Analysis of *Arabidopsis thaliana* small RNA-mediated defence responses to infestation by the green peach aphid *Myzus persicae*

Graeme J Kettles

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Abstract

The green peach aphid *Myzus persicae* is an economically important plant pest worldwide. Infestations deprive hosts of photoassimilates and aphids are primary vectors for hundreds of viruses. In plants, RNA silencing pathways have been implicated in resistance to viral, bacterial and fungal pathogens, as well as to a lepidopteran insect pest. I aimed to uncover any involvement of these pathways in aphid resistance.

I found that in the model plant *Arabidopsis*, miRNA pathway mutants are more resistant to aphid infestation than wild-type plants or siRNA pathway mutants. MiRNA mutant resistance is independent of the Salicylic Acid (SA) and Jasmonic Acid (JA) signalling pathways. In contrast, miRNA mutants have elevated levels of *PAD3* following infestation and accumulate the antimicrobial compound camalexin. Camalexin reduces aphid fecundity when fed by artificial diet, indicating a novel role for this compound in modifying insect reproductive development.

In addition, I uncovered a role for the WRKY33 transcription factor and the miRNA miR393 in aphid resistance. Aphids are more fecund on *wrky33* mutants and *WRKY33* is differentially regulated between wild-type and silencing-deficient plants during aphid infestation. WRKY33 positively regulates *PAD3*, and is likely to positively regulate camalexin production during aphid infestation. Furthemore, aphids are more successful on plants overexpressing miR393. MiR393 is responsive to aphid infestation and is known to regulate camalexin production through auxin signalling. There is evidence that miR393 may have additional roles in direct regulation of *WRKY33* and *PAD3*. This is the first report of a plant miRNA mediating resistance against a phloem-feeding insect pest.

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List of Abbreviations

bp	base pair
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
GFP	green fluorescent protein
hpi	hours post inoculation
nt	nucleotide
OD	optical density
PCR	polymerase chain reaction
GUS	beta-glucuronidase
siRNA	small interfering RNA
vsiRNA	virus-derived small interfering RNA
miRNA	microRNA

"Twenty years from now, you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbour. Catch the trade winds in your sails. Explore. Dream. Discover."

- *Mark Twain*

Chapter 1 - Introduction

1.1 Aphids and agriculture

The world's population is predicted to rise to around nine billion by the year 2050 (United Nations. Dept. of & Social Affairs. Population, 2004). In order to adequately feed this increasing population, agriculture must adapt to produce an increased amount of food from a similar area of land and without a significant increase in chemical input. In addition, the effect of global climate change will have unpredictable impact on regions devoted to food production.

Every year, pests and pathogens reduce productivity across all crop species. In particular, sap-sucking insects are some of the most destructive pests in temperate regions throughout the world (Blackman & Eastop, 2000). Of this group, aphids are of specific importance as they negatively impact plant productivity in three ways. Firstly, heavy infestations deprive host plants of photoassimilates which would otherwise have been used for growth and development. Secondly, aphids secrete large volumes of sticky fluid called honeydew. This allows growth of soot moulds on plant surfaces and reduces photosynthetic efficiency. Finally, the primary agricultural concern is that aphids act as carriers of plant viruses (Fig. 1.1). Hemipteran insects such as aphids and whiteflies are known to vector over one-third of the ~800 plant viruses described to date (Ng & Perry, 2004; Hogenhout *et al.*, 2008). In light of this role in the spreading of disease, aphids can be thought of as the mosquitoes of the plant world.

The green peach aphid (*Myzus persicae* Sulz.) is one of the most agriculturally important aphid pests. The Swiss entomologist Johann Heinrich Sulzer described this species in the latter half of the 18th century, with its species name derived from the latin, "of the peach." Although it is found and lays eggs on peach trees, it has one of the broadest host ranges of any aphid species, encompassing 40 plant families and including many

economically relevant crops such as tobacco, potato, tomato, and rape seed. Of all aphid species, *M. persicae* is the most important virus vector (Blackman & Eastop, 2000). It can transmit over a 100 different plant viruses of which the majority are potyviruses (Ng & Perry, 2004), but also the economically important luteoviruses, including *Beet western yellows virus* (Turnip yellows virus) and *Potato leafroll virus*. Additionally, it can also quickly acquire and transmit non-persistent viruses such as the broad range *Cucumber mosaic virus* (Fig. 1.1) (Palukaitis & García-Arenal, 2003).

For the last 60 years, aphid populations on agricultural crops have been largely controlled through the spraying of chemical insecticides. As a result of widespread and continuous exposure, *M. persicae* has developed resistance to several classes of insecticides, including organophosphates, carbamates and pyrethroids (Field *et al.*, 1988; Martinez Torres *et al.*, 1999). Until recently, neonicotinoids which target nicotinic acetylcholine receptors remained an effective control measure against *M. persicae* as well as many other insect pests. However, reports of resistance against this chemical class have emerged in *M. persicae* lineages on several continents which could have long-term impacts for aphid control in agriculture (Nauen & Denholm, 2005; Foster *et al.*, 2008; Puinean *et al.*, 2010). In light of increased insecticide resistance, alternative strategies must be developed for the control of aphid infestations. The use of aphid predators, such as the parasitic wasps *Aphidius colemani* and *Aphidius ervi,* which oviposit inside aphid bodies, is an alternative strategy to control aphid numbers. However, there is evidence that again, aphid lineages emerge which have increased resistance to this control measure (Li *et al.*, 2002). Intriguingly, the mechanisms responsible for parasitoid resistance appear distinct from those responsible for insecticide resistance and may involve the acquisition of beneficial bacterial endosymbionts (Oliver *et al.*, 2003). In commercial environments, nethouses are used to protect virus-susceptible crops, such as cucurbits. However, nethouses only cover small areas of land, must be replaced relatively frequently and offer

no protection should a small founder population of insects reach the crop under protection. A greater understanding of how plants detect and respond to aphids, and conversely, the strategies employed by aphids to overcome plant defences would allow novel strategies to be developed to counter aphid infestations. This would ultimately lead to development of aphid-resistant crop varieties to negate the economic, environmental and social costs of increased insecticide use.

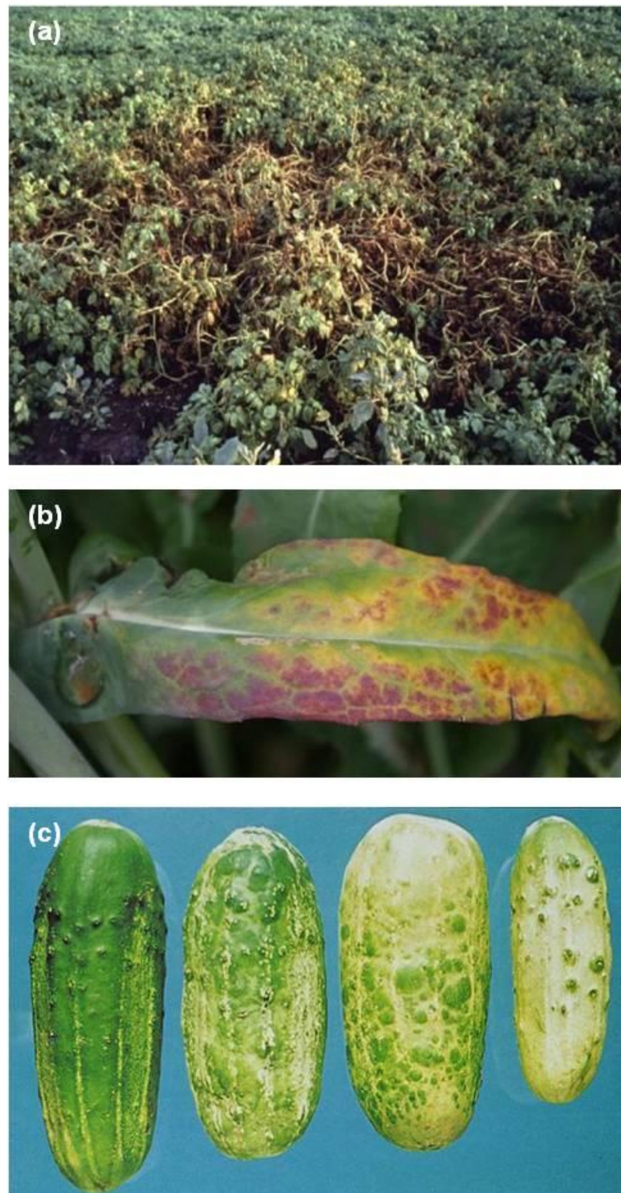

Figure 1.1 – Aphid infestations and the viruses they vector cause significant economic losses.

(a) Aphid infestations can occassionally be so severe that "aphid holes" develop in crop production fields. In this example, aphid infestation has caused potato vines to prematurely senesce and die. Image adapted from (Suranyi *et al.*, 2009). **(b)** *Turnip yellows virus* (TuYV) is an agricultural problem in the UK as it infects *Brassica napus* (oilseed rape). Here virus infection is characterized by yellow and purple mottled leaves and results in reduced productivity and poorer quality oil (photo by M. Stevens, Brooms Barn). **(c)** *Cucumber mosaic virus* (CMV) is one of the most common aphid-vectored viruses. It is able to infect >150 different plant species, and aphids can acquire the virus within minutes of feeding on an infected plant. Here CMV symptoms can be observed on cucumbers ranging from least severe (left) to most severe (right) (Image adapted from Texas Plant Disease Handbook (http://plantdiseasehandbook.tamu.edu/)).

1.2 The aphid life cycle

The aphid group consists of over 4000 species, and within this group, there is considerable diversity in life cycles. Many aphids have an cyclically parthenogenic life cycle which accommodates distinct sexual and asexual reproductive phases (Fig. 1.2) (Blackman & Eastop, 2000). This reproductive strategy is required for species that are not tolerant to cold winters and must seek out primary host plants in autumn to mate and lay eggs to overwinter. Aphids are much more selective in selecting hosts (primary hosts) for oviposition than feeding (secondary hosts). Many aphid species use a single primary host for oviposition, but may feed on plants from a wider range of families during other parts of the year. For these cyclically parthenogenic aphids, individuals hatch from overwintered eggs in spring. Throughout this period, the population consists of only females and reproduction occurs by parthenogenesis, with adults giving birth to large numbers of genetically identical offspring (nymphs). This reproductive strategy is generationally telescopic, with the embryos of the next generation already beginning to develop within the bodies of newly-born nymphs. Nymphs are also born alive (viviparous reproduction), which is unusual amongst insects and facilitates a more rapid development to reproductive maturity. During spring, and also in overcrowded conditions, significant numbers of aphids are winged (alates). Despite limited flying ability, they are able to disperse passively on the wind to colonise secondary hosts. Alates are known to be particularly restless, and will seed small numbers of nymphs on a large number of secondary host plants. These seeded nymphs will then proceed through multiple generations of asexual reproduction throughout the summer months. Given favourable environmental conditions, such as high levels of heat and humidity and an absence of predators, this strategy can result in explosive population growth. Towards the end of summer, shortening day length and cooler temperatures trigger both physical and behavioural changes in insects. These give rise to

larger proportions of both alates and male insects in the population, and trigger a return to the sexual phase of the life cycle. Alates disperse from summer hosts and seek primary hosts on which to mate and lay eggs to survive winter.

Under some circumstances however, such as regions with mild winters or in artificial glasshouse conditions, it is possible for aphids to exist by year-round parthenogenesis (anholocycly) (Turl, 1983; Fenton *et al.*, 1998). In addition, aphids are most common in temperate regions, indicating that many species have evolved some degree of cold-adapted lifestyles. In these cases, aphids may exist purely by asexual reproduction and may even have lost ability to reproduce sexually. *M. persicae* colonies of northern England and Scotland are commonly anholocyclic, probably as a result of a lack of peach trees, the primary oviposition/sexual reproduction host for this species (Fenton *et al.*, 1998). It is likely that the relatively mild winters coupled with a lack of primary hosts in these regions allow populations of mobile parthenogenic adults to survive without the need for sexual reproduction and egg laying.

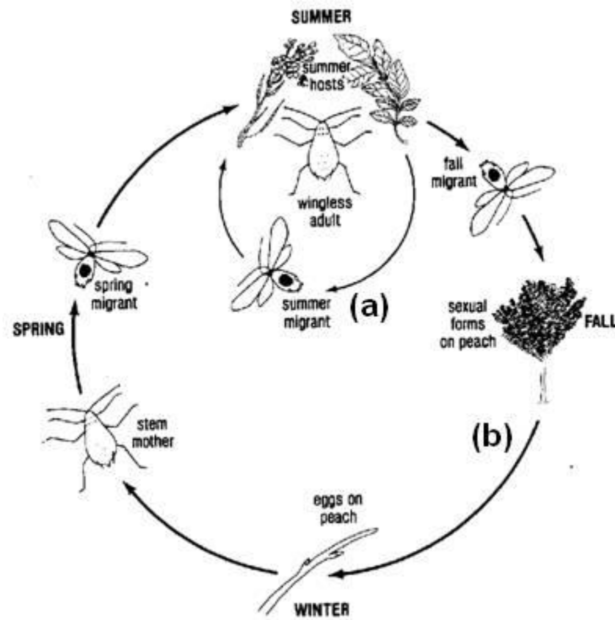

Figure 1.2 – An aphid life-cycle can accommodate two distinct phases.

In locations with mild winters and a lack of primary hosts (peach), *M. persicae* can exist with a monocyclical life cycle **(a)**. In these environments, reproduction is purely asexual and there is no egg-laying stage. In locations with colder winters and/or where the primary host is more abundant, a bicyclical life-cycle may be apparent **(a+b)**. In this life cycle, aphids reproduce sexually in autumn (fall) and lay eggs to survive winter. In spring, winged aphids (alates) migrate to many species of secondary host where aphids reproduce asexually over the summer. Image adapted from (Suranyi *et al.*, 1998).

1.3 The concept of immunity

As sessile organisms, plants have evolved sophisticated means of protecting themselves against the myriad of attackers they encounter in nature. These assailants include the innumerable strains of viruses, bacteria or fungi that occur in the environment, or one of many species of insects and nematodes that feed on plants. In addition, defence responses must also be flexible enough to respond to multiple infections or infestations concurrently.

The underlying principle in immunity is how an organism is able to differentiate between “self” and “non-self”. Plants, like animals, are able to recognise characteristic signatures of pathogens that are variably termed Pathogen-Associated Molecular Patterns (PAMPs) or Microbe-Associated Molecular Patterns (MAMPs) (Segonzac & Zipfel, 2011). These patterns are molecules or parts of molecules key to pathogen viability, and as such are retained by the pathogen through evolution. Examples of PAMPs perceived by plants include the bacterial motor protein flagellin (or its active epitope flg22) and the fungal cell wall protein chitin (Schwessinger & Zipfel, 2008). PAMPs are recognised on cell surfaces by a specific pattern recognition receptor (PRR) (Fig. 1.3a). PRRs identified to date are all transmembrane proteins and examples include the flagellin recognition protein FLS2 and the elongation factor Tu (EF-Tu) receptor EFR (Chinchilla *et al.*, 2006; Zipfel *et al.*, 2006). Recognition of a PAMP by its PRR represents the initial perception event in plant immunity and this triggers responses intended to stop pathogen colonisation of the host (Fig. 1.3a). Some PRRs have intracellular kinase domains which facilitate downstream signalling events that lead to appropriate defence responses (Segonzac & Zipfel, 2011). Some of these responses can be activated within minutes of PAMP perception, such as the generation of reactive oxygen species (ROS) or release of a burst of calcium ions. Additionally, PAMP recognition triggers transcriptional reprogramming and the induction of defence-related genes which is only detected several hours later. The response generated following PRR-mediated perception of PAMPs is in most cases enough to prevent colonisation by pathogens. This is referred to as PAMP-triggered immunity (PTI) and represents the basal layer of plant defence.

In order to usurp PTI in compatible interactions in which the pathogenic bacteria and fungi can colonise a plant species, the pathogens produce proteinaceous virulence factors referred to as effectors (Fig. 1.3b). These molecules interfere with various aspects of host physiology and signalling to block induction of PTI and increase host susceptibility

(Jones & Dangl, 2006). How effectors are delivered into host cells is very much dependent on the lifestyle of the pathogen. Intracellular pathogens such as members of the *Candidatus* Phytoplasma genus use conventional secretory pathways as they are already contained within host cells (Bai *et al.*, 2009). The majority of extracellular Gram-negative bacterial pathogens introduce effectors into host cells by the Type III Secretion System (T3SS), a delivery mechanism whereby molecules are injected directly into the cytoplasm of host cells via a needle-like protrusion embedded in the bacterial cell membrane (Alfano & Collmer, 2004) (Fig. 1.3b). In contrast to PAMPs, pathogen effectors are not essential for pathogen viability; however they can be essential for determining the success of colonisation on a specific host species. Effector genes are fast evolving, and can be deleted, altered or suppressed when they no longer serve a function as a virulence factor (Jones & Dangl, 2006). The reason underlying this is that hosts are constantly evolving to recognise effector molecules to activate a second level of immunity, referred to as Effector Triggered Immunity (ETI) (Fig. 1.3c). This response is mediated by resistance (R) genes. R gene products are often characterised by their nucleotide binding site and leucine-rich repeat (NBS-LRR) structures (Belkhadir *et al.*, 2004). Their function is to either directly recognise pathogen effectors, or more commonly, to recognise the action of effectors on host proteins. This latter function is referred to as the guard hypothesis. This recognition prompts a much stronger defence response than that of PTI, and is often associated with a form of programmed cell death called the hypersensitive response (HR) (Jones & Dangl, 2006). This localised cell death response isolates pathogens, limiting their ability to colonise other parts of the host.

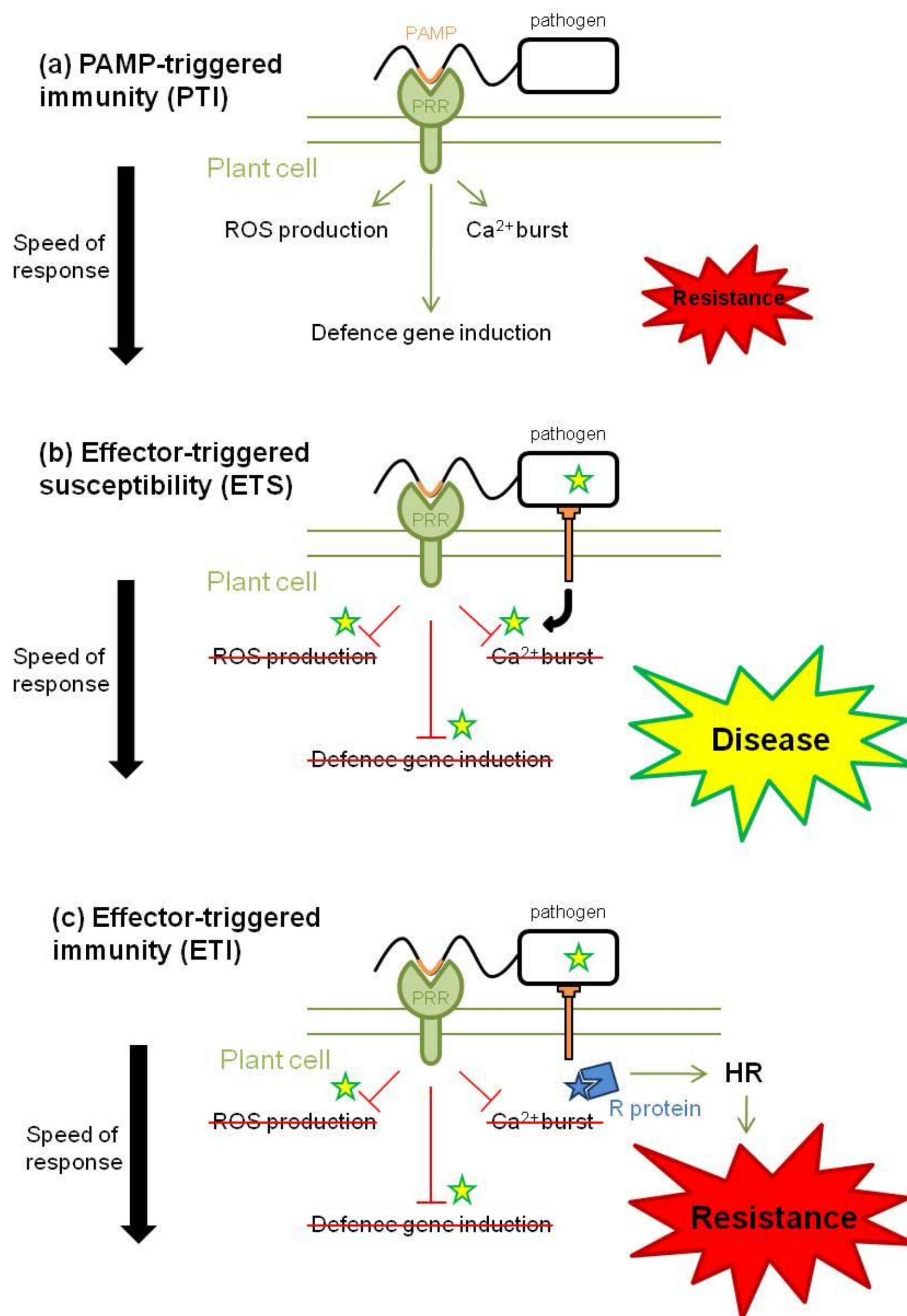

Figure 1.3 – A model of plant immunity.

(a) PAMPs are recognised by their respective pattern recognition receptors (PRRs), which triggers multiple defensive processes resulting in PAMP-triggered immunity (PTI). **(b)** Successful pathogens overcome PTI by introducing effectors into host cells that disrupt the defence response resulting in effector-triggered susceptibility (ETS). **(c)** Hosts may evolve R genes which encode proteins able to either recognise effectors directly or the damage caused by effectors. This triggers a more aggressive reaction termed the hypersensitive response (HR) which is a stronger form of resistance.

The suites of genes and proteins involved in both PTI and ETI have largely been identified through use of the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* and their interactions with several species of pathogenic bacteria, fungi and oomycetes (Segonzac & Zipfel, 2011). It is less clear how accurately this working model describes the plant defense response to insect pests. Nonetheless, there are examples of elicitors of plant defences present in insect saliva which can be classified as Herbivory-Associated Molecular Patterns (HAMPs) (Wu & Baldwin, 2010; Hogenhout & Bos, 2011). In addition, several studies have identified aphid salivary proteins, which suppress similar defence responses as those targeted by bacterial or fungal effectors (Will *et al.*, 2007; Mutti *et al.*, 2008; Bos *et al.*, 2010). Resistance genes are also involved in crop resistance to aphids (Dedryver *et al.*, 2010; Dogimont *et al.*, 2010). In general, aphid resistance appears to be polygenic although there are examples of single dominant *R* genes (Dedryver *et al.*, 2010; Dogimont *et al.*, 2010). Similar to *R* gene-mediated resistance against bacteria and fungi, plant *R* genes are only effective against some aphid species and even between species biotypes (Fig. 1.4). Two *R* genes (*Mi-1* and *Vat*) that confer aphid resistance in tomato and melon respectively have been isolated (Rossi *et al.*, 1998; Dogimont *et al.*, 2010). Both of these aphid *R* genes are members of the NBS-LRR family of resistance genes. Curiously, *Mi-1* provides resistance against several root-knot nematode species and a biotype of *Macrosiphum euphorbiae* (potato aphid) in tomato, indicating that there may be some functional overlap in defence against these distinct plant attackers. Additionally, other putative *R* genes that are members of the NBS-LRR family and confer resistance to aphids have been identified (Dogimont *et al.*, 2010). Therefore, whilst it remains to be elucidated whether all the molecular players identified in PTI and ETI against bacteria and fungi have roles in insect defence, the underlying principles hold true.

