] B. C. Meyers, A. Kozik, A. Griego, H. Kuang, and R. W. Michelmore, "Genome-wide analysis of NBS-LRR–encoding genes in *Arabidopsis*," *The Plant Cell*, vol. 15, no. 4, pp. 809–834, 2003.

[526] D. Leister, "Tandem and segmental gene duplication and recombination in the evolution of plant disease resistance genes," *Trends in Genetics*, vol. 20, no. 3, pp. 116–122, 2004.

[527] F. Shao, C. Golstein, J. Ade, M. Stoutemyer, J. E. Dixon, and R. W. Innes, "Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector," *Science*, vol. 301, no. 5637, pp. 1230–1233, 2003.

[528] Z. Zhang, Y. Wu, M. Gao, J. Zhang, Q. Kong, Y. Liu, H. Ba, J. Zhou, and Y. Zhang, "Disruption of PAMP-induced MAP kinase cascade by a *Pseudomonas syringae* effector activates plant immunity mediated by the NB-LRR protein SUMM2," *Cell Host & Microbe*, vol. 11, no. 3, pp. 253–263, 2012.

[529] S. Goritschnig, K. V. Krasileva, D. Dahlbeck, and B. J. Staskawicz, "Computational prediction and molecular characterization of an oomycete effector and the cognate *Arabidopsis* resistance gene," *PLoS Genetics*, vol. 8, no. 2, p. e1002502, 2012.

[530] J. Staal, M. Kaliff, S. Bohman, and C. Dixelius, "Transgressive segregation reveals two *Arabidopsis* TIR-NB-LRR resistance genes effective against *Leptosphaeria maculans*, causal agent of blackleg disease," *The Plant Journal*, vol. 46, no. 2, pp. 218–230, 2006.

[531] M. Narusaka, K. Shirasu, Y. Noutoshi, Y. Kubo, T. Shiraishi, M. Iwabuchi, and Y. Narusaka, "RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens," *The Plant Journal*, vol. 60, no. 2, pp. 218–226, 2009.

[532] A. Steinbrenner, S. Goritschnig, K. Krasileva, K. Schreiber, and B. Staskawicz, "Effector recognition and activation of the *Arabidopsis thaliana* NLR innate immune receptors," in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 77, pp. 249–257, Cold Spring Harbor Laboratory Press, 2012.

[533] G. Farnham and D. C. Baulcombe, "Artificial evolution extends the spectrum of viruses that are targeted by a disease-resistance gene from potato," *Proceedings of the National Academy of Sciences*, vol. 103, no. 49, pp. 18828–18833, 2006.

[534] T. M. Burch-Smith, M. Schiff, J. L. Caplan, J. Tsao, K. Czymmek, and S. P. Dinesh-Kumar, "A novel role for the TIR domain in association with pathogen-derived elicitors," *PLoS Biology*, no. 5, 2007.

[535] L. McHale, X. Tan, P. Koehl, and R. W. Michelmore, "Plant NBS-LRR proteins: adaptable guards," *Genome Biology*, vol. 7, no. 4, p. 212, 2006.

[536] L. Navarro, C. Zipfel, O. Rowland, I. Keller, S. Robatzek, T. Boller, and J. D. Jones, "The transcriptional innate immune response to flg22. interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis," *Plant Physiology*, vol. 135, no. 2, pp. 1113–1128, 2004.

[537] M. Hinsch and B. Staskawicz, "Identification of a new *Arabidopsis* disease resistance locus, RPS4, and cloning of the corresponding avirulence gene, AvrRps4, from *Pseudomonas syringae* pv. pisi," *Molecular Plant–Microbe Interactions*, vol. 9, no. 1, pp. 55–61, 1996.

[538] W. Gassmann, M. E. Hinsch, and B. J. Staskawicz, "The *Arabidopsis* RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes," *The Plant Journal*, vol. 20, no. 3, pp. 265–277, 1999.

[539] K. H. Sohn, R. K. Hughes, S. J. Piquerez, J. D. Jones, and M. J. Banfield, "Distinct regions of the *Pseudomonas syringae* coiled-coil effector AvrRps4 are required for activation of immunity," *Proceedings of the National Academy of Sciences*, vol. 109, no. 40, pp. 16371–16376, 2012.

[540] J. E. Rebers and L. M. Riddiford, "Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes," *Journal of Molecular Biology*, vol. 203, no. 2, pp. 411–423, 1988.

[541] J. H. Willis, N. C. Papandreou, V. A. Iconomidou, and S. J. Hamodrakas, "5 cuticular proteins," in *Insect Molecular Biology and Biochemistry*, pp. 134–166, Academic Press San Diego, 2012.