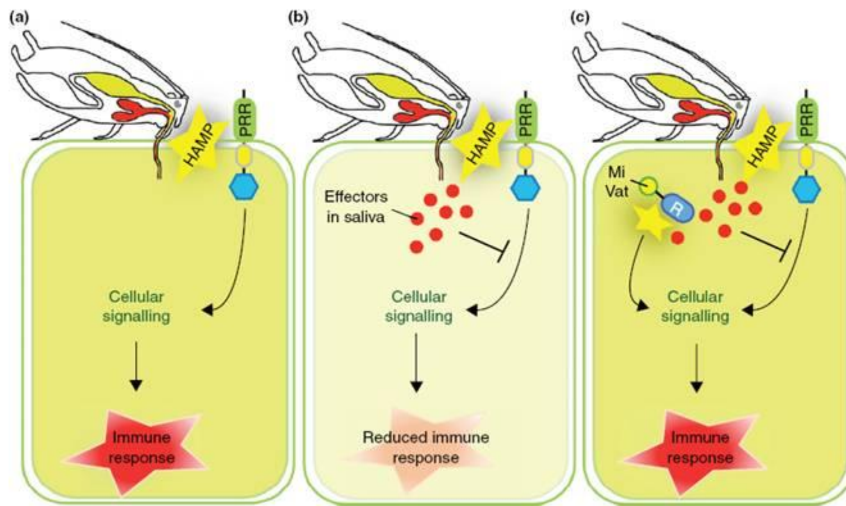

Figure 1.4 – Model of plant defence against aphid feeding.

(a) Plants are able to perceive herbivory-associated molecular patterns (HAMPs) via aphid PRRs that leads to HAMP-triggered immunity (HTI). **(b)** Effectors present in aphid saliva allow insects to suppress HTI. This leads to a reduced immune response and enhanced aphid colonisation. **(c)** *R* genes present in resistant hosts are able to recognize aphid effectors, resulting in a strong immune response and enhanced level of resistance. Two such effectors (*Mi-1* and *Vat*) confer resistance to biotypes of the potato and melon aphid respectively. Image taken from (Hogenhout & Bos, 2011).

1.4 The role of phytohormones in plant defence responses

A crucial determinant of whether invasion by a pathogen or pest is repelled is how successfully a host can modify its gene expression pattern to counteract the threat. Plants have evolved to produce a plethora of metabolites, many of which have defensive benefits against pathogens or pests. These metabolites may have functions in other non-defensive capacities, or they may be specifically produced, often in a highly localized fashion, following pathogen perception. The induction of some metabolic pathways may effectively restrict the colonisation of some pathogens but not others. Therefore, resources must be

channelled through pathways which are most effective against a certain pathogen. Additionally, plants should also balance growth and development versus defence response.

Many metabolic pathways are governed by a set of phytohormones that are involved to some degree in almost all aspects of plant physiology. These hormones play varied and important roles in regulating the responses that plants make following perception of a pathogen or pest. Salicylic acid (SA) and jasmonates (JA) are perhaps the best studied as they are involved in immune responses against many biotrophic and necrotrophic pathogens and also against both chewing and piercing-sucking insect pests (Bari & Jones, 2009). Ethylene (ET) has been the focus of extensive investigation due to its role in modulating JA-dependent defence mechanisms (Lin *et al.*, 2009). More recently, auxin, gibberellic acid (GA), brassinosteroids and cytokinins have emerged as additional regulators of plant defence processes (Robert-Seilaniantz *et al.*, 2007). Many of these pathways communicate in a highly intricate fashion to further tailor defence responses to maximally utilise resources at the plants disposal.

Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two related mechanisms in plants. Both mechanisms utilise hormone pathways and mobile signals to increase resistance levels in tissues that may be some distance from the site of pathogen perception. Classic SAR is defined as a whole plant response to an earlier and localised challenge by a pathogen (Grant & Lamb, 2006). In addition, some degree of immunity to pathogens can be conferred by an earlier exposure to non-pathogenic rhizobacterial strains (Pieterse *et al.*, 1998). In some circumstances, these previous exposures may also have growth promoting effects on plants. Immunity conferred by SAR and ISR is generally broad spectrum and long-lasting. However, there are distinct molecular mechanisms behind these resistances.

SA accumulation in both local and systemic tissues is required for effective SAR. SA is a small molecule which can be synthesised from phenylalanine, although the bulk of SA is produced from chorismate by isochorismate synthase (ICS) (Wildermuth *et al.*, 2001; Chen *et al.*, 2009). A central regulator of SA signalling is non-expressor of PR gene 1 (NPR1), which interacts with TGA MOTIF-BINDING FACTOR (TGA) transcription factors involved in the activation of many *pathogenesis-related* (*PR*) genes that are induced following pathogen challenge (Dong, 2004; Bari & Jones, 2009). Both SA levels and SA-responsive gene transcripts increase when plants are challenged with many biotrophic or hemi-biotrophic pathogens or piercing-sucking insects such as aphids and whiteflies (de Vos *et al.*, 2005; Bari & Jones, 2009). Many *PR* genes are also activated when plants are directly treated with SA in the absence of pathogens. *Arabidopsis* mutants impaired in SA signalling are often more susceptible to biotrophic and hemibiotrophic pathogens.

The JA signalling pathway is a lipid-derived signalling mechanism that is involved in multiple facets of plant responsiveness to the environment (Wasternack & Kombrink, 2009). Specifically for defence, JA-related compounds are candidates to be the mobile signals in SAR and are involved to some extent in the establishment of ISR (Pieterse *et al.*, 1998). JA signalling is complex and requires multiple components many of which were identified in pathogen-susceptibility screens in *Arabidopsis*.

JA is synthesised through multiple enzymatic reactions in chloroplasts and peroxisomes. Initially, galactolipids are converted to OPDA in a series of reactions involving the LOX, AOS and AOC enzymes in chloroplasts (Wasternack & Kombrink, 2009). OPDA is subsequently transported to peroxisomes where a further series of six mostly well characterised reactions result in production of JA. JA is activated in the cytoplasm by conjugation to the amino acid L-isoleucine (Ile) by JAR1 to produce the highly bioactive molecule JA-Ile (Schaller & Stintzi, 2009; Wasternack & Kombrink,

2009). Perception of JA-Ile perception involves another set of proteins that ultimately control the transcription of JA-responsive genes. In the absence of JA-Ile, the suppressor JASMONATE ZIM DOMAIN (JAZ) directly binds and represses the activity of the transcription factor MYC2, preventing transcription of JA-responsive genes (Chini *et al.*, 2007). In the presence of JA-Ile, the Skp1-Cul1-F-box protein (SCF) ubiquitin ligase complex containing CORONATINE INSENSITIVE 1 (COI1) sequesters JAZ proteins resulting in their polyubiquitylation and degradation (Sheard *et al.*, 2010). This interaction frees MYC2 to activate transcription of a suite of JA-responsive genes.

SA and JA often act antagonistically, where activation of one leads to suppression of the other (Bari & Jones, 2009; Verhage *et al.*, 2010). JA-mediated defence mechanisms are generally induced by necrotrophic pathogens such as the ascomycete fungi *Alternaria* and by insects with chewing mouthparts that cause large amounts of tissue damage such as *Manduca sexta* (tobacco hornworm) on tobacco or *Pieris rapae* (cabbage butterfly) on *Arabidopsis* (de Vos et al., 2005; Diezel et al., 2009). In contrast, biothrophic pathogens and piercing-sucking insects such as aphids induce SA and suppress JA. However, JA versus SA induction appears to differ between generalist and specialist insects (Diezel *et al.*, 2009). Both JA levels and levels of JA-related transcripts increase following challenge by some pathogens or insects (de Vos *et al.*, 2005). Many of these JA-related transcripts can also be induced by direct application of JA to a healthy plant (Ellis *et al.*, 2002).

A proportion of the genes that are responsive to JA are also responsive to the gaseous hormone ethylene (ET) (Wang *et al.*, 2002; Robert-Seilaniantz *et al.*, 2007; Bari & Jones, 2009; Verhage *et al.*, 2010). As such, ET signalling is generally associated with defence against necrotrophs and chewing insects. Besides defence, ET also regulates numerous plant processes such as fruit development and response to some abiotic stresses (Lin *et al.*, 2009). Its role in pathogen responses is borne from observations that *Arabidopsis* mutants unable to respond to ethylene are often more susceptible to disease

(Hoffman *et al.*, 1999; Thomma *et al.*, 1999). ETHYLENE INSENSITIVE 2 (EIN2) represents a crucial signalling node in ET perception (Alonso *et al.*, 1999). In the absence of ET, its activity is negatively regulated by CTR1 (Lin *et al.*, 2009). ET is perceived by a number of ETR receptors which relieve EIN2 from CTR1 suppression. This frees EIN2 to activate the EIN3 and EIN3-like families of transcription factors (Guo & Ecker, 2003; Stepanova & Alonso, 2009). The degree of communication and crosstalk between the JA and ET pathways varies depending on pathogen or pest. However, full understanding of these mechanisms remains elusive.

The hormone auxin is involved to some extent in almost all processes that occur in plant cells (Chapman & Estelle, 2009). How auxin influences defence processes is not fully understood, however there is evidence that it may function through antagonism of the SA signalling pathway (Bari & Jones, 2009). Transcription of auxin-responsive genes is regulated by a large family of Auxin Response Factors (ARFs). The activation of ARF family members is reminiscent of the mechanism controlling JA pathway responses. In the absence of auxin, ARFs are bound by Auxin/Indole-3-acetic acid (Aux/IAA) repressors. Auxin is perceived by Transport Inhibitor Response 1 (TIR1) which is an F-box protein that interacts with the Skp1-Cul1-F-box protein (SCF) ubiquitin ligase complex (Dharmasiri *et al.*, 2005). This complex directs the ubiquitination and degradation of Aux/IAA repressors, thus allowing ARFs to direct transcription of auxin-responsive genes. Studies in bacterial pathosystems indicate that increased levels of auxin tend to promote disease progression (Navarro *et al.*, 2006; Chen *et al.*, 2007). As one mechanism of auxin synthesis is linked to the indole pathway (Sugawara *et al.*, 2009), auxin may also (in)directly regulate production of defensive glucosinolates and phytoalexins.

1.5 Phytoanticipants and phytoalexins

Phytohormone pathways are present in all plant families studied to date, and as such have likely been conserved through the course of evolution. Plants however produce a galaxy of secondary metabolites which whilst not crucial to the functioning of individual cells, enhance fitness of the whole organism. Many of these metabolites are unique to specific plant families and some have potent antibacterial or insecticidal properties. In *Arabidopsis*, some of the best studied metabolite families are the glucosinolates and phytoalexins.

Glucosinolates are synthesized from tryptophan (indole glucosinolates), methionine (aliphatic glucosinolates) and phenylalanine. Glucosinolates are phytoanticipants as they are already present in cells and function as a pre-existing defence mechanism (Koroleva *et al.*, 2000). Upon tissue damage, glucosinolates are brought into contact with myrosinases which catalyse the production of toxic breakdown products such as nitriles, thiocyanates and isothiocyanates (Halkier & Gershenzon, 2006). There are examples however where glucosinolate production is induced following mechanical wounding, treatment with a bacterial elicitor or challenge with a fungal pathogen or insect pest (Halkier & Gershenzon, 2006; Bednarek *et al.*, 2009; Schlaeppi *et al.*, 2010). Glucosinolates are characteristic to the brassica family and deter feeding of generalist insects (Kim & Jander, 2007; Kim *et al.*, 2008). However, they also serve as attractants to brassica specialists which are highly adapted to processing the toxic degradation products (Pratt *et al.*, 2008).

The predominant *Arabidopsis* phytoalexin is camalexin. This compound was first identified over 20 years ago, but how it functions in antibacterial and antifungal defence is not yet fully understood. Camalexin is derived from tryptophan and is produced by a metabolic pathway that branches from the indole glucosinolates (Glawischnig, 2007).

Camalexin production is induced following challenge by a wide variety of pathogens and some insects, including aphids (Glawischnig, 2007; Kuśnierczyk *et al.*, 2008). It has inhibitory effects on the growth of some pathogens, mainly gram-negative bacteria and fungal necrotrophs (Rogers *et al.*, 1996; Kliebenstein *et al.*, 2005; Bednarek *et al.*, 2009; Sanchez Vallet *et al.*, 2010; Schlaeppi *et al.*, 2010). The major regulators of camalexin biosynthesis were identified in a screen of *Arabidopsis* mutants deficient in camalexin accumulation (Glazebrook & Ausubel, 1994). These *PHYTOALEXIN DEFICIENT* (*PAD*) genes are often induced in a highly-localised fashion following pathogen challenge (Kliebenstein *et al.*, 2005). Unlike glucosinolates, camalexin is not a pre-formed defence. It is present in miniscule quantities in unchallenged plants but accumulates in high quantities within 24-48h after inoculation. This suggests that it is metabolically expensive compound to produce and is only synthesised when required by the host.

1.6 Plant deterrents specific to insects

In addition to the pathways and reactions that occur in plants in response to pathogens, there is a range of plant defense responses that deter insect pests. These include the physical barriers erected by plants, such as leaf trichomes, which hinder the movement or feeding ability of insects or their larvae (Levin, 1973). Not only do trichomes act as physical barriers, some classes of glandular trichomes can secrete sticky or poisonous substances that act as feeding deterrents. In addition, the substances composing the waxy cuticle of leaves can hinder insect feeding attempts or reduce the ability to digest consumed tissue and impact insect fitness.

To counter the physical and chemical arsenal of plants, many insects have adopted a specialist feeding strategy. This strategy has a trade-off, in that it limits the number of species that are compatible hosts. However, it allows the development of counter-defence strategies against particular hosts. For example, the cabbage aphid (*Brevicoryne brassicae*) is a specialist of cruciferous plants which produce glucosinolates. Rather than being deterred from feeding, cabbage aphids are able to use these compounds for their own benefit by storing and using as a deterrent against their own predators (Pratt *et al.*, 2008).

Generalist insects have adopted a survival strategy whereby they have adapted to feed on a wide variety of plants. *M. persicae* is an example of a generalist insect, as it can colonise species belonging to 40 plant families (Blackman & Eastop, 2000). Whilst this strategy may increase the likelihood of finding a suitable host in the wild, it means that insects are generally less well adapted to each specific host they encounter and less likely to overcome species-specific defence mechanisms.

A further determinant of the outcome of any plant-insect interaction is the feeding mode of the insect. Many orders of insects, such as coleopterans or lepidopterans possess chewing or crushing mouthparts designed for breaking apart plant tissues prior to digestion. Hemipteran insects such as aphids and whiteflies have piercing-sucking mouthparts (Fig. 1.5) and therefore adopt a very different and more subtle feeding strategy. Compared to chewing insects, hemipterans cause considerably less damage to plant tissues. They insert a long narrow tube (stylet) into plant tissue and attempt to navigate between plant cells causing minimal damage (Fig. 1.5) (Tjallingii, 2006). Plant-feeding hemipterans attempt to reach and puncture the xylem or phloem tissue where they can obtain water or sugar-rich sap (Tjallingii & Esch, 1993; Tjallingii, 1995). Despite this stealthy mode of feeding, phloem- or xylem-feeding insects do induce immune responses in plants (de Vos *et al.*, 2005). However the composition and magnitude of these responses vary

considerable from those generated against either mechanical wounding or from feeding by chewing insects (de Vos *et al.*, 2005).

A final factor to consider with respect to insect feeding compared to pathogen colonisation is the attractiveness of a host to insects. Plants emit a varied bouquet of chemicals that function as signals to insects. The majority of angiosperms attempt to attract pollinating insects to their flowers to assist pollination, an aim that is realised partly through the production of attractive chemical signals. The basal production of some volatiles can discourage herbivorous insects from settling on a plant (Snoeren *et al.*, 2010), and in response to insect attackers, some plants emit volatiles that function in attracting insect predators (de Vos & Jander, 2010). Some viruses and bacterial pathogens are known to modify the volatiles emitted by infected plants and it is thought this may function in increasing the attractiveness of the plant to the virus's insect vector (Eigenbrode *et al.*, 2002; Mauck *et al.*, 2010).

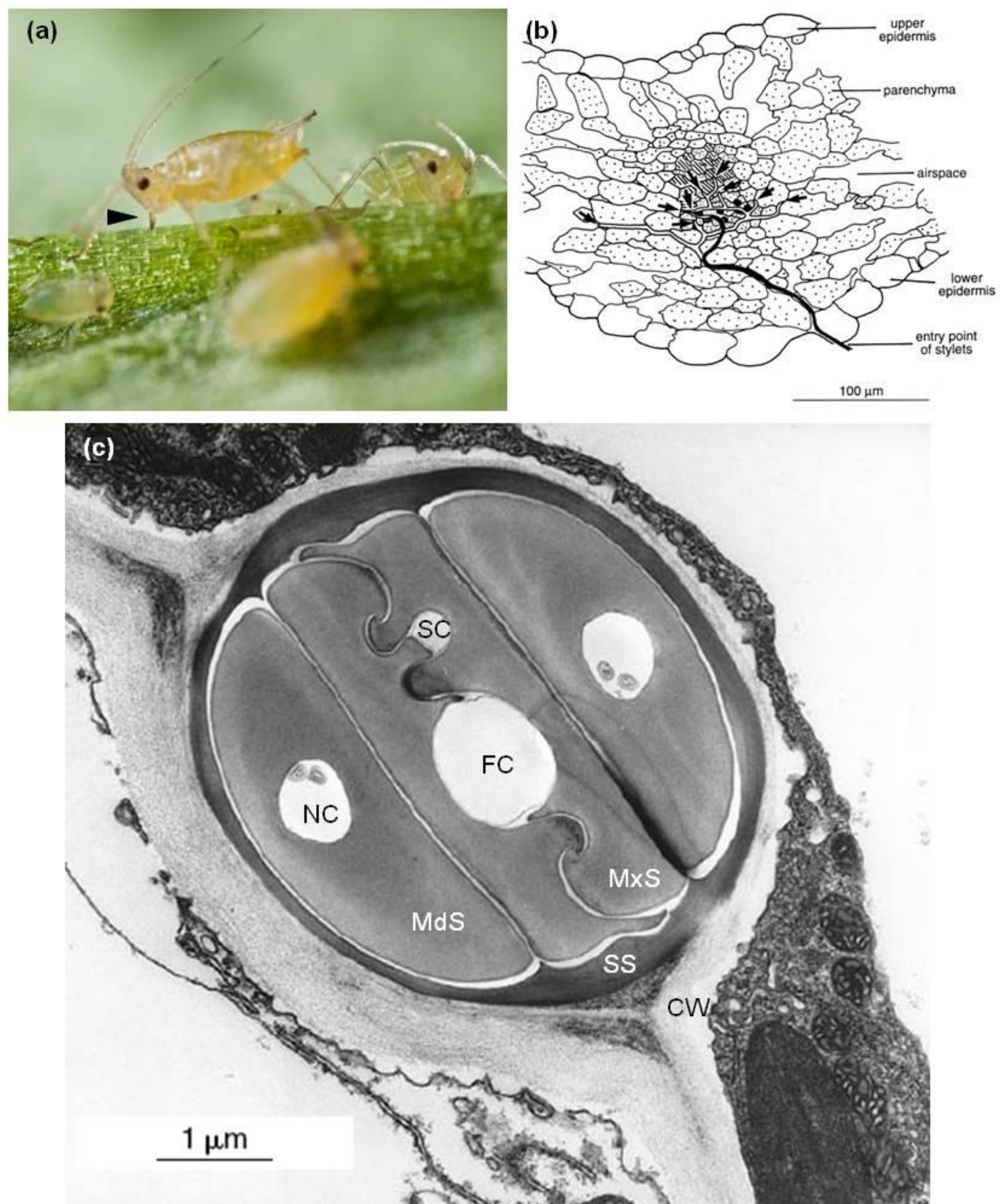

Figure 1.5 - Aphids puncture plant vascular tissues using stylets.

(a) *M. persicae* feeding from the midvein of an *Arabidopsis* rosette leaf. The proboscis (black arrow) houses multiple stylets which are used to penetrate plant tissues and feed on phloem sap (photo by A.Davis). **(b)** Aphid stylets navigate through multiple tissues before terminating in the phloem sieve element. Final stylet track is depicted in black. Aborted stylet tracks are shown in white (black arrows). Phloem sieve tubes (black), xylem (hatched) and parenchyma (dotted) are illustrated. Image adapted from (Chapman, 1998) **(c)** Cross-section of the mouthparts from *Aphis fabae* (black bean aphid) feeding on *Vicia faba* (broad bean). The two maxillary stylets (MxS) interlock to form the food canal (FC) and the salivary canal (SC). Two mandibular stylets (MdS) enclose neuronal canals (NC) housing mechanoreceptor dendrites. Dark material

encircling the stylets is the salivary sheath (SS). White material outside of this is the plant cell wall (CW) Image adapted from (Chapman, 1998).

1.7 Post-transcriptional regulatory processes in eukaryotes

RNA silencing is a collective term to describe regulatory mechanisms that occur in most eukaryotic organisms whereby RNA can direct the sequence-specific inhibition of gene expression. This regulation can occur either at the transcriptional level through RNA-induced modification of chromatin, or post-transcriptionally, where small segments of RNA bind to complementary sequences in mRNAs, guiding either the destruction of those molecules or preventing their translation.

The phenomenon of RNA silencing, or RNA interference (RNAi) had been unknowingly observed for many years, with particular relevance for those studying the progression of viral disease in plants. In 1928, S.A.Wingard described how tobacco plants presenting symptoms of ring-spot virus on older foliage developed new growth and leaves that were apparently resistant to virus infection (Wingard, 1928; Baulcombe, 2004) (Fig. 1.6a). He noted, “this masking of symptoms, or development of immunity, or whatever it is, seems to hold under greenhouse conditions for practically all the plants tested.” At the time, Wingard could not have guessed at the mechanisms underlying the resistance he had observed. In 1928, it was not even known that ring-spot virus was an RNA virus. However, his work serves to highlight one of the evolutionary pressures that resulted in the development and maintenance of RNA silencing machinery that would go largely unappreciated for the next 60 years.

It wasn't until the late 1980s, with the advent of advanced molecular techniques and plant transformation that further light was shed on this phenomenon. Plant virologists were experimenting with the insertion of viral genes into plants in an attempt to understand disease resistance. These experiments had revealed that tobacco expressing the *Tobacco mosaic virus* (TMV) coat protein, were more resistant to TMV infection that non-transgenic controls (Abel *et al.*, 1986). It later emerged that plants expressing untranslatable forms of the *Tobacco etch virus* (TEV) coat protein were more resistant to TEV infection that non-transgenic controls (Lindbo & Dougherty, 1992b; Lindbo & Dougherty, 1992a) These were the first confirmations that RNA and not protein was the agent responsible for the resistance phenotype.

Around the same time, experiments conducted using transgenic petunia shed light on another manifestation of gene silencing (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990) (Fig. 1.7a-d). Introduction of a chalcone synthase (CHS) transgene into purple-flowered petunia plants silenced expression of the endogenous CHS gene, leading to production of flowers that were either white (totally silenced) (Fig. 1.7d) or variegated (partially silenced) (Fig. 1.7b,c). Upon examination, investigators found that introduction of the transgene resulted in a 50-fold reduction in mRNA levels of the endogenous CHS gene (Napoli *et al.*, 1990). Although the mechanism responsible for this phenotype eluded the authors at the time, they did state that "the erratic and reversible nature of this phenomenon suggests the possible involvement of methylation". In subsequent years, the characterisation of heterochromatic silencing would validate this prophecy.

The experiments described above serve to illustrate the evolutionary pressures that favoured the maintenance of a system we know refer to as RNA silencing. The genomes of plants, like many other organisms, are composed of a large number of repetitive elements. These elements often transpose into other genomic regions and disrupt gene function. In addition, plants live in an environment populated with viruses. Stopping these two related

threats can be achieved with an RNA-based immune system which operates by sequence specificity. In many of the pioneering experiments in the field, including those described by both Napoli *et al.* and van der Krol *et al.*, disarmed versions of the naturally tumour-inducing plant pathogen *Agrobacterium tumefaciens* (*A. tumefaciens*) were used to introduce transgenes into plants. As transgenes were inserted along with the T-DNA element of *Agrobacterium*, the subsequent “genetic invader” inadvertently accessed the RNA silencing pathways to cause unexpected phenotypes.

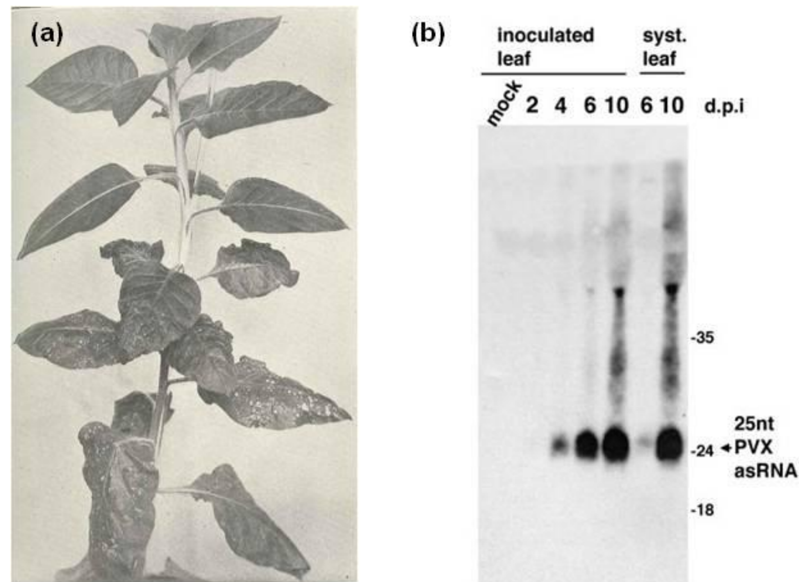

Figure 1.6 - An RNA-based immune system in plants.

(a) In 1928, S.A.Wingard observed that tobacco plants infected with ringspot virus on lower leaves produced new growth that was immune to infection. This was most probably the first description of RNA silencing in an antiviral immune capacity. Image adapted from (Wingard, 1928). **(b)** Seventy years later, it was established using *Potato virus X* (PVX) that viral infections elicit production of antiviral small RNAs both in virus-inoculated leaves and later in systemic leaves, leading to plant immunity. Image adapted from (Hamilton & Baulcombe, 1999).

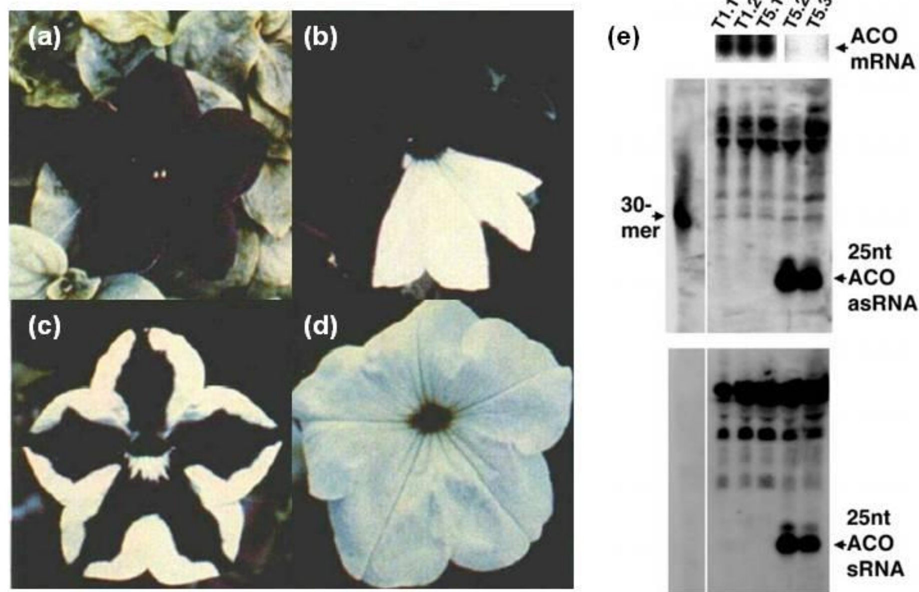

Figure 1.7 – Transgene-induced silencing is guided by small RNAs.

(a-d) Agrobacterium-mediated transformation of wild-type petunia **(a)** with a chalcone synthase (CHS) transgene unexpectedly led to silencing of the endogenous CHS gene, resulting in varying degrees of pigment-free flowers **(b-d)**. Image adapted from (Napoli *et al.*, 1990). **(e)** Hamilton and Baulcombe later revealed that this phenomenon of cosuppression was also guided by small RNAs. Using tomato plants transformed with a 1-aminocyclopropane-1-carboxylate oxidase (ACO) transgene, only plants silenced for ACO mRNA expression (top panel) produced ACO gene-specific small RNAs (mid and bottom panels). Image adapted from (Hamilton & Baulcombe, 1999).

We now have a much clearer understanding of how these related pathways operate in many different organisms. The work of Fire and Mello identified that gene silencing is caused by double-stranded RNA (dsRNA) (Fire *et al.*, 1998), a discovery for which they were subsequently awarded the 2006 Nobel Prize for Medicine. A rapid series of discoveries from several labs, working with plants, worms and flies rapidly identified the molecular machines involved in gene silencing. Together, these discoveries established that dsRNA is cleaved into pieces of small interfering RNA (siRNA) by an endoribonuclease called Dicer (Bernstein *et al.*, 2001). SiRNAs are subsequently incorporated into an RNA-induced

silencing complex (RISC) containing another ribonuclease with slicing activity called Argonaute (Hammond *et al.*, 2000; Hammond *et al.*, 2001). Direction of RISC to mRNAs with sufficient homology results in suppression of gene expression through either transcript cleavage or translational repression (Baumberger & Baulcombe, 2005; Brodersen *et al.*, 2008; Lanet *et al.*, 2009; Huntzinger & Izaurralde, 2011). Although many organisms enact variations of this process, the core fundamentals are retained across species. In recent years, the discoveries underlying gene silencing have allowed extensive investigation of many endogenous sRNA classes such as micro-RNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) that play important roles in many eukaryotic cellular processes, such as the precise control of gene expression and maintenance of genome integrity in the germline (Bartel, 2004; Rajagopalan *et al.*, 2006; Voinnet, 2009; Siomi *et al.*, 2011).

Plants are particularly interesting organisms to investigate, as RNA silencing appears to have expanded into multiple pathways that even now are not fully understood (Vazquez *et al.*, 2010). In *Arabidopsis*, there are six RNA-dependent RNA polymerases (RDRs) which act to produce dsRNA from a single-stranded RNA (ssRNA) template and serve to both generate and amplify silencing signals (Dalmay *et al.*, 2000; Ruiz-Ferrer & Voinnet, 2009). There are four Dicer-like (DCL) endoribonucleases which produce distinct sRNA size classes from dsRNA templates. DCL1 produces 21nt sRNAs and is involved with miRNA processing (Park *et al.*, 2002; Kurihara & Watanabe, 2004). DCL2 produces 22nt sRNAs and is involved with antiviral defence (Deleris *et al.*, 2006). DCL3 synthesizes 24nt sRNAs which feed into the RNA-directed DNA methylation (RdDM) pathway (Xie *et al.*, 2004). DCL4 produces 21nt sRNAs involved in both antiviral defence and the trans-acting small interfering RNA (tasiRNA) pathway (Vazquez *et al.*, 2004b; Xie *et al.*, 2005; Deleris *et al.*, 2006; Henderson *et al.*, 2006). This latter pathway is known to play a significant role in *Arabidopsis* organ development (Schwab *et al.*, 2009). There is also

considerable diversification amongst the Argonaute (AGO) gene family. *Arabidopsis* encodes ten Argonautes which are divided across three clades (Vaucheret, 2008; Vazquez *et al.*, 2010; Czech & Hannon, 2011). AGO1 is the predominant slicer of the miRNA pathway, although there are reports that this also involves AGO7 and AGO10 (Baumberger & Baulcombe, 2005; Brodersen *et al.*, 2008; Montgomery *et al.*, 2008; Zhu *et al.*, 2011). It has recently emerged that AGO2 may preferentially bind the miR* strands from some miRNA duplexes and that this may direct distinct functions from that encoded by the miR strand (Zhang, X *et al.*, 2011). AGO4 is involved in a process by which 24nt siRNAs dependent on RDR2 and DCL3 for their generation direct the methylation of genomic loci from which they were produced (Zilberman *et al.*, 2003; Matzke *et al.*, 2007; Vazquez *et al.*, 2010). AGO3 is known to play a role in plant immunity against a bacterial pathogen in a partially redundant process involving AGO2 and AGO7 (Zhang, X *et al.*, 2011). In comparison, the functions of AGO5, 6, 8 and 9 remain poorly understood. Even so, the breadth and scope for sRNA pathway involvement in many important cellular processes is clear.

1.8 Small RNA involvement in plant-virus interactions

Small RNAs play important roles in dictating the outcome of interactions between phytopathogens and their hosts. This is particularly important during viral infections (Ding & Voinnet, 2007). The genomes of RNA viruses and the replication intermediates of DNA viruses are substrates for enzymes involved in RNA silencing pathways. The ribonucleases DCL2 and DCL4 in particular are involved in cleaving viral RNA into virus-derived siRNA (vsiRNA) (Deleris *et al.*, 2006; Ruiz-Ferrer & Voinnet, 2009) (Fig. 1.8). These

vsiRNAs guide the RISC to seek and destroy other viral nucleic acids. An important property of this RNA-based immune system is the ability to amplify a silencing signal. Plants encode multiple RNA-dependent RNA polymerases (RDRs) that can generate dsRNA from ssRNA templates (Ruiz-Ferrer & Voinnet, 2009). RDRs ensure that a significant population of sRNA species can be produced from a small amount of trigger RNA. The antiviral siRNAs produced during an infection are also mobile. It is known that sRNAs can move to neighbouring cells via plasmodesmata and over longer distances via the vascular system (Dunoyer *et al.*, 2010; Molnar *et al.*, 2010). This feature allows inoculation of uninfected tissues with anti-viral siRNAs before the infection has a chance to develop. This ability to spread and confer immunity in distant tissues explains the ring-spot virus resistance first observed by Wingard in 1928.

As a response, the majority of plant viruses have evolved suppressors of RNA silencing mechanisms (Ding & Voinnet, 2007). There are several points at which suppressors can interfere with silencing pathways. For example, the 2b protein from *Cucumber mosaic virus* (CMV) can specifically bind the 21nt size class of sRNAs and is also reported to interact directly with AGO1 (Zhang *et al.*, 2006; González *et al.*, 2010). Other viral suppressor proteins also interact directly with either siRNAs themselves or the protein complex which effect silencing (Vargason *et al.*, 2003; Bortolamiol *et al.*, 2007). Viral infections or transgenic expression of suppressors in plants often results in significant developmental abnormalities (Chapman *et al.*, 2004). As most suppressors do not distinguish between endogenous sRNAs and vsiRNAs, there is scope for significant interference with endogenous silencing pathways, such as the miRNA and tasiRNA pathways (Chapman *et al.*, 2004; Jay *et al.*, 2011).

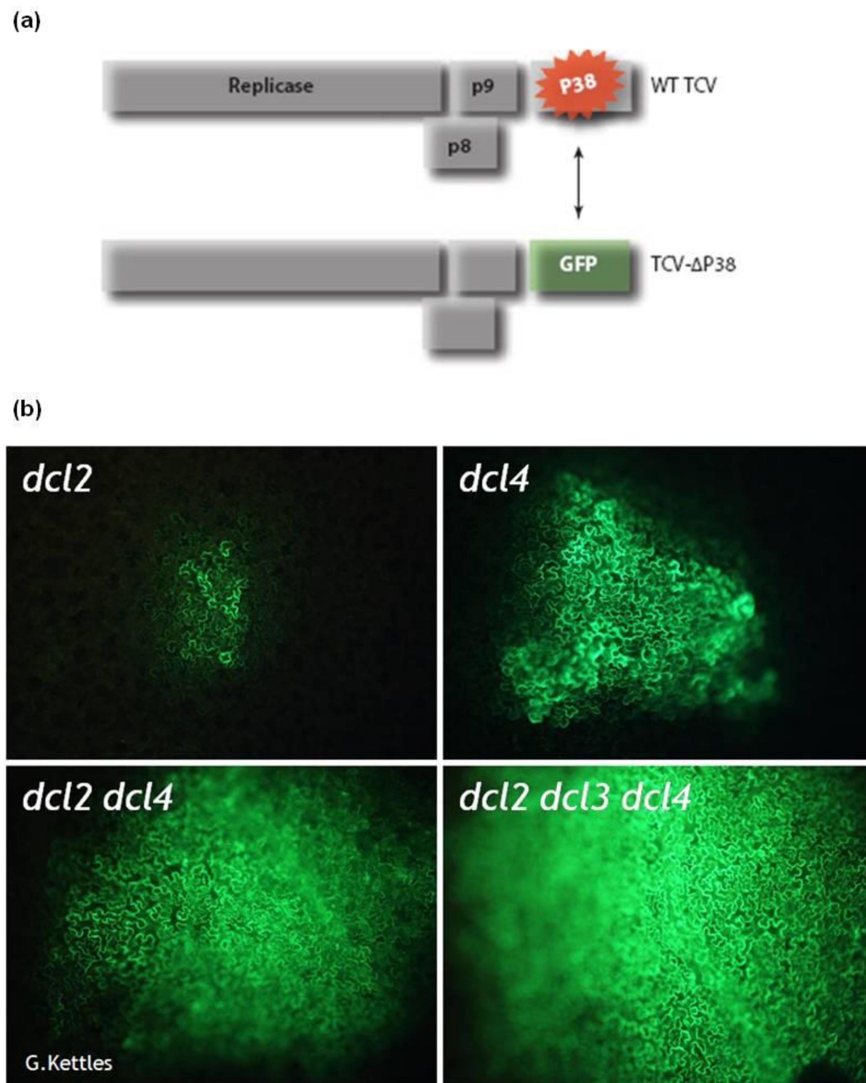

Figure 1.8 – Hierarchical involvement of *Arabidopsis* Dicer-like (DCL) genes in antiviral defence.

(a) A modified *Turnip crinkle virus* (TCV) was generated by replacing the coat protein and silencing suppressor (P38) with a GFP transgene. This recombinant virus (TCV-ΔP38) subsequently loses the ability to infect *Arabidopsis* Col-0 (figure modified from (Ruiz-Ferrer & Voinnet, 2009)). **(b)** TCV-ΔP38 regains virulence when rub-inoculated onto leaves of *Arabidopsis dcl* mutants. We now know that DCL4 in particular is crucial to antiviral silencing, although its activity is partially redundant with DCL2.

1.9 Small RNA involvement in resistance to cellular plant pathogens and pests

The relationship between host silencing pathways and viruses is very direct. Viruses replicate intracellularly and so their genomes and transcripts are exposed to DCL and AGO enzymes. The association between the silencing machinery and cellular pathogens is not so well developed. It is known that elements of some siRNA pathways are involved in bacterial resistance (Katiyar-Agarwal *et al.*, 2006; Katiyar-Agarwal *et al.*, 2007; Katiyar-Agarwal & Jin, 2010). It has been reported that several *Arabidopsis* miRNAs are involved in regulating resistance against *Pseudomonas syringae* (Li *et al.*, 2010; Zhang, W *et al.*, 2011). The miRNA miR393 is of particular relevance through its association with transcripts involved in auxin signalling (Navarro *et al.*, 2006). The auxin and SA signalling pathways act antagonistically so regulation of auxin may control activity of an SA-based defence response (Bari & Jones, 2009). More recently, miR393 was also shown to influence how resources are allocated between the glucosinolate and camalexin defence pathways in response to *P. syringae* infection (Robert Seilaniantz *et al.*, 2011).

Like viruses, some phytopathogenic bacteria produce effectors capable of interfering with aspects of sRNA signalling (Navarro *et al.*, 2008). Several *Arabidopsis* miRNAs are induced following avirulent *P. syringae* infection or induction with the PAMP flg22 (Li *et al.*, 2010; Zhang, W *et al.*, 2011). Some virulent strains possess effectors that prevent this induction and enhance colonisation (Navarro *et al.*, 2008). The *Arabidopsis* SUC-*SUL* line expresses an inverted repeat of the *SUL (SULPHUR)* gene under the control of the phloem-specific SUC2 promoter. These plants display a chlorotic phenotype in vasculature tissue and also in the neighbouring 10-15 cells. Stable expression of some bacterial effectors in SUC-*SUL* plants results in a loss of vein-centered chlorosis and induces stunting reminiscent of miRNA pathway mutants (Navarro *et al.*, 2008). These

observations indicate that there is significant scope for both miRNA and siRNA pathway interference by bacterial effector proteins.

Small RNA pathways coordinate resistance against the cyst nematode *Heterodera schachtii* (Hewezi et al., 2008). Expression of several miRNAs is altered following nematode infection, and in general, silencing pathway mutants are less susceptible to colonisation (Hewezi *et al.*, 2008). The fact the nematodes alter developmental processes in the root cells that they infect to establish feeding sites suggests that nematode-responsive miRNAs may play important roles in root development. This may share some features with *Agrobacterium*-induced tumour formation. *Arabidopsis dcl1* plants, which lack most miRNAs, are resistant to tumour formation following *Agrobacterium* inoculation (Dunoyer *et al.*, 2006). In addition, GFP expression is induced in tumours of *Agrobacterium*-infected GFP-silenced plants. These results suggest that manipulation of miRNA and siRNA activity may be a recurring theme during infection with a broad range of plant colonisers.

With regard insect resistance, work carried out in *Nicotiana attenuata* (native tobacco) has shown there to be significant involvement of sRNAs and the processes they control in mediating resistance against a chewing insect pest (Pandey & Baldwin, 2007; Pandey *et al.*, 2008). Transgenic plants silenced for *NaRdR1 (irRdr1)* are more susceptible to larvae of *M. sexta* compared to plants silenced for other *NaRdR* genes or a wild-type control. The *irRdr1* plants do not accumulate either nicotine or the precursors of nicotine biosynthesis following insect attack (Pandey & Baldwin, 2007). Subsequent deep sequencing analysis revealed that a considerable portion of tobacco sRNAs are responsive to insect feeding and require RDR1 for their biogenesis (Pandey *et al.*, 2008). Many of these changes were attenuated in *irRdr1* plants and accounted for a proportion of the susceptibility phenotype of those plants.

1.10 Focus and aims of research described in this thesis

In comparison to leaf-chewing insects that cause considerable damage to host plants, aphids and other phloem-feeders are comparatively stealthy and cause little damage at feeding sites. Despite this, aphid feeding is known to elicit considerable transcriptional reprogramming in infested tissue. Given the known involvement of sRNAs in coordinating defence responses to phytopathogenic viruses, bacteria, fungi and chewing insects, we set out to examine the roles of sRNAs in resistance against a phloem-feeding insect pest. The primary aim of this research was to identify how sRNAs may regulate the complex and large-scale response to aphid attack.

1.11 Overview of thesis contents

To realise this aim, we performed a resistance screen of a collection of *Arabidopsis* RNA silencing mutants against the green peach aphid *M. persicae* (chapter 3). This screen revealed that plants deficient in the miRNA pathway, but not other sRNA pathways, were more resistant to aphid infestation than either wild-type plants, or plants deficient in other siRNA-mediated silencing pathways. Aphids raised on miRNA pathway mutants displayed no change in survival over the 14-day test period, but produced significantly fewer offspring, suggesting that the miRNA mutant resistance phenotype interferes with aphid reproductive development or prevents the aphid from establishing a proper feeding site leading to less nutrient uptake and subsequent decline in aphid reproduction ability.

To characterise this resistance phenotype, we used quantitative real-time PCR (qRT-PCR) to compare defence pathway induction between wild-type (Col-0), miRNA

mutant (*dcl1*) and siRNA pathway mutant (*dcl2/3/4*) following aphid exposure (chapter 4). The *dcl1* plants have greater induction of several genes involved in secondary metabolism, including the camalexin biosynthetic gene *PAD3*. This data correlated with a significant increase in camalexin levels in aphid-infested *dcl1* plants relative to Col-0 and *dcl2/3/4*. When fed camalexin by artificial diet, there was no effect on adult aphid survival, but a strong and inhibitory effect on reproductive success, consistent with the reduction in the number of aphid offspring (but not survival) observed on the miRNA pathway deficient plants.

WRKY33 is a pathogen-responsive transcription factor that positively regulates *PAD3*. Aphids produced more offspring on *wrky33* mutants relative to wild type Col-0, but the numbers of offspring on 35S:WRKY33 and Col-0 were similar (chapter 5). *WRKY33* is also differentially expressed in both *dcl1* and *dcl2/3/4* plants relative to Col-0 during aphid infestation. Computational predictions suggested that both the *WRKY33* and *PAD3* transcripts are direct targets of miR393. To validate these predictions, 5`RACE experiments were conducted. The *WRKY33* transcript is not cleaved at the putative miR393 target site, but was cleaved ~100nt upstream of this site. This other potential cleavage site is homologous to several other sRNA species in *Arabidopsis* leaves, but these sRNAs were not detected on northern blots. The *PAD3* transcript is cleaved at two positions towards the 3` end of the transcript from the miR393 site. These products are detected in aphid-exposed leaves but not in mock-infested controls. Aphids produced more offspring on some plants overexpressing the miRNA miR393 (chapter 5). This indicates that WRKY33 is involved in aphid resistance, most likely through its known role in camalexin production and is regulated by sRNA pathways. As aphid fecundity is also increased on the *arf1* and *arf9* mutants, miR393 may play a role in aphid resistance through its role in auxin perception and also direct regulation of *PAD3* during aphid exposure.

To discover the particular miRNAs involved in regulating the aphid response, sRNA libraries generated from mock and aphid-exposed *Arabidopsis* rosette leaves were deep-sequenced (chapter 6). In addition, a *M. persicae* sRNA library was sequenced. This experiment illustrated that few *Arabidopsis* miRNAs were differentially regulated following aphid exposure. Nonetheless, detection of the apparent differentially regulated sRNAs on northern blots confirmed that some plant miRNAs were indeed aphid-responsive. Of these, miR393 expression appeared to be altered the most upon aphid infestations although some aphid-exposed plants had increased and others decreased miR393 levels compared to mock-treated Col-0.