[542] M. V. Karouzou, Y. Spyropoulos, V. A. Iconomidou, R. Cornman, S. J. Hamodrakas, and J. H. Willis, "*Drosophila* cuticular proteins with the R&R consensus: annotation and classification with a new tool for discriminating RR-1 and RR-2 sequences," *Insect Biochemistry and Molecular Biology*, vol. 37, no. 8, pp. 754–760, 2007.

[543] C. K. Magkrioti, I. C. Spyropoulos, V. A. Iconomidou, J. H. Willis, and S. J. Hamodrakas, "cuticleDB: a relational database of Arthropod cuticular proteins," *BMC Bioinformatics*, vol. 5, no. 1, p. 138, 2004.

[544] A. Dombrovsky, H. Huet, H. Zhang, N. Chejanovsky, and B. Raccah, "Comparison of newly isolated cuticular protein genes from six aphid species," *Insect Biochemistry and Molecular Biology*, vol. 33, no. 7, pp. 709–715, 2003.

[545] Y. Jia, S. A. McAdams, G. T. Bryan, H. P. Hershey, and B. Valent, "Direct interaction of resistance gene and avirulence gene products confers rice blast resistance," *The EMBO Journal*, vol. 19, no. 15, pp. 4004–4014, 2000.

[546] S. B. Saucet, Y. Ma, P. F. Sarris, O. J. Furzer, K. H. Sohn, and J. D. Jones, "Two linked pairs of *Arabidopsis* TNL resistance genes independently confer recognition of bacterial effector AvrRps4," *Nature Communications*, vol. 6, no. 6338, 2015.

[547] K. H. Sohn, C. Segonzac, G. Rallapalli, P. F. Sarris, J. Y. Woo, S. J. Williams, T. E. Newman, K. H. Paek, B. Kobe, and J. D. Jones, "The nuclear immune receptor RPS4 is required for RRS1 SLH1-dependent constitutive defense activation in *Arabidopsis thaliana*," *PLoS Genetics*, 2014.

[548] T.-H. Kim, H.-H. Kunz, S. Bhattacharjee, F. Hauser, J. Park, C. Engineer, A. Liu, T. Ha, J. E. Parker, W. Gassmann, and J. Schroeder, "Natural variation in small molecule–induced TIR-NB-LRR signaling induces root growth arrest via EDS1-and PAD4-complexed R-protein VICTR in *Arabidopsis*," *The Plant Cell*, vol. 24, no. 12, pp. 5177–5192, 2012.

[549] S. Dinesh-Kumar and B. J. Baker, "Alternatively spliced N resistance gene transcripts: their possible role in Tobacco Mosaic Virus resistance," *Proceedings of the National Academy of Sciences*, vol. 97, no. 4, pp. 1908–1913, 2000.

[550] X.-C. Zhang and W. Gassmann, "Alternative splicing and mRNA levels of the disease resistance gene RPS4 are induced during defense responses," *Plant Physiology*, vol. 145, no. 4, pp. 1577–1587, 2007.

[551] D. A. Halterman, F. Wei, and R. P. Wise, "Powdery mildew-induced Mla mRNAs are alternatively spliced and contain multiple upstream open reading frames," *Plant Physiology*, vol. 131, no. 2, pp. 558–567, 2003.

[552] S. Bhattacharjee, M. K. Halane, S. H. Kim, and W. Gassmann, "Pathogen effectors target *Arabidopsis* EDS1 and alter its interactions with immune regulators," *Science Signalling*, vol. 334, no. 6061, p. 1405, 2011.

[553] D. Birker, K. Heidrich, H. Takahara, M. Narusaka, L. Deslandes, Y. Narusaka, M. Reymond, J. E. Parker, and R. O'Connell, "A locus conferring resistance to *Colletotrichum higginsianum* is shared by four geographically distinct *Arabidopsis* accessions," *The Plant Journal*, vol. 60, no. 4, pp. 602–613, 2009.

[554] S. T. Chisholm, G. Coaker, B. Day, and B. J. Staskawicz, "Host-microbe interactions: shaping the evolution of the plant immune response," *Cell*, vol. 124, no. 4, pp. 803–814, 2006.

[555] R. L. Allen, P. D. Bittner-Eddy, L. J. Grenville-Briggs, J. C. Meitz, A. P. Rehmany, L. E. Rose, and J. L. Beynon, "Host-parasite coevolutionary conflict between *Arabidopsis* and downy mildew," *Science*, vol. 306, no. 5703, pp. 1957–1960, 2004.

[556] L. E. Rose, P. D. Bittner-Eddy, C. H. Langley, E. B. Holub, R. W. Michelmore, and J. L. Beynon, "The maintenance of extreme amino acid diversity at the disease resistance gene, RPP13, in *Arabidopsis thaliana*," *Genetics*, vol. 166, no. 3, pp. 1517–1527, 2004.