This study reports for the first time that the miRNA pathway is involved in the orchestration of the plant defense response to aphids and may guide the identification of other defence pathways involved in aphid resistance. Furthermore, this work highlights a previously undocumented role for the phytoalexin camalexin in regulating plant defence to an economically important aphid pest. It was found that multiple sRNA-mediated regulatory factors are likely to orchestrate the induction of camalexin production. These regulatory factors may also play a role in plant defence to other pathogens where camalexin is a contributory factor.

Chapter 2 – Materials & Methods

2.1 Plant & insect growth/maintenance conditions

Stock colonies of the green peach aphid *Myzus persicae* (RRes genotype O) (Bos *et al.*, 2010) were reared in 52cm x 52 cm x 50cm cages containing up to six Chinese cabbage (*Brassica rapa*, subspecies *chinensis*) plants with a 14-h day (90μmol m^{-2} sec^{-1} at 18°C) and a 10-h night (15°C) photoperiod.

The following *Arabidopsis thaliana* mutants are all in the Col-0 background and were obtained from various laboratories as indicated. The *ago1-25*, *1-26* and *1-27* mutants (Morel *et al.*, 2002) were supplied by Hervé Vaucheret (Laboratoire de Biologie Cellulaire, INRA Centre de Versailles, France). The *dcl1-9*, *hen1-5*, *rdr1-1*, *rdr2-1* and *rdr6* (Jacobsen *et al.*, 1999; Mourrain *et al.*, 2000; Vazquez *et al.*, 2004a; Xie *et al.*, 2004) were kindly provided by Dr. Fuquan Liu (Cell & Developmental Biology, John Innes Centre, Norwich, UK). The *dcl2*, *dcl3*, *dcl4*, *dcl2/3*, *dcl2/4* and *dcl2/3/4* mutants (Xie *et al.*, 2004; Xie *et al.*, 2005; Henderson *et al.*, 2006) were obtained from Prof. Olivier Voinnet (Swiss Federal Institute of Technology, Zurich, Switzerland). The *hst*, *se1*, *ago2*, *ago4*, *ago7*, 35S::LOX2 and 35S::LOX2 antisense lines (35S::LOX2as) (Bell *et al.*, 1995; Bollman *et al.*, 2003; Zilberman *et al.*, 2003; Vazquez *et al.*, 2004b; Lobbes *et al.*, 2006) were provided by the Nottingham Arabidopsis Stock Centre (NASC) (http://arabidopsis.info/). Arabidopsis *dcl1.fwf2* and *fwf2* plants (Katiyar-Agarwal et al., 2007) were kindly provided by Dr. Rebecca Mosher (The School of Plant Sciences, University of Arizona, AZ, USA). The *pad3*, *npr1*, *sid2* mutants as well as 35S::miR393a and miR393a-p:GFP transgenic plants (Cao *et al.*, 1994; Glazebrook & Ausubel, 1994; Nawrath & Metraux, 1999; Navarro *et al.*, 2008) were obtained from Dr. Alexandre Robert-Seilaniantz (Prof. Jonathan Jones's group, The Sainsbury Laboratory (TSL), Norwich, UK). The 35S::miR393b lines (Zhang, X *et al.*, 2011) were provided by Dr. Hailing Jin (Department of Plant Pathology and Microbiology, University of California Riverside, Riverside, CA, USA). The

cyp79b2/cyp79b3 double mutant (Zhao *et al.*, 2002) was obtained from Prof. Jean-Pierre Métraux (Department of Biology, Plant Biology, University of Fribourg, Switzerland). The *coi1-35* and *jar1* mutants (Staswick *et al.*, 1992) were provided by Prof. Jonathan Jones (TSL, Norwich, UK) and *ein2-5* and *etr1-1* mutants (Bleecker *et al.*, 1988; Alonso *et al.*, 1999) were from Dr. Freddy Boutrot (Dr. Cyril Zipfel's group, TSL, Norwich, UK). 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 72h and grown in a controlled environment room (CER) with an 8-h day (90µmol m^{-2} sec^{-1} at 18°C) and a 16-h night (16°C). Two-week old seedlings were transferred to seedling trays containing twenty-four modules. Plants were used for experiments after a further two weeks when plants were four weeks old.

Seed of the *Arabidopsis* hormone/secondary metabolite pathway mutants were vernalized for one week at 5-6°C and grown in a CER with a 10-h day (90µmol m^{-2} sec^{-1} at 22°C) and a 14-h night (22°C) photoperiod. Plants were used for experiments at four weeks old.

2.2 Aphid survival and fecundity assays

All aphid survival and fecundity assays were carried out in a CER with an 8-h day (90µmol m^{-2} sec^{-1} at 18°C) and a 16-h night (16°C) photoperiod. Four-week old plants were potted into one litre round black pots (13cm diameter, 10cm tall) containing fresh compost and were caged in clear plastic tubing (10cm diameter, 15cm tall) (Jetran tubing, Bell Packaging, Luton, UK) capped at the top with white gauze-covered plastic lids. Each plant was seeded with four adult *M. persicae* from the stock colony and plants were

returned to the CER. After 48h, all adults were removed from test plants (day 0) and plants were returned to the growth room. On day 3, excess nymphs were removed leaving 5 nymphs per plant. On day 11 when most nymphs reached adulthood and started to produce their own offspring, the numbers of these new nymphs were counted. The newly produced nymphs were removed while the adults remained on the plant. On day 14, a second nymph count was carried out along 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 5 plants per genotype that were arranged in trays using a randomized block design and each experiment was repeated at least twice. Nymph production and adult survival data were analysed by Analysis of Deviance (ANODE) using GenStat v.11 (VSN International, Hemel Hempstead, UK).

2.3 Short exposure survival and fecundity assay

The experiments to assess aphid performance on wild-type *Arabidopsis* and the *dcl1* and *dcl2/3/4* mutants over two days (as opposed to 14 days as described above) were done following a method described elsewhere (Pegadaraju *et al.*, 2005). In this method, five four-week old plants of each genotype were seeded with 15 unaged adult *M. persicae* from the stock colony using a fine paintbrush. Plants were returned to the CER with the same growth conditions as for the 14-day assay for a period of 48h. At the end of this period, the total number of aphids on each plant (both adults and newly-born nymphs) was counted. The experiment was conducted twice with similar results. Data from both experiments were combined and statistically analysed as for the survival and fecundity assays described above.

2.4 Aphid camalaxin diet assay

To study the effect of camalexin on aphid survival and fecundity, aphids were fed on artificial diets containing various camalexin concentrations. Sachets for feeding aphids on artificial diets were constructed by cutting the top 2cm portion of a 50ml Corning tube and reattaching the lid. Parafilm was stretched over the open end to form a feeding sachet containing 100µl artificial diet. We used an artificial diet previously described for these experiments (Kim & Jander, 2007). This diet contained sucrose (440mM) and the following amino acids (Ala 10mM, Arg 16mM, Asn 20mM, Asp 10mM, Cys 3.3mM, Glu 10mM, Gln 10mM, Gly 10mM, His 10mM, Ile 6mM, Leu 6mM, Lys 10mM, Met 5mM, Phe 3mM, Pro 7mM, Ser 10mM, Thr 12mM, Trp 4mM, Tyr 2mM, Val 7mM). Synthetic camalexin was provided by Jean-Pierre Métraux (University of Fribourg, Switzerland) (Stefanato *et al.*, 2009). A camalexin stock solution (100mg/ml) was prepared by dissolving camalexin in DMSO. Aphids were fed either diet alone, diet containing 0.1% DMSO or diet containing variable concentrations of camalexin. The 0.1% DMSO control was the equivalent DMSO concentration in the highest camalexin treatment. 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 (90µmol m^{-2} sec^{-1} at 18°C) and a 16-h night (16°C) photoperiod. The number of surviving adults (from 10) and number of nymphs produced were assessed after 48h. Each experiment contained 5 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.

2.5 qRT-PCRs

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays were conducted to quantify *Arabidopsis* gene expression upon exposure to aphids. For these experiments, thirty non-aged *M. persicae* nymphs from the stock cage were transferred to a single clip-cage using a fine paintbrush and confined to single mature rosette leaves of five-week old plants at one clip-cage per plant. Plants were returned to the CER with an 8-h day (90µmol m^{-2} sec^{-1} at 18°C) and a 16-h night (16°C) photoperiod for either 6, 12, 24 or 48 hours. Four aphid exposed leaves per treatment were pooled to produce each sample and leaves caged with aphid-free clip cages were used as controls. All samples were snap-frozen in liquid nitrogen and stored at -80°C until further processing. The leaf samples were ground in chilled 1.5ml Eppendorfs using disposable pellet pestles (Sigma-Aldrich, St Louis, USA). Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) and included a DNaseI treatment (RQ1 DNase set, Promega, Madison, USA). RNA was purified using the RNA cleanup protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany), analyzed for purity on a 1% agarose gel and Picodrop spectrophotometer (Picodrop Ltd, Saffron Walden, UK). RNA samples with an A260/A280 ratio of between 1.85 and 2 were taken forward for qRT-PCR analyses. cDNA was synthesised from 500ng RNA using the MMLV-RT Kit (Invitrogen, Carlsbad, USA) and oligo dT primer following the manufacturer's instructions. cDNA from these reactions was diluted 1:20 with dH_2O prior to qRT-PCR.

The 20µl reactions were setup in 96-well white ABgene PCR plates (Thermo Scientific, Loughborough, UK) in a CFX96 Real-Time System with a C1000 Thermal Cycler (Biorad, Hemel Hempstead, UK) using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich).

All reactions were carried out using the following thermocycle: 3 mins at 95°C, followed by 40 cycles of (30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C), followed by melt curve analysis: 30 sec at 50°C, (65°C - 95°C at 0.5°C increments, 5 sec for each).

Reference genes for this study were chosen by selecting nine candidates previously identified as superior reference genes (Czechowski *et al.*, 2005). These were ACT2 (At3g18780), UBQ10 (At4g05320), GAPDH (At1g13440), Clathrin adapter complex subunit (At5g46630), TIP41-like protein (At4g34270), SAND-family protein (At2g28390), PEX4 (At5g25760), At4g26410 and PTB1 (At3g01150). Using geNORM (Vandesompele *et al.*, 2002), it was established that ACT2, Clathrin adapter complex subunit and PEX4 were the most stable across a range of mock and *M. persicae*-exposed *Arabidopsis* rosette leaf RNA samples and hence are suitable reference genes for this study. Mean Ct values for each sample/primer pair combination were calculated from two or three replicate reaction wells. Mean Ct values were then converted to relative expression values using the formula $2^{-\Delta Ct}$ (Pfaffl, 2001) such that the sample with the lowest mean Ct value (most concentrated) was assigned a value of 1. The geometric mean of the relative expression values of the three reference genes was calculated to produce a normalisation factor unique to each sample (Vandesompele *et al.*, 2002). Relative expression values for each gene of interest were normalised using the normalisation factor for each sample. The normalised expression values for each gene of interest were then compared between mock and aphid-exposed samples across all plant genotypes tested in the experiment. Analysis of Variance (ANOVA) was performed to assign variance attributable to plant genotype, block and replicate using a general linear model (GLM) in GenStat. Means were compared by calculating t-probabilities within the GLM. For display of data, mean expression values were rescaled such that mock-infested Col-0 represents a value of 1. The primer sequences used for both reference and target gene quantification are listed below.

2.6 *Arabidopsis* qRT-PCR reference gene primer sequences

Gene Name	Primer Name	Gene Identifier	Sequence (5`>3`)
ACT2	Actin2-newF*	At3g18780	GATGAGGCAGGTCCAGGAATC
	Actin2-newR*		GTTTGTCACACACAAGTGCATC
UBI10	UBI10 F1	At4g05320	AATACGCCTGCAAAGTGACTC
	UBI10 R1		CCAACAGCTCAACACTTTCG
GAPDH	GAPDH F1	At1g13440	AGGTCAAGCATTTTCGATGC
	GAPDH R1		AACGATAAGGTCAACGACACG
CLATH	CLATH F1	At5g46630	TGCGTTTTGGTTAATCTGTCTC
	CLATH R1		CCGTGTTGTAACCGCTCTTC
TIP41	TIP41 F1	At4g34270	TCCATCAGTCAGAGGCTTCC
	TIP41 R1		AAGAAAGCTCATCGGTACGC
SAND	SAND F1	At2g28390	TGTGCCAAAGGGTAAAAGATG
	SAND R1		AGCACAATATAGGGGGTCAAAC
PEX4	PEX4 F1	At5g25760	TGCAACCTCCTCAAGTTCG
	PEX4 R1		CACAGACTGAAGCGTCCAAG
AT4G26410	AT4G26410 F1	At4g26410	GCAGGAAATTGCTTGAGAGG
	AT4G26410 R1		AGTCCGTCCATCAAATCAGC
PTB1	PTB1 F1	At3g01150	ACCCATCATCTGATCCCAAC
	PTB1 R1		AACATGGAAATACTCCCCATTG

*Primers designed by Akiko Sugio (JIC)

2.7 *Arabidopsis* qRT-PCR target gene primer sequences

Gene Name	Primer Name	Gene Identifier	Sequence (5`>3`)
PR1	PR1 F5**	At2g14610	GTTGCAGCCTATGCTCGGAG
	PR1 R5**		CCGCTACCCCAGGCTAAGTT
LOX2	AT3G45140-lox2-RTF*	At3g45140	GCAAGCTCCAATATCTAGAAGGAGTG
	AT3G45140-lox2-RTR*		CGGTAACACCATGCTCAGAGGTAG
VSP2	VSP2 F3**	At5g24770	GTTAGGGACCGGAGCATCAA
	VSP2 R3**		AACGGTCACTGAGTATGATGGGT
PDF1.2	PDF1.2 F4**	At5g44420	CCATCATCACCCTTATCTTCGC
	PDF1.2 R4**		TGTCCCACTTGGCTTCTCG
HEL	HEL F3	At3g04720	TGTGAGAATAGTGGACCAATGC
	HEL R3		ATGAGATGGCCTTGTTGATAGC
PAD3	PAD3 F3***	At3g26830	TGCTCCCAAGACAGACAATG
	PAD3 R3***		GTTTTGGATCACGACCCATC
CYP79B2	CYP79B2 F5	At4g39950	TCTCCGGTTTATCTCGTTCAGTA

	CYP79B2 R5		CGTGTCTCATTCTCAGGTAGCTT
CYP83B1	CYP83B1 F2	At4g31500	TGCTGGTAGATATGGCGTGAC
	CYP83B1 R2		AAGGGACCCGAATATTAAACATC
CYP81F2	CYP81F2 F2	At5g57220	TGGCTATGCGTAAACTCGTG
	CYP81F2 R2		GGTAAACTTCAAAATGGTGGTCA
CYP89A2	CYP89A2 F2	At1g64900	AGTACTACGTCGCCAACATGG
	CYP89A2 R2		ATCTCCTAGGAACAGCAAGAGC
TIR1	TIR1 F2	At3g62980	TTCTTGTTCCGTGAGTTTTGG
	TIR1 R2		CCAGCCACTGTTCGGTATATG
AT4G12980	AT4G12980 F3	At4g12980	GAGGCATCACGTTACTCTTGG
	AT4G12980 R3		TACCAACACCATTAGGCCTTG
BDL-IAA12	BDL-IAA12 F3	At1g04550	TGTTCCATGGAGAATGTTTATCAAC
	BDL-IAA12 R3		CTATCCTTCTGCTCTTGACGTC
MPK3	MPK3 F5	At3g45640	TCCGAATGGCTACTTAGTATCTTTG
	MPK3 R5		TGGAGCTACACTTAATCACTAGCAG
WRKY33	WRKY33 miR F1	At2g38470	GCTGTGTACAATGCCAGTTTG
	WRKY33 miR R1		TTCGTCTCTGCTGACAATCG

*Primers designed by Akiko Sugio (JIC).

**Primer sequences previously published (Abe *et al.*, 2008)

***Primer sequences previously published (Chassot *et al.*, 2008)

2.8 *Arabidopsis* camalexin extraction and quantification

To determine if *Arabidopsis* camalexin levels increase upon aphid exposure, we measured camalexin content in aphid-exposed whole plants. For these measurements, five-week old *Arabidopsis* plants were infested with 30 adult *M. persicae*, harvested after 24h, snap-frozen in liquid nitrogen and stored at -80 C. Plants without aphids were used as controls. Camalexin extractions were carried out using a method based on work described previously (Meuwly & Métraux, 1993). Briefly, total camalexin was extracted from whole plants using 70% ethanol and 90% methanol in the presence of 200ng *o*-anisic acid as an

internal standard. Following evaporation of organic solvent in a speedvac, plant debris was precipitated using 5% trichloroacetic acid before recovery of phenolic compounds/camalexin using a 1:1 ethyl acetate/cyclohexane mix. Samples were dried using a speedvac.

Samples were analysed by High-performance liquid chromatography (HPLC) on a Surveyor instrument (Thermo Scientific) attached to a DecaXP^{plus} ion trap mass spectrometer (MS) (Thermo Scientific). Camalexin and *o*-anisic acid were separated on a 50×2mm 3µ Luna C18(2) column (Phenomenex). All peak areas were integrated using the Xcalibur software Genesis algorithm (Thermo Scientific). Each experiment contained four biological replicates of each treatment and the experiment was conducted twice.

2.9 GUS staining

To assess the spatial aspect of *PAD3* induction during aphid infestation, transgenic *Arabidopsis* lines expressing *CYP71B15p::GUS* (*PAD3p::GUS*) were obtained from Erich Glawischnig (Technische Universität München, Germany). These plants were grown in a CER with an 8-h day (90µmol m^{-2} sec^{-1} at 18°C) and a 16-h night (16°C) photoperiod. Leaves of four week old plants were clip caged with 30 *M. persicae* nymphs. Leaves clip caged without aphids were used as controls. After 48h, aphids were carefully removed with a dry paintbrush and leaves were immediately submerged in GUS staining solution (0.2M Na_2HPO_4, 0.2M $NaH_2PO_4.2H_2O$, 10% Triton X-100, 10mM EDTA, pH7) containing 50mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl-beta-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 16h

at 37°C in the dark before destaining in 70% ethanol for two nights at 37°C. Destaining solution was changed twice during this period. Leaves were mounted on glass microscope slides in 40% glycerol and viewed under a Nikon Eclipse 800 light microscope attached to a Pixera Pro ES600 digital camera.

2.10 Aphid symbiont qRT-PCR

To assess the relative symbiont populations in aphids raised on plants of various camalexin concentrations, we conducted a qRT-PCR experiment using symbiont-specific primers. Primer sequences for both *M. persicae* reference genes and bacterial symbiont target genes are listed below. Five-week old Col-0, *dcl1*, *dcl2/3/4* and *pad3 Arabidopsis* were used for this experiment. All plants were grown as described for the aphid survival/fecundity assay. Four plants of each genotype were individually seeded with 15-35 *M. persicae* nymphs from the stock colony and returned to the CER for five days. At this point, ten aphids from each Col-0 plant were transferred to one of four artificial feeders containing the artificial diet described previously. Ten more aphids from each Col-0 plant were transferred to one of four artificial feeders containing artificial diet plus camalexin (100µg/ml). Artificial feeders were then returned to the CER alongside test plants for a further two days. At the end of this period, all adult aphids from both test plants and artificial feeders were harvested and immediately frozen in liquid nitrogen.

Genomic DNA was extracted from aphids using the OmniPrep for Plant DNA extraction kit (G Biosciences) following the manufacturer's instructions. DNA was resuspended in 50µl TE buffer and quantified on a Picodrop spectrophotometer. All samples were diluted 1:10 with dH_20 after quantification and prior to qPCR.

qPCR was carried out as previously described with the exception that the aphid reference genes *EF1α* and *Tub1* were used for normalization. These have previously been used to normalize symbiont genome copy number in the pea aphid *A. pisum* (Oliver *et al.*, 2003; Oliver *et al.*, 2006; Dunbar *et al.*, 2007).

2.11 *M. persicae* qRT-PCR reference gene primer sequences

Gene Name	Primer Name	Gene Identifier	Sequence (5`>3`)
EF1α	Ap EF1α F1*	NCBI ID 100165786	CTGATTGTGCCGTGCTTATTG
	Ap EF1α R1*		TATGGTGGTTCAGTAGAGTCC
Tub1	Ap tub1 F1	NCBI ID 100168148	AGCCGGTCAGTGCGGAAACC
	Ap tub1 R1		ACCCGACCGCCATGTTTGAGC

*These primer sequences were previously used for normalization of *B. aphidicola*, *H. defensa* and *S. symbiotica* genome copy number in pea aphids (Oliver *et al.*, 2003; Oliver *et al.*, 2006; Dunbar *et al.*, 2007).

2.12 Bacterial symbiont qRT-PCR target gene primer sequences

Gene Name	Primer Name	Gene Identifier	Sequence (5`>3`)
dnaK (*B. aphidicola*)	Ba dnaK F1*	BB0091**	ATGGGTAAAATTATTGGTATTG
	Ba dnaK R1*		ATAGCTTGACGTTTAGCAGG
gyrB (*H. defensa*)	Hd gyrB F1*	NC_012751.1	CAAACGCAACGATCAAGAAA
	Hd gyrB R1*		GGAACGATGGATTTCAGGAA
gyrB (*S. symbiotica*)	Ss gyrB F1*	NZ_GL636117.1	CGCTGAACAGCTACATGGAA
	Ss gyrB R1*		GCCGACCACAATTTTAGCAT

*Primer sequences were previously used to assess genome copy number of several symbiotic bacterial species in pea aphids (Oliver *et al.*, 2003; Oliver *et al.*, 2006; Dunbar *et al.*, 2007).

**Gene identifier refers to BuchneraBASE gene ID (http://www.buchnera.org/)

2.13 Small RNA Northern blot

Northern blot hybridisations were conducted to compare sRNA abundance in aphid-exposed and mock-exposed *Arabidopsis* rosette leaves. Leaves of 5-week old plants were clip caged with 30 aphids or no aphids for 12 hours as described above. Samples consisted of a total of 6 leaves at one leaf per plant. Leaves were harvested and combined as samples before being snap-frozen in liquid nitrogen and stored at -80°C until further processing. Three samples were generated for each the aphid and no aphids (control) treatments. RNA was purified using Tri-Reagent (Sigma-Aldrich) as described in qRT-PCR methods with the following exceptions. Tissue was homogenised in a chilled TissueLyser LT (Qiagen) for 2 mins at 50 oscillations/sec using chilled metal ball-bearings. RNA was precipitated overnight at -20°C to increase recovery of the small RNA fraction. RNA was not DNase-treated or purified on Rneasy columns as the size exclusion limits of these columns are too large and the small RNAs would have been largely lost. Unless otherwise stated, 10µg total RNA was loaded per lane. RNA was separated on a 15% polyacrylamide gel (7M urea, 20mM MOPS) before blotting onto a Hybond-NX membrane (GE Healthcare) by semi-dry electrotransfer. RNA was crosslinked to the membrane using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) dissolved in 1-methylimidazol for 1h at 60°C. Membranes were pre-hybridized for at least 2h in ULTRAhyb-Oligo hybridization buffer (Ambion) at 37°C with rotation. Probes were produced by terminal labelling of oligonucleotides complementary to the miRNA of interest with γ-^{32}P-ATP in the presence of T4 polynucleotide kinase at 37°C for 1h. Labelling reactions were cooled on ice prior to recovery of radiolabelled probe from unincorporated reagents on a QIAquick Nucleotide Removal Kit column (Qiagen) as per the manufacturer's instructions. Probes were eluted in 100µl dH_2O which was added directly to the hybridisation solution. Probes were

hybridised to the membrane for 16-24h at 37°C with rotation. Membranes were washed (2 x 30min) with washing buffer (0.1x SSC, 0.1% SDS) at 37°C with rotation before being wrapped in Saran wrap and exposed to storage phosphor screens (GE Healthcare) at room temperature. Exposure times varied due to differing miRNA abundance, but were typically between 1-2h. Images were recovered from storage phosphor screens using a Typhoon 9200 Variable Mode Imager (GE Healthcare) and ImageQuant TL software (GE Healthcare). Membranes were stripped by addition of boiling stripping buffer (10mM Tris/HCl pH8.5, 5mM EDTA, 0.1% SDS) directly to the membrane surface for 5mins at room temperature with shaking. This was repeated until no signal could be detected from the membrane. Blots were re-hybridised for all miRNAs of interest including the U6 blotting control.

2.14 RNA-seq

To identify sRNAs that may be induced in Arabidopsis upon aphid exposure, an RNA-seq experiment was conducted. Leaves of 5-week old plants were clip caged with 30 aphids or no aphids for 12 hours as described above. Samples consisted of a total of 5 leaves at one leaf per plant. Leaves were pooled into samples which were snap-frozen in liquid nitrogen immediately upon harvesting and stored at -80°C until further processing. Three samples were generated for each the aphid and no aphids (mock) treatments. All aphids that comprised the infestations (450 nymphs total) were pooled into a single seventh sample. RNA was double-extracted using Tri-Reagent as described above for qRT-PCR. All samples had A260/280 ratios of 1.85 or higher and yielded 8-12µg total RNA.

Libraries were prepared following the Illumina Small RNA v1.5 Sample Preparation protocol (Illumina Inc, San Diego, USA). Ligation of the 5` and 3` RNA adapters were conducted with 5µl (1.25-1.8µg) RNA of each sample. We followed the manufacturer's instructions with two exceptions. Firstly, reverse transcription was carried out with a 10mM dNTP mix as opposed to 12.5mM. Secondly, PCR was performed using 25µl reaction volumes instead of 40µl.

Following ligation of the 5` and 3` RNA adapters, cDNA synthesis and PCR amplification, fragments corresponding to adapter-sRNA-adapter ligations (93-100bp) were excised from polyacrylamide gels and eluted using the manufacturer's instructions.

The seven libraries were then validated by A-tailing a proportion of the recovered DNA fragments with GoTaq DNA polymerase (Promega) and cloning into the pGem-T easy vector system (Promega). Recombinant plasmids were transformed into electrocompetent *E. coli* (DH5α) and bacteria were grown on carbenicillin$^+$ (100µg/ml) agar plates for 16h at 37°C. Colony PCR was done using GoTaq and M13 forward and reverse primers to identify colonies containing the pGem-T easy vector and adapter-sRNA insert. Selected colonies were grown for 16h at 37°C in carbenicillin$^+$ L-broth (100µg/ml) and DNA purified using QIAprep Spin Miniprep Kit (Qiagen). The purified DNA (150 ng) from each colony were sequenced using the BigDye (v3.1) sequencing protocol and M13 forward and reverse primers. Ready reactions were submitted to Genome Enterprise Limited (The Genome Analysis Centre, Norwich) for sequencing on Life Technologies 3730XL capillary sequencers.

Following validation that the libraries contained sequences corresponding to their source organism, all libraries were submitted to The Sainsbury Laboratory (TSL, Norwich, UK) for 36nt single-end sequencing on an Illumina Genome Analyzer.

2.15 Bioinformatic analysis of small RNA-seq data

We used two data analysis pipelines to identify differentially regulated sRNAs in aphid-exposed and non-exposed plants. In a first analysis, raw reads were processed to remove reads of low quality and adapter-adapter ligations containing no sRNA sequence. Remaining reads were trimmed to the first 20nt, the region of sequence corresponding to cloned sRNAs. Trimmed sequences were then matched to known *Arabidopsis* mature miRNA sequences deposited in miRBase (http://www.mirbase.org/) as of February 2011. Matches greater than 10nt were accepted and counted as a read of a known miRNA. The number of matches was rescaled to reads/million and compared among the three control and three aphid-exposed libraries.

In a second analysis, raw reads were processed using the perl implementation of the UEA Plant sRNA toolkit (Moxon *et al.*, 2008) (http://srna-tools.cmp.uea.ac.uk). Firstly, raw reads were converted from FASTQ format to FASTA and adapter sequences removed using the adaptor removal tool. Low quality reads, or those not matching the *Arabidopsis* genome (TAIR10) (August 2011) or outside the size criteria for sRNAs (16-26nt) were removed using the filter tool. The miRProf tool was used to assess number of reads matching to known miRNAs across all samples (miRBase 17 - August 2011). The miRCat tool was used to identify novel miRNA sequences from our datasets.

2.16 5`RACE

To assess if *WRKY33* mRNA is cleaved by miR393, we conducted a modified 5` rapid amplification of cDNA ends (5` RACE) experiment (Llave *et al.*, 2002). In this method,

the calf intestinal phosphatase (CIP) and tobacco acid pyrophosphatase (TAP) steps are omitted so that an RNA oligo can be directly ligated to cleaved mRNA fragments.

50µg total RNA from both uninfested *Arabidopsis* Col-0 and aphid-infested (12h) Col-0 was used as starting material for 5`RACE. Polyadenylated RNA was purified from total RNA using the MicroPoly(A) Purist Kit (Ambion) as per the manufacturers instructions. Polyadenylated RNA was eluted from spin columns in 15µl of THE RNA Storage Solution (Ambion). Half of this (154ng and 291ng) RNA was used to ligate to the GeneRacer RNA oligo (250ng) from the GeneRacer Kit (Invitrogen). Reactions were catalyzed by T4 RNA ligase (5U) in 10µl reaction volumes at 37°C for 1h. RNA was recovered in phenol:chloroform and precipitated in 3M sodium acetate (pH 5.2) with 95% ethanol at -20°C for 1h. RNA pellets were subsequently washed in 70% ethanol and resuspended in 10µl dH_2O. RNA was then reverse transcribed using SuperScript II reverse transcriptase following the maker's instructions. Reactions were inactivated at 70°C for 15min before digestion of RNA using RNase H (2U) for 20min at 37°C. cDNA was stored at -20°C prior to PCR.

Touchdown PCR was performed to specifically amplify fragmented transcripts of interest ligated to the GeneRacer oligo. Primer sequences specific for the GeneRacer oligo and transcripts of interest are listed in the *Arabidopsis* 5`RACE primer sequences table below. Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) in accordance with guidelines provided with the GeneRacer Kit for touchdown PCR primer design. Reactions were carried out using the following thermocycle: 2 mins at 94°C, followed by 5 cycles of (30 sec at 94°C, 30 sec at 72°C), followed by 5 cycles of (30 sec at 94°C, 30 sec at 70°C) followed by 25 cycles of (30 sec at 94°C, 30 sec at 65°C, 30 sec at 68°C) with a final extension of 10 min at 68°C. Amplification products were visualized on a 1.5% agarose gel, excised using a sterile scalpel and recovered using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturers instructions. Fragments were cloned

into the pGem-T easy vector system, transformed into *E. coli* (DH5α) and sequenced as described for small RNA library preparation.

2.17 *Arabidopsis* 5`RACE primer sequences

Gene Name	Primer Name	Gene Identifier	Sequence (5`>3`)
PHABULOSA	PHABULOSA RACE R1*	At2g34710	TGCAGAAGTAAGCGACCTTTCACAAACC
	PHABULOSA RACE R2*		CGTCCCACCGTTTCCAGCAGGTATCAC
WRKY33	WRKY33 RACE R1	At2g38470	CATGTTTCCTCACTGGACAACCGATGG
	WRKY33 RACE R2		TCGGCTCTCTCACTGTCTTGCTTCCA
WRKY18	WRKY18 RACE R1	At4g31800	TTCATATACAGTATTCAGACGACATCTA
	WRKY18 RACE R2		ACAGTATTCAGACGACATCTAACTGGT
AT5G18370	At5g18370 RACE R1	At5g18370	CATGGTTGATCTCATGCGCTCTCAAA
	At5g18370 RACE R2		CCACTGAGTTTCTTTCGCCAATGCAG
PAD3	PAD3 miR393 RACE R1	At3g26830	GTGGTGAACTTGAGAGCATCTCCATCGT
	PAD3 miR393 RACE R2		GCTCTCTCTTCCAGGCTTAAGATGCTCGT
	PAD3 miR854 RACE R1		GAAGGGTGCCATCCCGATGTCTTTG
	PAD3 miR854 RACE R2		TCCGTATTGGAGAAGCGCCACTGGT
	GeneRacer 5` primer		CGACTGGAGCACGAGGACACTGA
	GeneRacer 5`nested primer		GGACACTGACATGGACTGAAGGAGTA

*Primers designed by Sara Lopez-Gomollon (UEA)

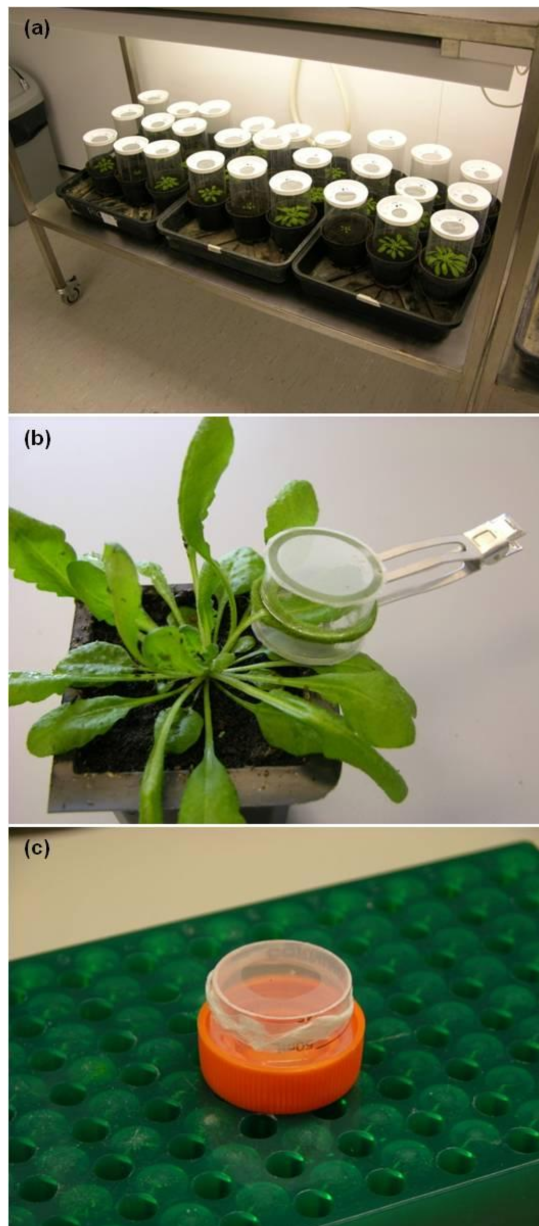

Figure 2.1 – Examples of three aphid assays.

(a) Aphid survival/fecundity assay. This experiment allows a longer-term assessment of aphid survival and fecundity on *Arabidopsis*. The 14-day test period allows effects on aphid development and performance during adulthood to be assessed. **(b)** Clipcages were used to confine aphid populations to single leaves. This allowed the generation of plant tissue samples that had been subject to relatively intense infestations. **(c)** Artificial feeders were used to assess the effect of camalexin on aphid survival and reproductive development over a 2-day test period.

Chapter 3 – The miRNA pathway is involved in *Arabidopsis* resistance to the green peach aphid *Myzus persicae*

3.1 Introduction

The green peach aphid *Myzus persicae* is one of the most destructive pests on cultivated crops worldwide (Blackman & Eastop, 2000). This species causes feeding damage and, more importantly, is the vector of many different plant viruses (Ng & Perry, 2004; Hogenhout *et al.*, 2008). However the mechanisms by which plants defend themselves against aphids 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 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 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 RNA (sRNA) molecules 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 were implicated in plant defense responses 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). Small RNAs modify gene expression by acting both at 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 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 2nt 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 ten Argonaute (AGO) proteins (Vazquez *et al.*, 2010). The sRNA guides the RISC to either cleave or repress translation of target transcripts bearing sufficient homology to the loaded sRNA.

Small RNAs 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). MicroRNA (miRNA) molecules are a class of largely 21nt 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 regulating the defence response that occurs following challenge by the bacterial biotroph *Pseudomonas syringae*

(Navarro *et al.*, 2006; Zhang, W *et al.*, 2011) or the pathogen associated molecular pattern (PAMP) flg22 (Li *et al.*, 2010). Evidence suggests that a substantial set of miRNAs are differentially regulated following perception of these challenges, and have impact on hormone pathways regulating resistance. The defence pathways activated in response to attack from chewing herbivores are also governed by sRNAs. Growth of *M. sexta* larvae is enhanced on *N. attenuata* lacking RNA-dependent RNA polymerase 1 (RDR1) (Pandey & Baldwin, 2007). In this interaction, RDR1-dependent siRNAs are required to coordinate a defence response involving nicotine biosynthesis and the JA and 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; Kuśnierczyk *et al.*, 2007; Kuśnierczyk *et al.*, 2008; Gao *et al.*, 2010). In one study, transcriptional 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 (Kuśnierczyk *et al.*, 2008; Gao *et al.*, 2010). Given these observations and the known involvement of sRNAs in defence responses against pathogens and a chewing herbivore, we hypothesised that sRNAs may play a similarly important role in coordinating plant processes in response to aphid attack.

In the work described here, we find that *M. persicae* fecundity is reduced on plants deficient in miRNA processing. In contrast, aphid performance is unchanged on plants deficient in other siRNA processing pathways. The resistance phenotype of the miRNA mutants is independent of their reduced stature phenotype. This indicates that the miRNA pathway is a negative regulator of *M. persicae* resistance in *Arabidopsis*.

3.2 Results

Establishing a reliable aphid survival and fecundity assay

Our initial aim was to develop an assay by which the success of aphids could be assessed across a range of *Arabidopsis* mutants. One measure of insect success is fecundity, which describes the capacity of an organism to produce offspring. A measurement of fecundity could include the age at which an aphid is first able to reproduce, or the total number of nymphs produced over a lifetime. Survival or mortality is another measure of aphid success that is distinct from reproductive capacity. Many examples of aphid assays in the literature make no distinction between these two parameters. Indeed, there is immense variation in the methodology used to assess aphid performance. There are studies where experiments are as short as two days (Pegadaraju *et al.*, 2005), whereas other laboratories have favoured a longer term approach where experiments are run for two to three weeks (Kuśnierczyk *et al.*, 2008). A longer exposure to test plants presumably allows detection of more subtle effects on aphid behaviour which might be missed in short term experiments.

In some initial experiments, we used non-aged adult *M. persicae* to assess aphid performance. These experiments did not make a distinction between survival of adult insects and their fecundity (Fig. 3.1). Experiments were run for seven days after inoculation, with total aphid counts conducted every day from day three up until day seven. In some of these trials, there was considerable variation within each treatment (Fig. 3.1a). In other trials, we could not reproduce results and in some cases obtained contrasting sets of data between replicate experiments (Fig. 3.1b).

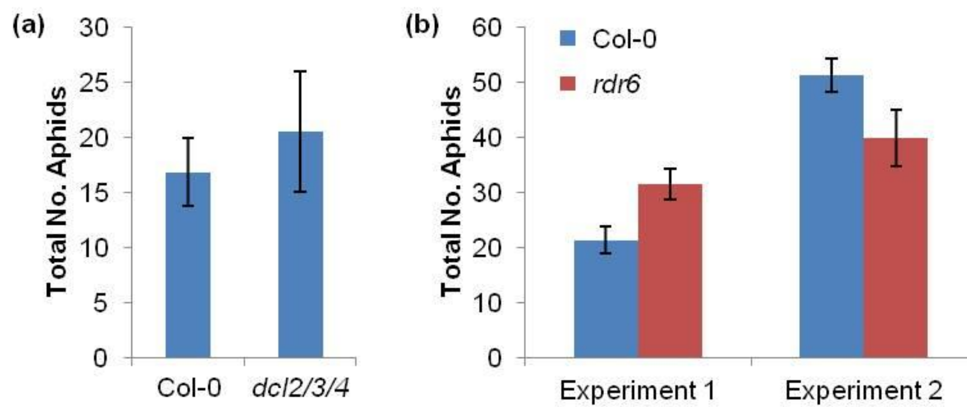

Figure 3.1 – Using non-aged adult aphids on plants produces inconsistent results.

(a) An early experiment comparing *M. persicae* fecundity on *Arabidopsis* Col-0 and the *dcl2/3/4* triple mutant. In this experiment, five non-aged adult aphids were added to each plant and total aphid counts were taken every day up to seven days. The graph indicates the total number of aphids counted when the experiment was terminated. Bars represent the mean (±SE) across six plants per genotype. This experimental protocol produced results with large degrees of variation within each treatment. **(b)** Non-aged adults were added to Col-0 and *rdr6* plants following the same method as in (a). In these trials, the variation within each treatment was lower, however results were highly variable between repeat experiments. Bars represent the mean (±SE) across 4-6 plants per genotype.

I suspected that these discrepancies were due to the use of non-aged adult insects, and that our initial trials did not take into account two factors which likely explain that majority of the variability. Firstly, insects taken from the stock colony as adults could have very different reproductive capacity. We discovered that upon reaching maturity, there is a peak in nymph production, which then stabilises with age (Fig. 3.2). Two adults separated by two or three days in age may therefore have very different levels of fecundity. In addition, the environment during juvenile aphid development has an impact on adult behaviour. It is known that the fecundity of adult insects has to an extent been pre-determined by

conditions within the stock cage. This may mask fecundity results when aphids are then raised on hosts of varying suitability.

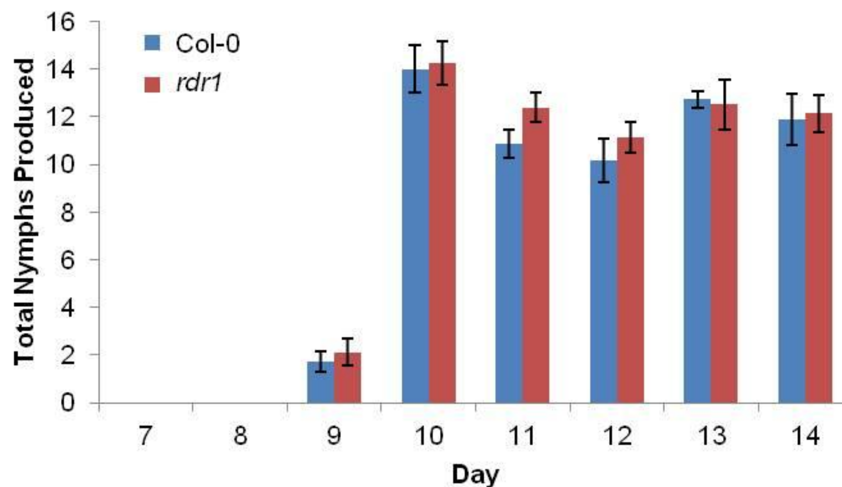

Figure 3.2 – Assessing aphid performance by two-week survival/fecundity assay.

In this example, nymph production begins on day nine, peaks on day ten before stabilising towards the end of the experiment on day fourteen. In our final experimental design, nymphs produced up to day eleven are removed to reduce crowding on test plants. There is then a further three days of nymph production before the final nymph count on day fourteen.

To counter these problems, we decided to progress using nymphs as test insects. These nymphs would be born and spend their entire lives on test plants. This hopefully minimises the influence of conditions within the stock cage. However, as *M. persicae* has telescoping generations, it is highly likely that a stressful or beneficial environmental condition will have bearing on multiple generations of unborn aphids in a female. This may have two consequences. Firstly, stress conditions that may have occurred in the aphid stock cage will be carried forward into experiments resulting in some variations in aphid performance

(survival plus fecundity) among biological replicates over time. Secondly, differences in aphid performance between treatments may be smaller as might be expected, because aphids have already generated embryos at various stages of development in the stock cage. Therefore, to increase the likelihood of finding *Arabidopsis* mutants that are disadvantageous or beneficial for the aphid, it appears to be essential that aphids are exposed to these plants as long as possible. For this reason, a new assay was developed in which aphids were exposed to the experimental plants from the time of birth until they bear their own young. To achieve this, adult aphids from the stock cage were allowed to produce young on the experimental plants during 48 hours and were then removed. Five nymphs remained on the experimental plants until they reached adulthood for maximally 14 days at which time they are past their peak of nymph production (Fig. 3.2). Nymphs were counted at days 11 and 14, and to avoid crowding becoming a problem, all nymphs are removed from plants on day 11. I predicted that this assay would produce more consistent and sensitive results, thereby increasing the likelihood of the discovery of *Arabidopsis* mutants that are disadvantageous or beneficial to the aphids compared to wild type plants. In an initial trial of this method, both aphid survival (the number of nymphs that developed into adults and were alive at 14 days) and fecundity (number of nymphs born from the adults at days 11 and 14) on wild type Col-0 and *rdr1* mutants were highly consistent (Fig. 3.3a,b). These results provided confidence for continuing with this 14-day aphid survival and fecundity assay.

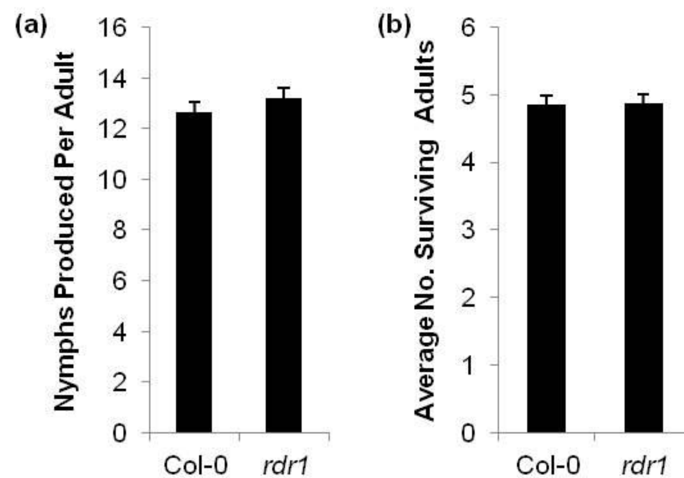

Figure 3.3 – Establishing performance readouts for the two-week survival/fecundity assay.

(a) In one of the earliest experiments using this method, we found that adult aphid fecundity was unchanged on the *rdr1* mutant relative to Col-0. In the majority of subsequent experiments, we found that on Col-0, an average of between ten and fourteen nymphs are produced per adult over a two-week period. **(b)** We found no change in levels of survival between aphids raised on Col-0 and *rdr1*. In no subsequent experiments did we find any change in survival due to plant genotype. On Col-0, adult survival is typically between four and five insects from the original five that were seeded at the start of the experiment.

In all subsequent experiments, I found no significant differences in aphid survival between *Arabidopsis* Col-0 and mutants (data not shown). However, significant differences in aphid fecundity were noticed on some *Arabidopsis* mutants compared to wild type Col-0. Hence I report on fecundity measurements only.