[557] M. T. Simonich and R. Innes, "A disease resistance gene in *Arabidopsis* with specificity for the avrPph3 gene of *Pseudomonas syringae* pv. phaseolicola.," *Molecular Plant-Microbe Interactions*, vol. 8, no. 4, pp. 637–640, 1994.

[558] J. Ade, B. J. DeYoung, C. Golstein, and R. W. Innes, "Indirect activation of a plant nucleotide binding site–leucine-rich repeat protein by a bacterial protease," *Proceedings of the National Academy of Sciences*, vol. 104, no. 7, pp. 2531–2536, 2007.

[559] D. Qi, B. J. DeYoung, and R. W. Innes, "Structure-function analysis of the coiled-coil and leucine-rich repeat domains of the RPS5 disease resistance protein," *Plant Physiology*, vol. 158, no. 4, pp. 1819–1832, 2012.

[560] P. N. Dodds and J. P. Rathjen, "Plant immunity: towards an integrated view of plant–pathogen interactions," *Nature Reviews Genetics*, vol. 11, no. 8, pp. 539–548, 2010.

[561] J. E. Rebers and J. H. Willis, "A conserved domain in arthropod cuticular proteins binds chitin," *Insect Biochemistry and Molecular Biology*, vol. 31, no. 11, pp. 1083–1093, 2001.

[562] K. J. Medina-Ortega and G. Walker, "Does aphid salivation affect phloem sieve element occlusion in vivo?," *Journal of Experimental Botany*, vol. 64, no. 18, pp. 5525–5535, 2013.

[563] H. Konishi, H. Noda, Y. Tamura, and M. Hattori, "Proteomic analysis of the salivary glands of the rice brown planthopper, *Nilaparvata lugens* (Stal)(Homoptera: Delphacidae)," *Applied Entomology and Zoology*, vol. 44, no. 4, pp. 525–534, 2009.

[564] S. R. Shames and B. B. Finlay, "Bacterial effector interplay: a new way to view effector function," *Trends in Microbiology*, vol. 20, no. 5, pp. 214–219, 2012.

[565] T. Tzfira, M. Vaidya, and V. Citovsky, "Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*," *Nature*, vol. 431, no. 7004, pp. 87–92, 2004.

[566] S. Magori and V. Citovsky, "Agrobacterium counteracts host-induced degradation of its effector F-box protein," *Science Signaling*, vol. 4, no. 195, pp. 69–69, 2011.

[567] A. Block, T. Y. Toruño, C. G. Elowsky, C. Zhang, J. Steinbrenner, J. Beynon, and J. R. Alfano, "The *Pseudomonas syringae* type III effector HopD1 suppresses effector-triggered immunity, localizes to the endoplasmic reticulum, and targets the *Arabidopsis* transcription factor NTL9," *New Phytologist*, vol. 201, no. 4, pp. 1358–1370, 2014.

[568] A. P. Macho, C. M. Guevara, P. Tornero, J. Ruiz-Albert, and C. R. Beuzón, "The *Pseudomonas syringae* effector protein HopZ1a suppresses effector-triggered immunity," *New Phytologist*, vol. 187, no. 4, pp. 1018–1033, 2010.

[569] L. Deslandes and S. Rivas, "Catch me if you can: bacterial effectors and plant targets," *Trends in Plant Science*, vol. 17, no. 11, pp. 644–655, 2012.

[570] Z.-Y. Wang, "Brassinosteroids modulate plant immunity at multiple levels," *Proceedings of the National Academy of Sciences*, vol. 109, no. 1, pp. 7–8, 2012.

[571] H. Lindner, L. M. Müller, A. Boisson-Dernier, and U. Grossniklaus, "CrRLK1L receptor-like kinases: not just another brick in the wall," *Current Opinion in Plant Biology*, vol. 15, no. 6, pp. 659–669, 2012.

[572] A. Boisson-Dernier, S. A. Kessler, and U. Grossniklaus, "The walls have ears: the role of plant CrRLK1Ls in sensing and transducing extracellular signals," *Journal of Experimental Botany*, vol. 62, no. 5, pp. 1581–1591, 2011.

[573] M. Haruta, G. Sabat, K. Stecker, B. B. Minkoff, and M. R. Sussman, "A peptide hormone and its receptor protein kinase regulate plant cell expansion," *Science*, vol. 343, no. 6169, pp. 408–411, 2014.

[574] C. Li, F.-L. Yeh, A. Y. Cheung, Q. Duan, D. Kita, M.-C. Liu, J. Maman, E. J. Luu, B. W. Wu, L. Gates, M. Jalal, A. Kwong, H. Carpenter, and H.-M. Wu, "Glycosylphosphatidylinositol-anchored proteins as chaperones and co-receptors for FERONIA receptor kinase signaling in *Arabidopsis*," *eLife*, vol. 4, p. e06587, 2015.