The miRNA pathway is involved in plant defence to aphids

To determine whether sRNAs are involved in *Arabidopsis* resistance to *M. persicae*, aphid performance was assessed on a collection of *rdr*, *dcl* and *ago* mutants and wild type Col-0 *Arabidopsis*. In an initial experiment, fecundity was unchanged among three RDR mutants (*rdr1*, *rdr2*, *rdr6*) compared to Col-0 (Fig. 3.4a). This indicates that RDRs are not involved in *Arabidopsis* resistance to *M. persicae,* unlike the *rdr1* mutant of *N. attenuata* that has decreased resistance to the herbivore *M. sexta* (Pandey & Baldwin, 2007). In contrast, aphids produced significantly fewer offspring on *dcl1* mutants relative to Col-0 (ANODE; $p<0.001, n=5$) but were not affected on *dcl2*, *dcl3* or *dcl4* mutants (Fig. 3.4b). In addition, aphid fecundity was significantly lower on the *ago1-25* mutant (ANODE; $p<0.001, n=5$) but was unchanged on *ago2*, *ago4* and *ago7* mutants (Fig. 3.4c). Aphid performance was also not affected on the *dcl2/3* and *dcl2/4* double mutants or the *dcl2/3/4* triple mutant (Fig. 3.4d). Because DCL1 and AGO1 both process sRNAs in the miRNA pathway, these data suggest that the miRNA pathway is involved in *Arabidopsis* resistance to *M. persicae*, whilst other small RNA processing pathways appear not to play a significant role.

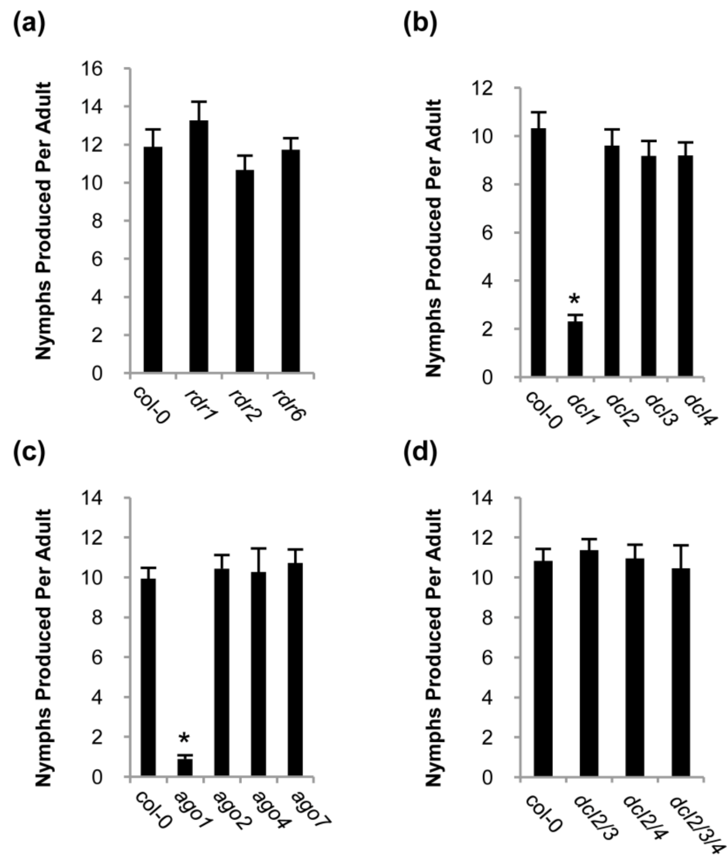

Figure 3.4 - **The *Arabidopsis* miRNA pathway is involved in aphid resistance.**

Aphid fecundity is reduced on miRNA pathway mutants (*dcl1*, *ago1*) (b,c) but not mutants in other 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).

miRNA mutant resistance to aphids is independent of plant stature

To investigate this further, I conducted *M. persicae* fecundity assays on other mutants in the miRNA pathway. Additionally, to determine whether the smaller stature of *dcl1* and *ago1* mutants affects aphid fecundity, I 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, but has no impairment in sRNA processing (Fig. 3.5a, 3.6). I observed that aphid fecundity was not significantly different between PDLP1 and Col-0, whilst aphids produced significantly fewer nymphs on the miRNA mutant *dcl1* and the *hen1* mutant which is deficient in all small RNA pathways (ANODE; $p<0.001$, $n=5$) (Fig. 3.5b). Similarly, aphids were significantly less fecund on *hst* and *se1* mutants compared to both Col-0 and PDLP1 (ANODE; $p<0.001$, $n=5$) (Fig. 3.5c). SERRATE is a zinc finger protein that assists DCL1 in the accurate excision of miRNAs from their precursors whilst 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, I 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. 3.5a, 3.6). Fecundity on these plants matched that of *dcl1*-raised aphids (Fig. 3.5d). I 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. 3.5e) although in our growth conditions the *ago1-26 and ago1-27* mutants were similar in size and stature compared to the *ago1-25* mutant analysed in Fig. 1C (Fig. 3.6). Nonetheless, these results suggest that the miRNA pathway is involved in regulating the plant resistance response to *M. persicae*, while other siRNA pathways are not involved. Furthermore, the resistance exhibited by miRNA pathway mutants is independent of the dwarfism phenotype.

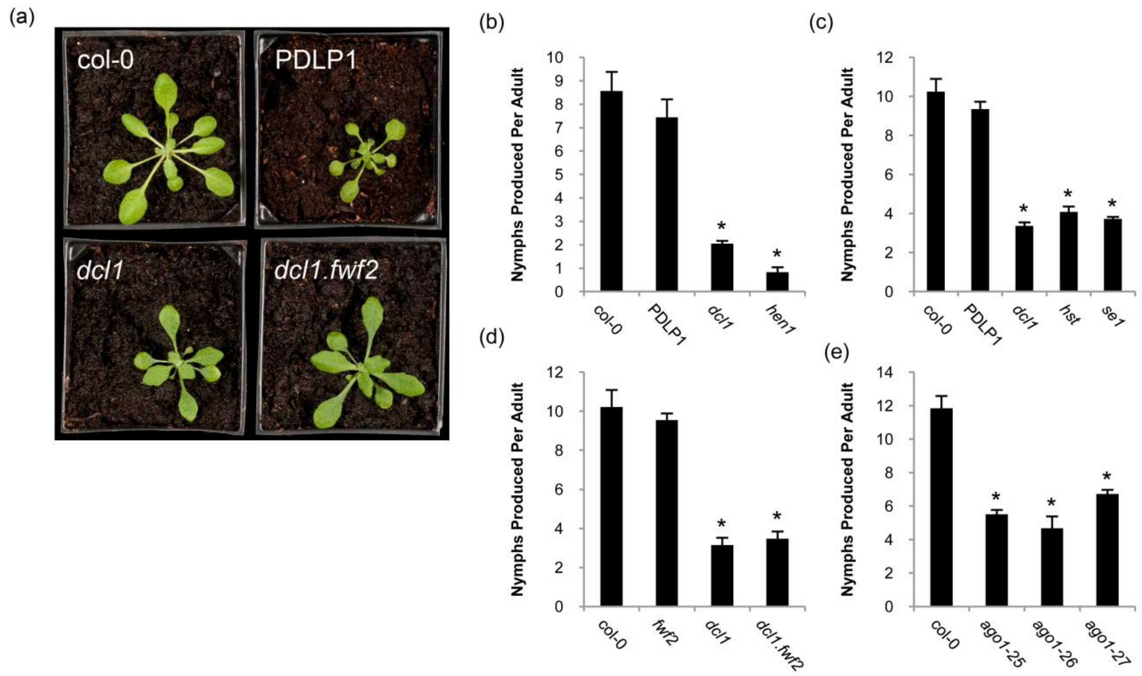

Figure 3.5 - Plant stature has no effect on aphid fecundity.

Aphid fecundity is reduced on *Arabidopsis* lines that aberrantly process miRNA (*hen1, hst, se1*) (b,c) but remains high on the unrelated dwarf PDLP1 (a,b,c). Reduced fecundity is also observed on the partial *dcl1* rescue line *dcl1.fwf2* (a,c) and across several *ago1* alleles (e). 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 ANODE.

Figure 3.6 - Phenotypes of all plants used in the silencing mutants fecundity screen.

Plants are 4-weeks old in these photographs, the same age as plants that are first exposed to aphids in the 2-week survival/fecundity trial.

miRNA mutant resistance is absent in a short-term fecundity trial

There is considerable variation in the methods employed by different laboratories to assess aphid performance. Using a 14-day assay, I observed that aphids were less fecund on miRNA pathway mutants compared to either wild-type plants or plants deficient in aspects of siRNA processing. To further characterise this resistance phenotype, I exposed Col-0, *dcl1* (miRNA mutant) and *dcl2/3/4* (siRNA mutant) to aphids using a previously published short-term protocol (Pegadaraju *et al.*, 2005). In this experiment, plants are seeded with 15

non-aged adults and exposed for two days. At the end of this period, the total number of aphids on test plants is recorded. This method makes no distinction between survival and fecundity of test insects. Following this protocol, I found that the total numbers of aphids on Col-0, *dcl1* and *dcl2/3/4* plants were similar (Fig. 3.7). Therefore, the resistance phenotype exhibited by miRNA pathway mutants is not present during a 2-day experimental protocol, and is only revealed when insects are exposed to plants for a longer period.

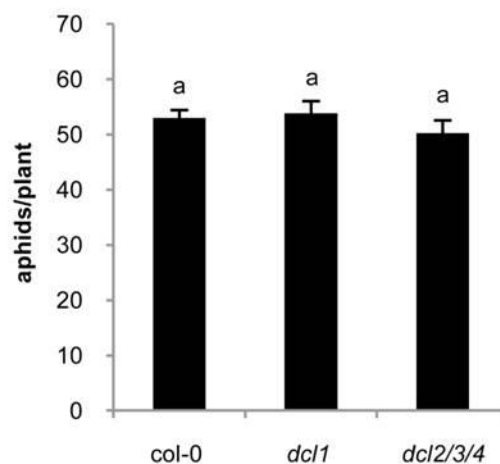

Figure 3.7 – miRNA mutant resistance is absent in a short-term performance assay.

Aphid performance was assessed on wild-type (Col-0), miRNA mutant (*dcl1*) and siRNA mutant (*dcl2/3/4*) following the method described by (Pegadaraju *et al.*, 2005). Bars represent the mean (±SE) of ten plants from two independent experiments. Data was analysed by ANODE (GenStat) and the letters indicate no statistically significant differences in aphid performance across these genotypes.

3.3 Discussion

In these experiments, I show that the green peach aphid *M. persicae* produces significantly less progeny on *Arabidopsis* plants that aberrantly process miRNAs. I found no change in aphid performance on plants deficient in siRNA-mediated pathways. I continue to show that miRNA mutant resistance is independent of the developmental phenotype of these plants, as aphid fecundity is close to wild-type levels on 35S::PDLP1a:GFP transgenic plants which also exhibit a dwarf stature. Furthermore, aphid fecundity was similar between *dcl1* and the *dcl1.fwf2* line which has a partially-rescued phenotype. Interestingly, the resistance phenotype of a miRNA pathway mutant (*dcl1*) was absent when non-aged adult aphids were exposed to plants in a short-term performance assay.

The finding that aphids were less successful on *dcl1* plants was initially unexpected, as pathogen and insect performance has 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 reproduce poorly on *Arabidopsis*), show increased proliferation on miRNA pathway mutants, but not on plants defective in other silencing pathways (Navarro *et al.*, 2008). Similarly, *P. fluorescens* and *Escherichia coli*, which do not normally infect *Arabidopsis*, can multiply on miRNA pathway mutants (Navarro *et al.*, 2008). In addition, some RNA silencing mutants are hyper-susceptible to infection by the vascular fungi *Verticillium* (Ellendorff *et al.*, 2009). More specifically for insects, an RDR1-silenced line of *N. attenuata* (irRdR1) is more susceptible to larvae of the solanaceous specialist *M. sexta* (Pandey & Baldwin, 2007). Nonetheless, there are several examples of increased resistance of *Arabidopsis* miRNA mutants to pathogens and pests. Both *Arabidopsis* miRNA or 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 tumourigenic *Agrobacterium*

(Dunoyer *et al.*, 2006). This may be expected as miRNAs are integral players in plant development, and cyst nematodes and *Agrobacterium* reprogram plant development to generate cysts and galls, respectively, that provide feeding and replication sites for these plant colonisers. Thus, our observation that aphids do less well on *Arabidopsis* miRNA mutants may be a consequence of the highly specialised feeding mode of aphids. *M. persicae* 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 while 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) may induce these modulations. We propose that *M. persicae* colonisation efficiency of *Arabidopsis* is enhanced by the ability of this aphid to modulate specific plant processes that are regulated by miRNAs.

In a two-week fecundity assay, I demonstrated that significantly fewer nymphs are produced by aphids raised on miRNA pathway mutants relative to either wild-type plants or siRNA pathway mutants. When I repeated this experiment using a short two-day method described by Pegadaraju and colleagues, performance was similar across all genotypes. This inconsistency may involve several contributory factors. Firstly, as exposure times in these experiments were ~14-days and 2-days respectively, it could be that the resistance mechanism requires an exposure longer than two days before measureable effects can be observed on aphids. Secondly, as one method uses nymphs as test insects and the other adults, it could be that the resistance mechanism is effective against developing aphids whereas adults have some level of immunity. The data indicates that the miRNA mutant resistance phenotype interferes with aphid reproductive development and not the survival of insects as they mature from nymph to adult. It remains a possibility that the longevity of aphids is altered on some of plants assessed in these experiments, however I did not investigate this as part of the screen.

There are currently ~240 known miRNA species in *Arabidopsis*. Of these, only a small proportion has so far been implicated in plant defence signalling (Navarro *et al.*, 2006; Li *et al.*, 2010; Zhang, W *et al.*, 2011). In the miRNA mutants tested in this set of experiments (*dcl1*, *ago1*, *se1*, *hst*, *hen1*), the quantity of fully functional mature miRNAs is globally depleted. However, it is likely that the majority of miRNAs are not involved in aphid resistance. The crucial questions leading on from this exploratory work are to identify the suite of miRNAs that are important for aphid resistance, and the metabolic or other defence pathways that they regulate. This may facilitate the identification of novel mechanisms involved in aphid resistance in *Arabidopsis*. Additionally, there may exist aphid-inducible miRNAs. To our knowledge, no study has investigated responses of the sRNA transcriptome to a phloem-feeding insect. This work therefore presents an opportunity for novel miRNA discovery.

Chapter 4 – The camalexin pathway is involved in *Arabidopsis* miRNA mutant resistance to *Myzus persicae*

4.1 Introduction

In higher plants, miRNAs negatively regulate gene expression at the transcript level. Additionally, it is known that in the moss *Physcomitrella patens*, miRNAs are involved in a process that guides DNA methylation (Khraiwesh *et al.*, 2010), although it is currently unknown whether this phenomenon occurs in other plant species. The post-transcriptional targets of some miRNAs have been identified in *Arabidopsis* and include conserved transcription factors (Sunkar & Zhu, 2004; Wang *et al.*, 2004). As some miRNAs are known to be involved in plant defence responses (Navarro *et al.*, 2006; Zhang, W *et al.*, 2011; Zhang, X *et al.*, 2011) and miRNA pathway mutants are more resistant to aphid infestation (Chapter 3), we hypothesised that the miRNA pathway mutants have altered defence pathway regulation upon aphid attack.

Arabidopsis responses to aphid attack have been extensively investigated and involve suites of genes involved in numerous biochemical pathways (Moran *et al.*, 2002; de Vos *et al.*, 2005; Couldridge *et al.*, 2007; Kuśnierczyk *et al.*, 2007; Kuśnierczyk *et al.*, 2008). The magnitude and composition of the response vary between studies. In one example, the response to *M. persicae* involved a greater number of genes than defence responses against a bacterial biotroph (*Pseudomonas syringae*), a fungal necrotroph (*Alternaria brassicicola*), a cell-content feeding insect (*Frankliniella occidentalis*) or a leaf-chewing insect (*Pieris rapae*) (de Vos *et al.*, 2005). Given that among all these attackers, aphids induce the least visible damage or necrosis, this result is somewhat remarkable. However, extensive aphid-induced transcriptional reprogramming has not been observed in all studies. Couldridge and coworkers found that only two genes were differentially expressed in leaves following two hours aphid exposure, increasing to twenty-five following a thirty-six hour infestation (Couldridge *et al.*, 2007). It is likely that

plant response to aphids depends on experimental conditions and the temporal aspects, and both conditions must be considered carefully when assessing host responses.

In some of the earliest studies using *Arabidopsis* as a model to study plant-aphid interactions, pathogenesis-related genes such as *PR1* and *BGL2* were found to be upregulated during aphid attack (Moran & Thompson, 2001; Moran *et al.*, 2002), suggesting that there may be considerable overlap between the aphid response and the response generated against some biotrophic pathogens. Genes such as *PR1* are SA-responsive, raising the possibility that the aphid response may be dependent on a SA signal to trigger transcriptional reprogramming. However, no change has been recorded in SA levels of aphid-infested tissue relative to aphid-free controls (de Vos *et al.*, 2005). Furthermore, aphids are no more successful, and in some cases are less successful, on plants deficient in SA signalling compared to wild-type plants (Mewis *et al.*, 2005; Pegadaraju *et al.*, 2005; Mewis *et al.*, 2006).

Aphid-responsive genes also involve those in the JA and ET defence pathways (Moran *et al.*, 2002; Kuśnierczyk *et al.*, 2007). JA appears particularly important, as plants supplied with exogenous JA are more resistant to aphid infestation (Ellis *et al.*, 2002). Furthermore, aphid performance increases on JA pathway mutants, but was reduced on plants with constitutively activated JA signalling (Ellis *et al.*, 2002). It has been suggested that aphids preferentially induce SA signalling as a means to dampen a more effective JA response. This model has been termed the decoy hypothesis (Zhu-Salzman *et al.*, 2004).

The role of glucosinolates in insect resistance has received much attention, specifically on the indolic forms synthesized from the amino acid Tryptophan. Glucosinolates have potent defensive benefits against chewing herbivores. Upon tissue disruption, glucosinolates are hydrolyzed by endogenous myrosinases to produce a range of compounds that act as insect deterrants (Koroleva *et al.*, 2000). Aphids do not cause

significant amounts of feeding damage, but there is persuasive evidence to suggest that some glucosinolates possess powerful defensive properties against these pests.

In *Arabidopsis*, aphid feeding catalyzes the conversion of indol-3-ylmethylglucosinolate (I3M) to 4-methoxyindol-3-ylmethylglucosinolate (4MI3M) (Kim & Jander, 2007). In artificial diet experiments, 4MI3M exhibited a greater deleterious effect on aphid reproductive success than I3M did (Kim & Jander, 2007). It has therefore been suggested that this conversion is an adapted response in plants to produce glucosinolates that offer the greatest defensive benefit against aphids. Conversion of I3M to 4MI3M depends on activity of the cytochrome P450 CYP81F2 (Pfalz *et al.*, 2009). Mutants lacking functional CYP81F2 do not accumulate either 4MI3M or the intermediate 4-hydroxyindol-3-ylmethylglucosinolate (4OHI3M) and are more susceptible to aphid colonization (Pfalz *et al.*, 2009). Thus, 4MI3M and 4OHI3MA play active roles in aphid resistance in *Arabidopsis*. However, aphid performance on the *cyp79b2/cyp79b3* double mutant, which is deficient in all indolic glucosinolates, and wild type plants are similar (Kim *et al.*, 2008). Whilst the double mutant does not support higher aphid numbers, they do senesce and die much faster than wild-type plants when aphid colonies are allowed to develop for an extended period of up to 30 days (Kim *et al.*, 2008). How glucosinolates exert protective effects in plants against aphids is not yet understood. The glucosinolates are unlikely to be hydrolysed by myrosinases prior to ingestion by the aphid as these insects cause minimal tissue disruption. The indolic glucosinolate fraction is less abundant while breakdown products of indolic forms are comparatively enriched in whole aphids and aphid honeydew than in whole plants or phloem exudate (Kim *et al.*, 2008). On the other hand, the aliphatic glucosinolates pass through aphids unaltered, and are found in similar quantities in aphid honeydew as in plants. Therefore, the indolic glucosinolates may be broken down in aphids in a manner that is myrosinase-independent or in the phloem prior to ingestion.

The role of phytoalexins in aphid resistance is less defined compared to that of glucosinolates. The pathway leading to camalexin synthesis, which branches from the indole glucosinolates, is also not fully described. However, both the camalexin and indole glucosinolate pathways compete for the common precursor indole-3-acetaldoxime (IAOx) which is derived from Tryptophan (Zhao *et al.*, 2002; Glawischnig *et al.*, 2004). CYP71A13 converts IAOx to indole-3-acetonitrile (IAN) which is the substrate for as yet uncharacterised reactions leading to the production of dihydrocamalexic acid (DHCA) (Nafisi *et al.*, 2007). DHCA is converted to camalexin by CYP71B15 (PAD3) (Schuhegger *et al.*, 2006; Böttcher *et al.*, 2009; Su *et al.*, 2011). Although *PAD3* is induced during infestations by the generalist aphid species *M. persicae* in some studies, survival of these aphids on *pad3* mutants and wild-type *Arabidopsis* plants were similar over a 2-day exposure to the experimental plants (Pegadaraju *et al.*, 2005). On the other hand, the crucifer-specialist aphid species *B. brassicae*, which also induces *PAD3* on *Arabidopsis*, is more successful on *pad3* mutants than on wild-type Col-0 over a 13-day exposure to the experimental plants (Kuśnierczyk *et al.*, 2008). Thus, the effectiveness of camalexin may differ between aphid species and/or may become more noticeable when aphids are exposed to it for longer periods of time.

Phytohormone synthesis and response pathways are also involved in the complex plant defense responses to aphid attack. Moreover, other components of the glucosinolate and camalexin synthesis pathways may influence aphid performance. To investigate why the miRNA pathway mutants are more resistant to aphids (chapter 3), the expression levels of various genes in the glucosinolate, camalexin and phytohormone pathways were analysed in the aphid-exposed miRNA mutant *dcl1,* the siRNA pathway mutant *dcl2/3/4* and wild-type Col-0.

4.2 Results

Transcriptional response to *M. persicae* infestation is first detected at 12hpi

To assess the temporal aspect of the host response to infestation, I used quantitative real-time PCR (qRT-PCR) to assess induction of a range of defence pathway-related transcripts in the Col-0 ecotype (Fig. 4.1, 4.2, 4.3). I measured defence induction at six, twelve, twenty-four and forty-eight hours and found that significant transcriptional changes are first detected at 12-hpi (Fig. 4.1a,d 4.2a,b 4.3c). The changes present at 12-hpi were generally maintained at the 24-hpi and 48-hpi time points and in many cases the magnitude of induction was higher at these later time points (Fig. 4.1a,c,d 4.2c 4.3c). Eight out of the eleven genes tested showed some degree of induction in aphid-infested samples relative to mock infestation controls. This is perhaps not surprising as all of these genes have previously been implicated in aphid defence responses. Of the genes induced in aphid-exposed samples, those involved in SA-response (*PR1*) (Fig. 4.1a), tryptophan-derived secondary metabolism (*PAD3, CYP79B2, CYP83B1, CYP81F2*) (Fig. 4.1d 4.2a,b,c) and JA/ET-response (*PDF1.2, HEL*) (Fig. 4.1c 4.3c) were most consistently induced. In contrast, genes involved in JA synthesis (*LOX2*) and response (*VSP2*) were either stable or repressed (Fig. 4.1b 4.3b). These results are consistent with the known antagonistic interaction between the SA and JA signalling pathways. As the transcriptional response against infestation appears to be mobilised at 12-hpi, we selected this time point for future experiments as it would be possible to detect decrease as well as increase in gene expression levels.

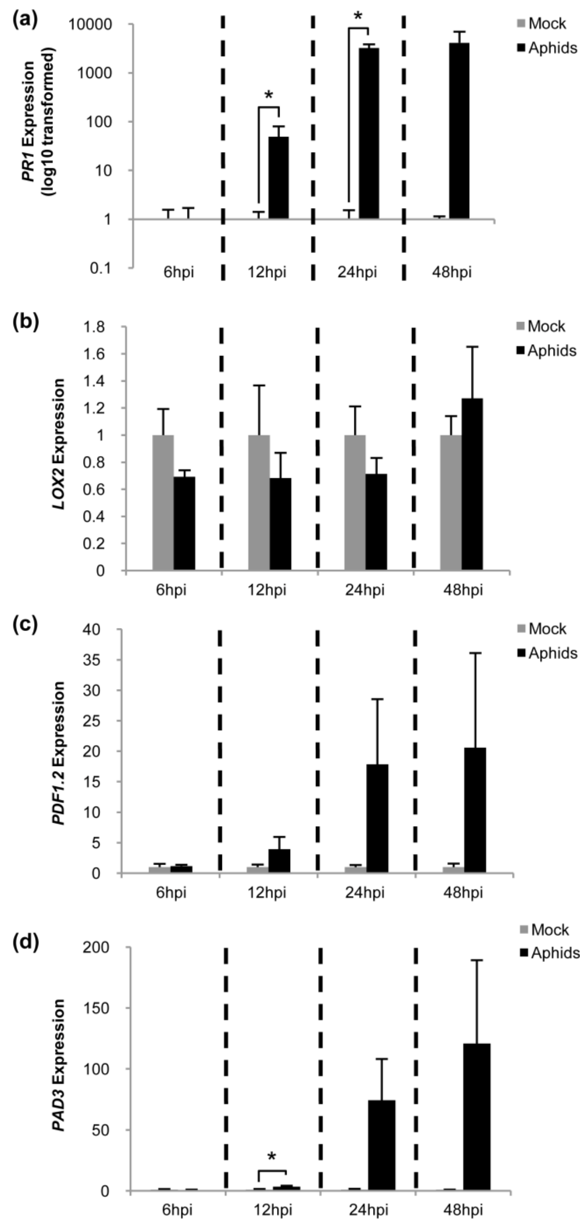

Figure 4.1 - Investigation into temporal response of Col-0 plants to *M. persicae* infestation.

qRT-PCR analysis of transcripts involved in **(a)** SA response (*PR1*), **(b)** JA production (*LOX2*), **(c)** JA/ET response (*PDF1.2*), and **(d)** camalexin production (*PAD3*) following 6, 12, 24 or 48h aphid infestation. Bars represent the mean expression levels (±SE) across four biological replicates. Leaves housed in empty cages (Mock) for the same period were used as controls. Asterisks indicate statistically significant differences at $p<0.05$ as determined by t-probabilities within a General Linear Model (GLM). The mock-treated sample at each timepoint is displayed as a value of 1.

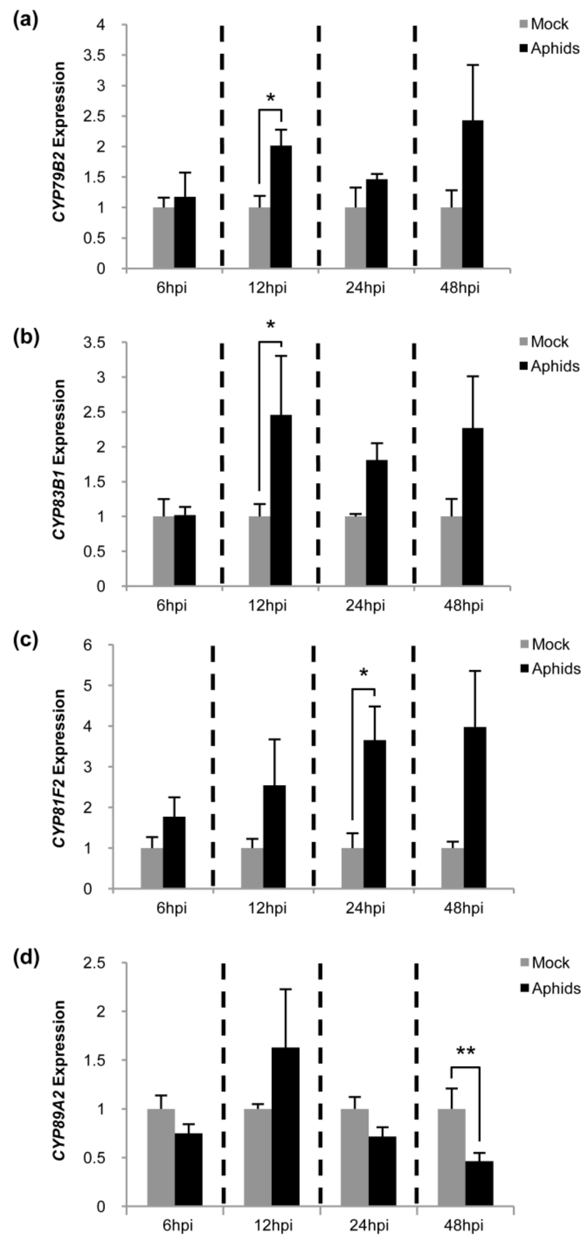

Figure 4.2 - Investigation into temporal response of Col-0 plants to *M. persicae* infestation.

qRT-PCR analysis of transcripts involved in production **(a)** glucosinolates and camalexin (*CYP79B2*), **(b)** glucosinolates (*CYP83B1*), and **(c)** aphid-relevant glucosinolate (*CYP81F2*). **(d)** *CYP89A2* has previously been found to be highly repressed following 48h aphid feeding (de Vos *et al.*, 2005). Expression levels were measured following 6, 12, 24 or 48h aphid infestation. Bars represent the mean expression levels (±SE) across four biological replicates. Leaves housed in empty cages (Mock) for the same period were used as controls. Single asterisks indicate statistically significant differences at $p<0.05$, double asterisks indicate differences at $p<0.01$ as determined by t-probabilities within a GLM. The mock-treated sample at each timepoint is displayed as a value of 1.

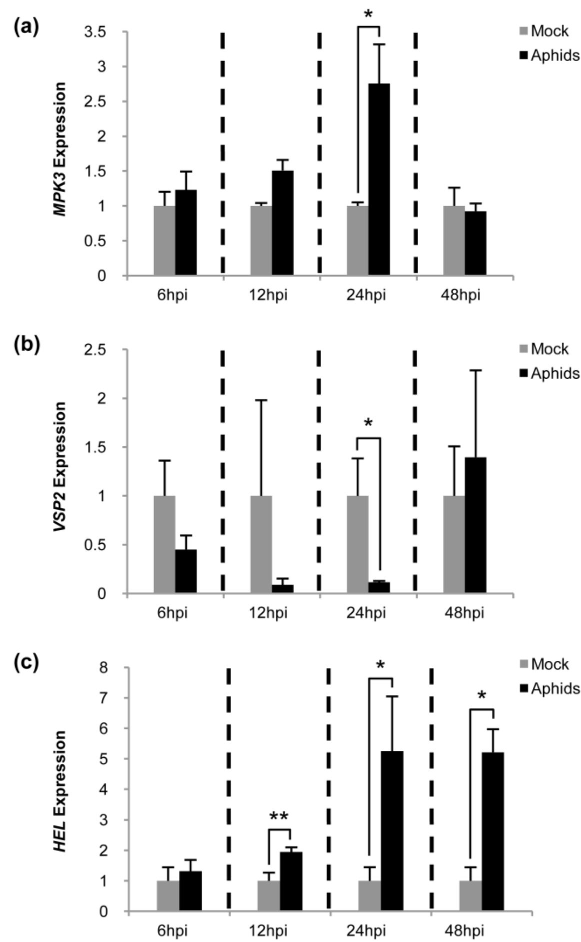

Figure 4.3 - Investigation into temporal response of Col-0 plants to *M. persicae* infestation.

qRT-PCR analysis of transcripts involved in **(a)** defence signalling (*MPK3*), **(b)** JA response (*VSP2*), and **(c)** ET response (*HEL*) following 6, 12, 24 or 48h aphid infestation. Bars represent the mean expression levels (±SE) across four biological replicates. Leaves housed in empty cages (Mock) for the same period were used as controls. Single asterisks indicate statistically significant differences at $p<0.05$, double asterisks indicate differences at $p<0.01$ as determined by t-probabilities within a GLM. The mock-treated sample at each timepoint is displayed as a value of 1.

Camalexin, indole glucosinolate, ET and JA pathway transcripts are upregulated in aphid-exposed *dcl1* mutants

To identify defence pathways involved in the miRNA mutant resistance phenotype, I exposed Col-0, *dcl1* and *dcl2/3/4* plants to 12h *M. persicae* infestations. *PAD3 (CYP71B15)*, a marker for the camalexin biosynthetic pathway (Chassot *et al.*, 2008; Xu *et al.*, 2008), was most strikingly induced upon exposure to aphids in the *dcl1* mutant compared to Col-0 and the *dcl2/3/4* triple mutant among all the genes tested (Fig. 4.4a). In addition, *CYP81F2*, a gene involved in the indolic glucosinolate pathway was significantly induced in aphid-infested *dcl1* plants compared to Col-0 and *dcl2/3/4* (Fig. 4.4d). The JA biosynthetic gene *LOX2* was also significantly upregulated in aphid-exposed *dcl1* compared to aphid-exposed Col-0 and *dcl2/3/4* (Fig. 4.5b). The defence-related gene *MPK3* was most strongly induced in *dcl1*, although the increase was not significantly different to aphid-exposed Col-0 or *dcl2/3/4* (Fig. 4.6). *PR1*, a marker for SA signalling (de Vos *et al.*, 2005; Kuśnierczyk *et al.*, 2007), is upregulated upon aphid exposure, however its induction was not significantly different among aphid-exposed Col-0, *dcl1* and *dcl2/3/4* plants (Fig. 4.5a). The basal expression levels of some genes, such as *CYP79B2* and *CYP83B1* of the indole glucosinolate/camalexin pathways were greater in *dcl1* compared to Col-0 and *dcl2/3/4*, but did not alter significantly upon exposure to aphids (Fig. 4.4b,c). *VSP2* and *PDF1.2* have been used as downstream markers of the JA and ET pathways (de Vos *et al.*, 2005). I found that the expression of these genes were either stable or repressed following aphid treatment and did not differ significantly across any of the lines tested (Fig. 4.5c,d). In contrast, the ethylene-responsive transcript *HEL (PR4)* was significantly induced in aphid-exposed *dcl1* plants compared to aphid-exposed Col-0 and *dcl2/3/4* (Fig. 4.5e). As genes involved in glucosinolate and camalexin biosynthesis and the JA- and ET-signaling pathways were differentially regulated in *dcl1* plants, I hypothesised that these

pathways may be responsible for the aphid-resistant phenotype of *Arabidopsis* miRNA pathway mutants.

To assess if differences in gene expression at 12-hpi were consistent at later time points, I exposed Col-0, *dcl1* and *dcl2/3/4* plants to 24- and 48h aphid infestations (Fig. 4.7). I found that *PAD3* and *HEL* remained significantly upregulated in aphid-exposed *dcl1* relative to aphid-exposed wild type plants at the later time points (Fig. 4.7). Many of the other genes were upregulated in both aphid-exposed Col-0 and *dcl1* mutants indicating that these do not contribute to the difference in aphid resistance of miRNA pathway mutants.

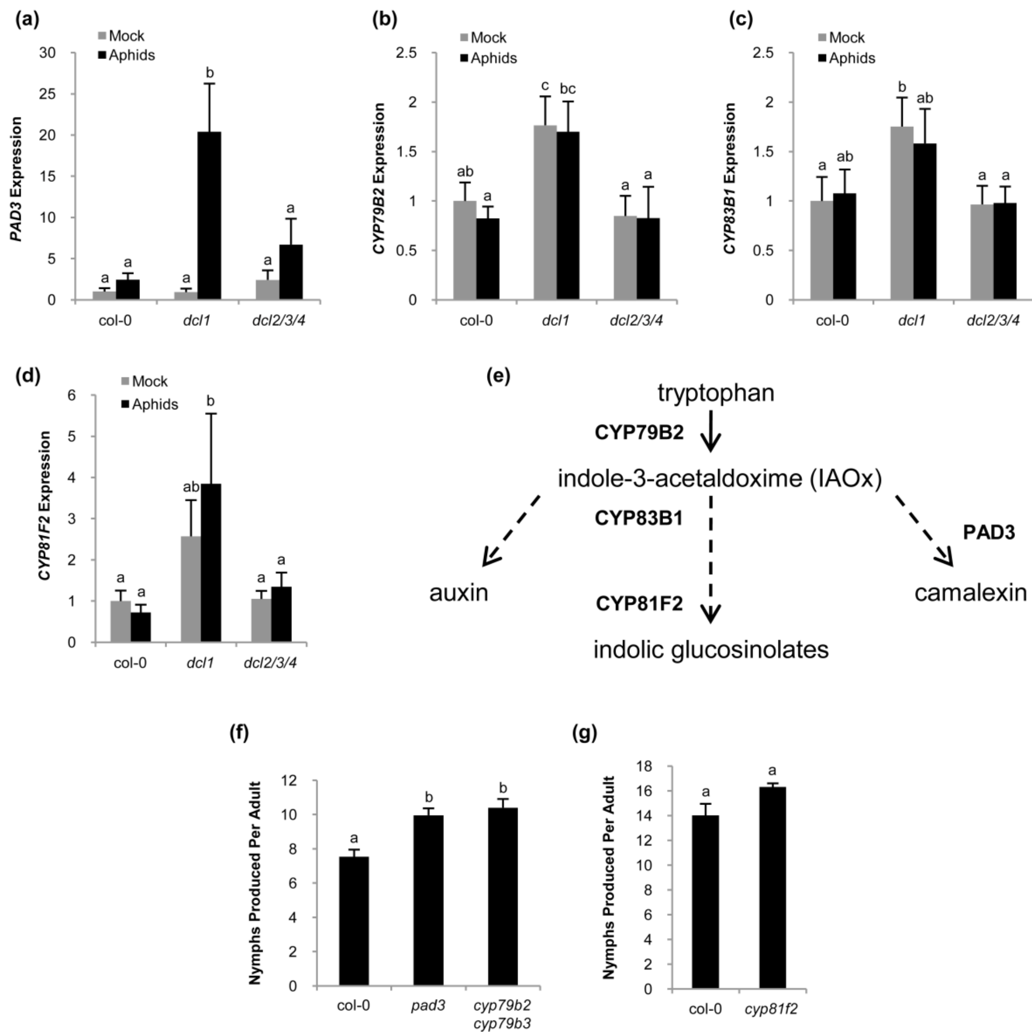

Figure 4.4 - MiRNA mutants have differential expression of enzymes involved in tryptophan-derived secondary metabolism.

qRT-PCR analysis of transcripts involved in production of **(a)** camalexin (*PAD3*), **(b)** camalexin/indole glucosinolates (*CYP79B2*), and **(c-d)** indole glucosinolates (*CYP83B1, CYP81F2*) following 12h aphid infestation. MiRNA mutants (*dcl1*) show greater induction of *PAD3* and *CYP81F2* compared to Col-0 and *dcl2/3/4* and also have increased basal expression of *CYP79B2* and *CYP83B1*. 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 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 ten plants of each genotype from two experiments. Letters indicate differences at $p<0.05$ as determined by ANODE.

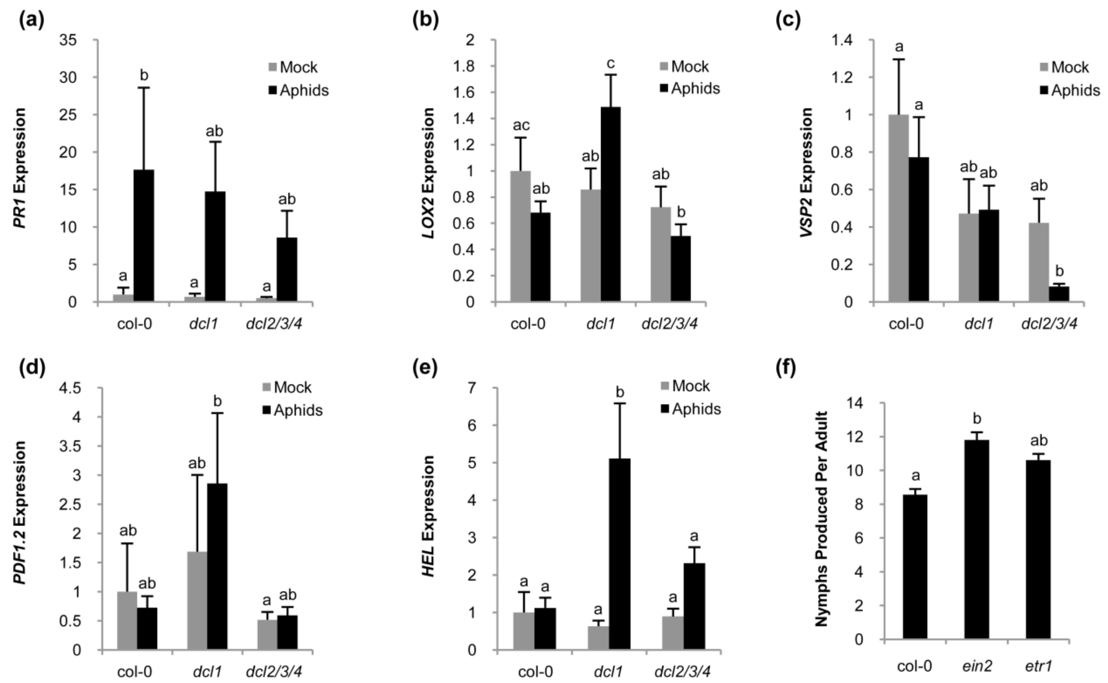

Figure 4.5 - MiRNA mutants have altered expression of genes involved in JA synthesis and ET response.

qRT-PCR analysis of transcripts involved in **(a)** SA (*PR1*), **(b,c)** JA (*LOX2*, *VSP2*), **(d)** JA/ET (*PDF1.2*) and **(e)** ET (*HEL*) pathways following 12h aphid infestation. Expression of *LOX2* and *HEL* are 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 GLM. **(f)** Aphid fecundity is increased on ethylene-insensitive *ein2* mutants. Bars represent the mean (±SE) of ten plants of each genotype from two experiments. Experiment was repeated with similar results. Letters indicate differences at $p<0.05$ as determined by ANODE.

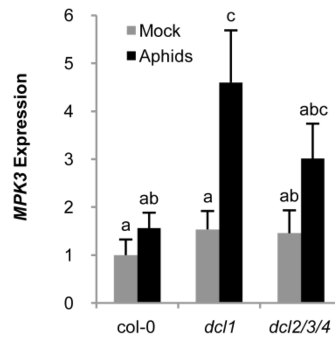

Figure 4.6 – Defence-related gene *MPK3* is induced by aphid exposure.

MPK3 is more highly induced in aphid-exposed *dcl1* compared to Col-0 and *dcl2/3/4*, although the increase is only statistically significant between Col-0 and *dcl1*. 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 GLM.

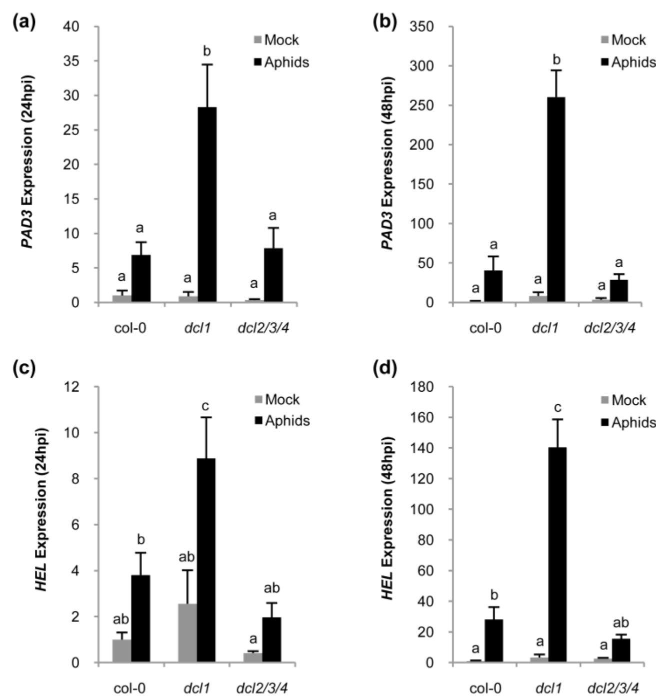

Figure 4.7 – Induction of *PAD3* and *HEL* at 24-hpi and 48-hpi.

Induction of *PAD3* at 24hpi **(a)** and 48hpi **(b)** is highest in *dcl1* compared to Col-0 and *dcl2/3/4*. Induction of *HEL* at 24hpi **(c)** and 48hpi **(d)** is also significantly higher in aphid-exposed *dcl1* relative to Col-0 and *dcl2/3/4*.

Some auxin-related transcripts respond to aphid infestation but are not differentially regulated in silencing pathway mutants

Because camalexin synthesis (mediated by PAD3) is a branch of the auxin synthesis pathway, I investigated other genes involved in auxin signalling (Fig. 4.8). Furthermore, the auxin and SA pathways can act antagonistically during infection with *P. syringae* (Bari & Jones, 2009). Two targets (*TIR1*, *BDL-IAA12*) have been used to assess auxin response with relevance to miRNA-based resistance against *P. syringae* (Navarro et al., 2006), whilst *At4g12980* is a putative auxin-responsive gene that is highly repressed following 48h aphid infestation (de Vos *et al.*, 2005). In our analysis, *TIR1* is expressed at a slightly higher basal level in *dcl1* relative to Col-0 and *dcl2/3/4* (Fig. 4.8a), although this was not statistically significant. *TIR1* was similarly repressed in all lines following aphid exposure. *BDL-IAA12* was similarly expressed in all lines both in the presence and absence of aphids (Fig. 4.8b). Therefore this gene appears to play no role in aphid defence. *At4g12980* was repressed in Col-0 and *dcl1* during aphid exposure although this change in expression was only significantly different for *dcl1* compared to non-exposed *dcl1* mutants, aphid-exposed wild type plants and non- and aphid-exposed *dcl2/3/4* mutants (Fig. 4.8c). These data indicates that similar to previous studies, some genes involved in auxin signalling are aphid-responsive. Although how the auxin pathway helps regulate resistance to *M. persicae* needs further investigation.