[575] Q. Duan, D. Kita, E. A. Johnson, M. Aggarwal, L. Gates, H.-M. Wu, and A. Y. Cheung, "Reactive oxygen species mediate pollen tube rupture to release sperm for fertilization in *Arabidopsis*," *Nature Communications*, vol. 5, p. 3129, 2014.

[576] Q. Duan, D. Kita, C. Li, A. Y. Cheung, and H.-M. Wu, "FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development," *Proceedings of the National Academy of Sciences*, vol. 107, no. 41, pp. 17821–17826, 2010.

[577] M. Wrzaczek, M. Brosché, and J. Kangasjärvi, "ROS signaling loops - production, perception, regulation," *Current Opinion in Plant Biology*, vol. 16, no. 5, pp. 575–582, 2013.

[578] G. Dubreuil, E. Deleury, D. Crochard, J.-C. Simon, and C. Coustau, "Diversification of MIF immune regulators in aphids: link with agonistic and antagonistic interactions," *BMC Genomics*, vol. 15, no. 1, p. 762, 2014.

[579] G. Coaker, A. Falick, and B. Staskawicz, "Activation of a phytopathogenic bacterial effector protein by a eukaryotic cyclophilin," *Science*, vol. 308, no. 5721, pp. 548–550, 2005.

[580] E. Russinova, J.-W. Borst, M. Kwaaitaal, A. Caño-Delgado, Y. Yin, J. Chory, and S. C. de Vries, "Heterodimerization and endocytosis of *Arabidopsis* brassinosteroid receptors BRI1 and AtSERK3 (BAK1)," *The Plant Cell*, vol. 16, no. 12, pp. 3216–3229, 2004.

[581] W. S. Leal, "Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes," *Annual Review of Entomology*, vol. 58, pp. 373–391, 2013.

[582] S. R. Uppalapati, Y. Ishiga, C.-M. Ryu, T. Ishiga, K. Wang, L. D. Noël, J. E. Parker, and K. S. Mysore, "SGT1 contributes to coronatine signaling and *Pseudomonas syringae* pv. tomato disease symptom development in tomato and *Arabidopsis*," *New Phytologist*, vol. 189, no. 1, pp. 83–93, 2011.

[583] C. An and Z. Mou, "Salicylic acid and its function in plant immunity," *Journal of Integrative Plant Biology*, vol. 53, no. 6, pp. 412–428, 2011.

[584] P. Bednarek, M. Piślewska-Bednarek, E. Ver Loren van Themaat, R. K. Maddula, A. Svatoš, and P. Schulze-Lefert, "Conservation and clade-specific diversification of pathogen-inducible tryptophan and indole glucosinolate metabolism in *Arabidopsis thaliana* relatives," *New Phytologist*, vol. 192, no. 3, pp. 713–726, 2011.

[585] M. E. Møldrup, B. Salomonsen, F. Geu-Flores, C. E. Olsen, and B. A. Halkier, "De novo genetic engineering of the camalexin biosynthetic pathway," *Journal of Biotechnology*, vol. 167, no. 3, pp. 296–301, 2013.

[586] S. Zhu, Y. Li, J. H. Vossen, R. G. Visser, and E. Jacobsen, "Functional stacking of three resistance genes against *Phytophthora infestans* in potato," *Transgenic Research*, vol. 21, no. 1, pp. 89–99, 2012.

[587] C. Halpin, "Gene stacking in transgenic plants–the challenge for 21st century plant biotechnology," *Plant Biotechnology Journal*, vol. 3, no. 2, pp. 141–155, 2005.

[588] C. B. Hill, L. Crull, T. K. Herman, D. J. Voegtlin, and G. L. Hartman, "A new soybean aphid (Hemiptera: Aphididae) biotype identified," *Journal of Economic Entomology*, vol. 103, no. 2, pp. 509–515, 2010.

[589] S. Lacombe, A. Rougon-Cardoso, E. Sherwood, N. Peeters, D. Dahlbeck, H. P. Van Esse, M. Smoker, G. Rallapalli, B. P. Thomma, B. Staskawicz, J. D. Jones, and C. Zipfel, "Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance," *Nature Biotechnology*, vol. 28, no. 4, pp. 365–369, 2010.

[590] H.-P. Dong, J. Peng, Z. Bao, X. Meng, J. M. Bonasera, G. Chen, S. V. Beer, and H. Dong, "Downstream divergence of the ethylene signaling pathway for harpin-stimulated *Arabidopsis* growth and insect defense," *Plant Physiology*, vol. 136, no. 3, pp. 3628–3638, 2004.

[591] D. Walters, D. Walsh, A. Newton, and G. Lyon, "Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors," *Phytopathology*, vol. 95, no. 12, pp. 1368–1373, 2005.