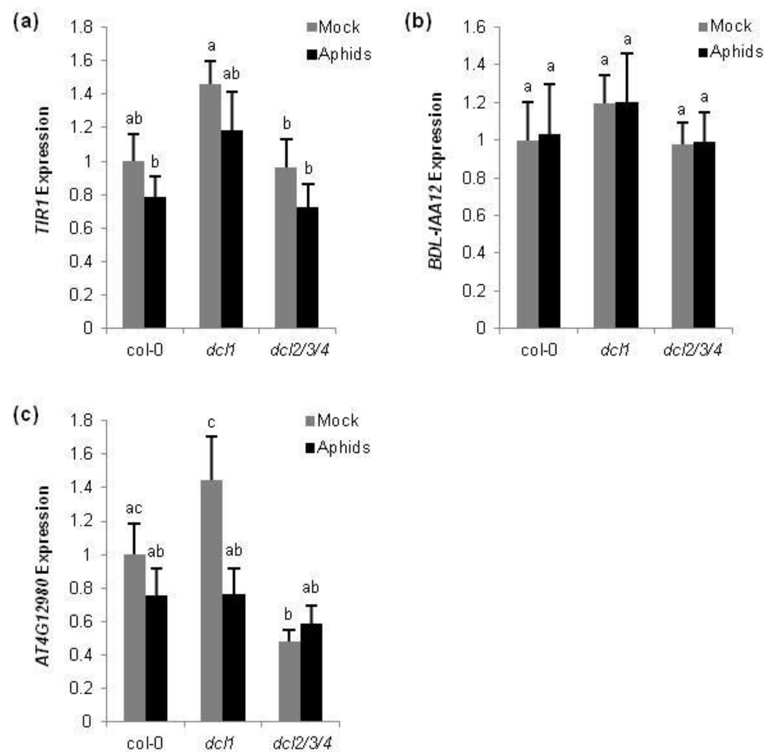

Figure 4.8 – Regulation of auxin-related transcripts during *M. persicae* infestation.

qRT-PCR analysis of transcripts involved in auxin signalling **(a)** *TIR1*, **(b)** *BDL-IAA12* and auxin response **(c)** *At4g12980* following 12h aphid infestation. *TIR1* and *At4g12980* are repressed during aphid treatment but the magnitude of repression was not different between any line tested. *BDL-IAA12* is not aphid reponsive nor differentially expressed between Col-0, *dcl1* 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 GLM.

M. persicae fecundity is increased on *pad3* mutants but not on *cyp81f2* plants

Results so far indicated that *PAD3* expression is specifically induced in aphid-exposed *dcl1* plants. Hence, PAD3 involvement in aphid resistance was further investigated. The cytochrome P450 PAD3 catalyzes the conversion of dihydrocamalexic acid to camalexin, the major *Arabidopsis* phytoalexin (Schuhegger *et al.*, 2006). CYP79B2 acts upstream of

the glucosinolate and camalexin pathways (Zhao *et al.*, 2002), and CYP81F2 is involved in a downstream part of the indolic glucosinolate pathway and acts parallel to camalexin syntheses (Pfalz *et al.*, 2009). Both are involved in aphid resistance. To investigate the contribution of PAD3 compared to CYP81F2 and CYP79B2, the *pad3* (camalexin-deficient), *cyp79b2/cyp79b3* (camalexin and indole glucosinolate-deficient) and *cyp81f2* (aphid-relevant glucosinolate-deficient) mutants were exposed to aphids. Aphid fecundity was significantly higher on both *pad3* and *cyp79b2/cyp79b3* mutants compared to Col-0 (ANODE; $p<0.05$, $n=10$) (Fig. 4.4f). However, aphid fecundity was not significantly different on *cyp79b2/cyp79b3* plants compared to *pad3*. Moreover, aphid fecundity was not significantly increased on the *cyp81f2* mutant relative to Col-0 (Fig. 4.4g). Together this indicates that the additional blocking of glucosinolate production had little additive effect on aphid resistance when the camalexin pathway was disabled. The finding that aphid performance was increased on *pad3* mutants is consistent with the induction of *PAD3* expression in aphid-exposed *dcl1* plants. Thus, under the experimental conditions used in these fecundity experiments, camalexin plays a more substantial role than indolic glucosinolates 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

My qRT-PCR data indicated that in *dcl1* plants, the JA pathway transcript *LOX2* is induced following aphid infestation (Fig. 4.5b). This is in contrast to aphid-infested Col-0 and *dcl2/3/4* where this transcript is repressed. Thus, JA signalling may be involved in miRNA mutant resistance. To assess this possibility, I exposed plants defective in JA signalling (*coi1*, *jar1*, 35S::*LOX2*) to aphids. Aphid fecundity was slightly increased on these lines

relative to controls (Fig. 4.9b) however the increase was not statistically significant. Therefore, whilst *dcl1* plants exhibit differential regulation of the JA pathway relative to Col-0 and *dcl2/3/4*, this pathway does not affect plant resistance to aphids in wild type Col-0. Aphid fecundity was also not significantly increased on plants deficient in SA signalling (*npr1*, *sid2*) relative to Col-0 (Fig. 4.9a). Thus, the SA pathway does contribute to the aphid resistance phenotype of wild-type plants either. It remains to be investigated if the JA and SA pathways contribute to resistance in the *dcl1* background.

As *dcl1* plants show increased induction of the ET-responsive *HEL* transcript following infestation (Fig. 4.5e 4.7c,d), I investigated whether ethylene signalling affects aphid performance by assessing aphid performance on the ethylene-insensitive *etr1-1* and *ein2-5* mutants. Aphid fecundity was significantly higher on *ein2* plants relative to Col-0 (ANODE; $p<0.05$, $n=10$) (Fig. 4.5f) but was not significantly changed on *etr1*.

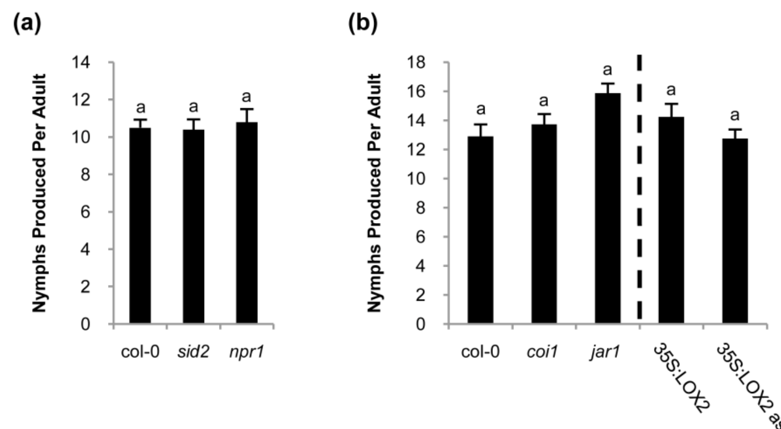

Figure 4.9 – Aphid fecundity assays on SA and JA pathway mutants.

(a) Aphid performance is unchanged on plants deficient in SA production (*sid2*) and signalling (*npr1*) compared to Col-0. **(b)** Aphid performance was equal on plants deficient in JA biosynthesis (*jar1*, 35S:LOX2) and perception (*coi1*). 35S:LOX2 antisense (as) plants served as a negative control for 35S:LOX2. Bars represent the mean (±SE) from ten plants per genotype. The experiments were conducted twice and the results combined for statistical analysis by ANODE (GenStat).

Camalexin accumulation is increased in miRNA pathway mutants

To assess whether increased *PAD3* expression in *dcl1* plants led to increased levels of camalexin, I exposed plants to 24h aphid infestation and measured camalexin content by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). I found that camalexin was present in similarly small quantities in Col-0, *dcl1* and *dcl2/3/4* plants without aphid challenge (Fig. 4.10a). Aphid-exposed *dcl1* plants showed noticeable camalexin accumulation whereas there was no significant accumulation observed in Col-0 or *dcl2/3/4* (Fig. 4.10a). This result indicates that elevated levels of *PAD3* expression correlate with increased amounts of camalexin.

Although I observed increased build-up of camalexin in aphid-infested *dcl1* relative to aphid-infested Col-0 and *dcl2/3/4*, the quantities measured were much lower than those previously found in plants challenged with bacterial or fungal pathogens. To better assess the absolute quantities of camalexin that accumulate in response to aphids, I used clipcages to confine aphids to single leaves for 48h. Single leaves were then harvested and processed as for the whole plant samples. Unfortunately, data from this experiment was not yet available for inclusion in this investigation. However, I predict that a similar pattern of camalexin content will be found in single leaf samples as for whole plant samples.

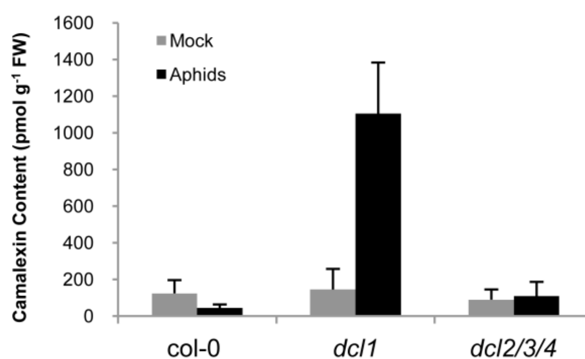

Figure 4.10 – Camalexin accumulates in aphid-exposed *dcl1* plants.

HP-LC (+MS) analysis of mock and aphid-infested Col-0, *dcl1* and *dcl2/3/4* indicates that *dcl1* accumulates more camalexin when exposed to aphids than Col-0 and *dcl2/3/4*. Bars represent the mean amount of camalexin (±SE) detected in whole plants exposed to 30 *M. persicae.* Experiments contained 4 biological replicates of each treatment and the experiment was conducted twice.

PAD3 induction is highly localised during *M. persicae* infestation

It is known that camalexin build up is highly localised to the site of attack by some necrotrophic pathogens (Kliebenstein *et al.*, 2005). I suspected this was also true during aphid infestation. To assess this, single leaves of plants expressing a PAD3p:GUS transgene were exposed to 48h aphid infestation. Leaves were harvested and infiltrated with X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) to detect areas of β-glucuronidase (GUS) production. GUS staining was absent in non-treated plants (Fig. 4.11a). Leaves exposed to spores of the necrotrophic fungus *Botrytis cinerea* showed GUS staining in a characteristic circular pattern surrounding the edge of the *Botrytis* lesion (Kliebenstein *et al.*, 2005) (Fig. 4.11b). GUS staining was also observed in leaves exposed to *M. persicae*, although the magnitude and pattern of staining differed considerably from *Botrytis*-exposed leaves. The staining patterns on aphid-exposed leaves were much less uniform than for *Botrytis*, and occurred mainly at aphid stylets penetration sites, but

resulted in one of three principle outcomes (Fig. 4.11c-h). First, in the majority of feeding sites, GUS staining was observed in small patches around sites of stylets penetrations (Fig. 4.11e,f). Secondly, in a smaller proportion of feeding sites, stylets tracks were observed without any GUS staining (Fig. 4.11c,d), indicating that aphids had either abandoned probing at this site, or had established a successful feeding site without activating a defence response involving *PAD3* induction. Thirdly, on some leaves, GUS staining was observed in an extremely localised fashion (Fig. 4.11g,h), appearing confined solely to the vasculature tissue at a site where feeding had been attempted. These data suggest that *PAD3* is expressed in the vasculature and raises the possible that camalexin is present in the phloem stream and is ingested by aphids when they feed.

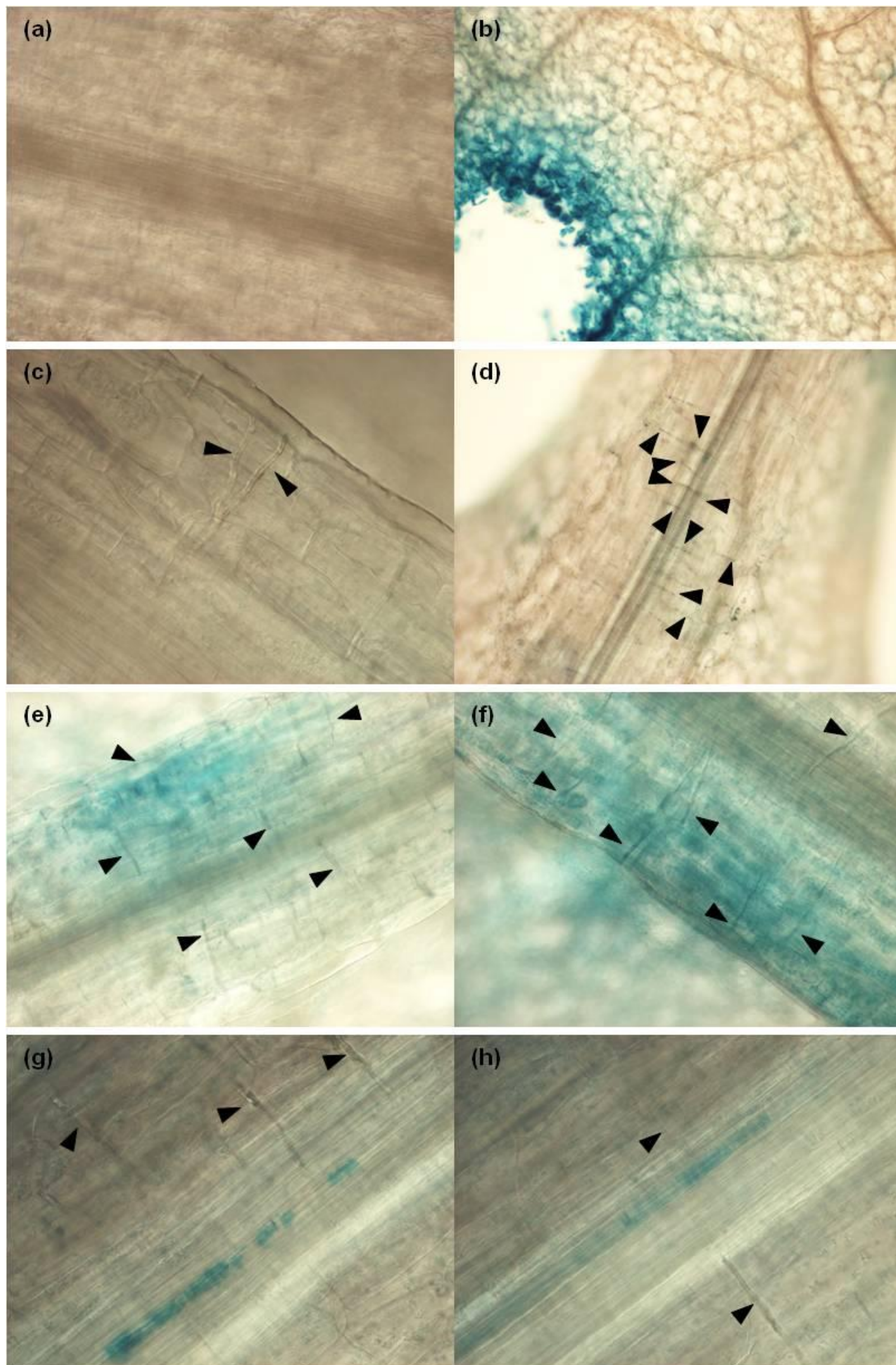

Figure 4.11 – *PAD3* induction by *M. persicae* feeding is localised.

Transgenic *Arabidopsis* (PAD3p:GUS) rosette leaves were exposed to either no treatment (a), *Botrytis cinerea* (B05.10) (b) or *M. persicae* (c-h) for 48h. Aphid feeding generally elicited three patterns of PAD3p induction. (i) Feeding sites with no PAD3p induction (c,d), (ii) Feeding sites with considerable localised PAD3p induction (e,f) and (iii) Feeding sites with highly localised PADp3 induction confined to the vasculature (g,h). Aphid stylet tracks are indicated with black arrows.

Camalexin inhibits adult aphid reproduction but not survival

To determine if camalexin is toxic for aphids, these insects were fed camalexin via an artificial diet. Ten adult aphids were transferred to parafilm sachet feeders containing a complex artificial diet previously used to examine aphid performance (Kim & Jander, 2007). Following two days feeding, the numbers of remaining live adults were recorded as adult survival, and the total number of nymphs produced was recorded as fecundity. I found that at all camalexin concentrations tested, fecundity was significantly reduced compared to both diet-only (Diet) and DMSO (0.1%) controls (t-probabilities within GLM; $p<0.01$, $n=10$) (Fig. 4.12a). The degree of fecundity loss observed correlated closely with increasing camalexin dosage. In contrast, I found that adult survival was unchanged at all camalexin doses relative to diet-only control (Fig. 4.12b). However at camalexin doses of 62.5μM and 500μM, adult survival was significantly lower than the DMSO control (t-probabilities within GLM; $p<0.05$, $n=10$) (Fig. 4.12b). Thus, camalexin affects the reproductive success of aphids but does not appear to reduce aphid survival.

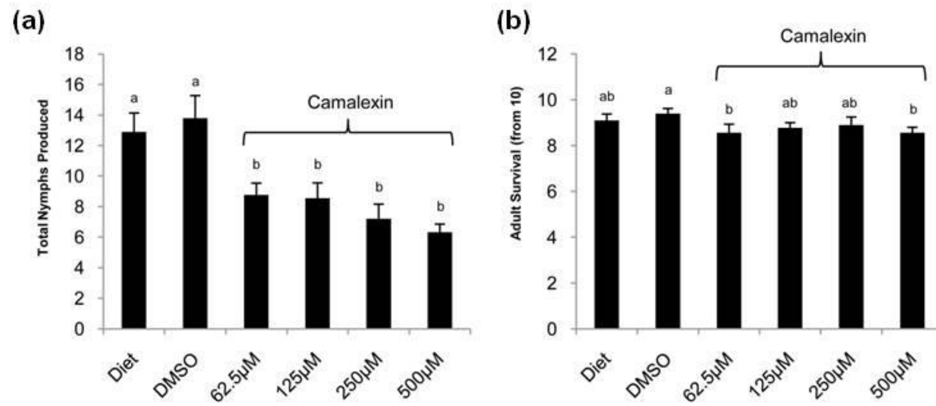

Figure 4.12 – Camalexin affects aphid reproductive development when fed by artificial diet.

(a) Feeding camalexin by artificial diet retards aphid fecundity. **(b)** Survival of adult insects was not affected at any concentration tested. DMSO (0.1%) served as a negative control. Each experiment contained five feeders at each condition. Bars represent the mean number of nymphs produced (a) or surviving adults (b) (±SE) from two independent experiments. Letters indicate differences at $p<0.05$ as determined by t-probabilities within a GLM.

Camalexin does not alter *B. aphidicola* numbers in aphids

To assess if camalexin alters the population of the bacterial endosymbiont *B. aphidicola*, I raised populations of aphids on plants of various abilities to produce camalexin (Col-0, *dcl1*, *dcl2/3/4* and *pad3*) for seven days. In addition, after five days, some Col-0 raised aphids were transferred to camalexin-free artificial diets and artificial diets containing 500µM camalexin for the final two days of the experiment. Genomic DNA was subsequently extracted from all aphids and endosymbiont populations were quantified by qPCR. In this experiment, relative populations of *B. aphidicola* were similar across all treatments (Fig. 4.13). Thus, camalexin does not impact the absolute number of the aphid primary endosymbiont.

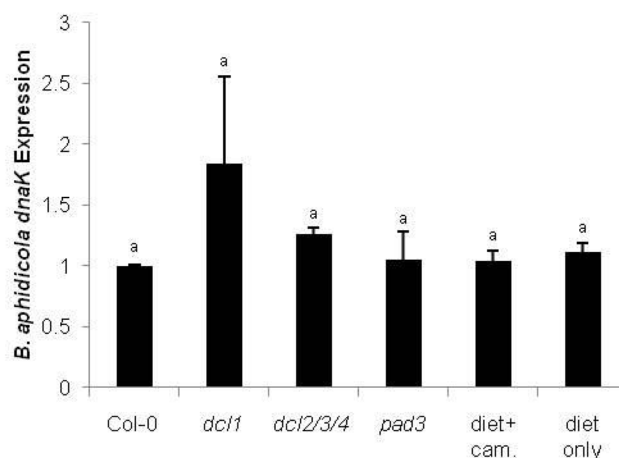

Figure 4.13 – Camalexin does not alter *B. aphidicola* populations.

Aphids were raised for seven days on the camalexin pathway mutant *pad3*, the low camalexin producers Col-0 and *dcl2/3/4*, and the high camalexin producer *dcl1*. After five days, some Col-0 raised aphids were transferred to camalexin-free diet (diet only) and 500µM camalexin-spiked diet (diet+cam.) for the final two days. *B. aphidicola* populations were examined by qPCR. Differences were assessed by calculating t-probabilities within the GLM. The experiment was conducted once and contained three biological replicates of each treatment.

4.3 Discussion

The data shows that *dcl1* plants display greater resistance to *M. persicae* infestation, and that this is in part due to hyper-activation of the camalexin defence pathway. In contrast, this pathway is only modestly induced in Col-0 and *dcl2/3/4* plants. One possibility is that factors that act as brakes or suppressors of defence hyper-activation in Col-0 or *dcl2/3/4* are ineffective or absent in *dcl1* plants. Suppressors of hyper-activation 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 management of this pathway may be targets for as yet

uncharacterised 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 high 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) are specifically related to auxin signalling (Zhang, W *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, W *et al.*, 2011). It has recently been reported that miR393 also has a role in resource allocation between the glucosinolate and camalexin pathways (Robert Seilaniantz *et al.*, 2011). It is possible that this group of miRNAs play important roles in aphid resistance.

Dcl1 plants are able to induce the JA biosynthetic gene *LOX2* following aphid attack, unlike Col-0 and *dcl2/3/4* where this transcript is repressed following aphid exposure. *LOX2* expression is regulated by TCP transcription factors which in turn are governed by miR319 (Schommer *et al.*, 2008). An absence or reduced quantity of functional miR319 may afford hosts a level of JA pathway induction when normally this is repressed. However the role of this mechanism in aphid resistance is unlikely to be major, as our fecundity assays indicate that aphids are not significantly more successful on plants deficient in this signalling pathway.

My qRT-PCR assays indicated that aphid-resistant *dcl1* plants increase transcription of an ET-responsive gene compared to susceptible Col-0 and *dcl2/3/4* plants following aphid colonisation. Fecundity assays confirmed 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, where performance of *M. persicae* and *B. brassicae* was either unaffected or reduced on *etr1* mutants (Mewis *et al.*, 2005; Mewis *et al.*, 2006). Other laboratories demonstrated that saliva-induced aphid resistance is independent of EIN2 and ethylene signalling (de Vos & Jander, 2009) while EIN2 is known to be critical for resistance to *M. persicae* 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 is increased on the *pad3* and *cyp79b2/cyp79b3* mutants relative to Col-0. In contrast, aphid performance was unchanged on the *cyp81f2* mutant. Taken together, these results indicate that under our experimental conditions, production of camalexin rather than indole glucosinolates is the major resistance factor. As both *PAD3* and camalexin production are highly inducible in *dcl1* plants, the data suggests that this defence pathway contributes substantially to the miRNA mutant resistance phenotype.

The finding that PAD3 is involved in aphid resistance is in contrast to Pegadaraju *et al.* who found no statistically significant increase in *M. persicae* colonisation ability on *pad3* mutants (Pegadaraju *et al.*, 2005). In addition, Kim and coworkers found no change in fecundity of aphids raised on *cyp79b2/cyp79b3* mutants relative to wild-type plants (Kim *et al.*, 2008). However, in both cases non-aged aphids were exposed to the mutant plants for a relatively short period, i.e. 2 to 5 days, whereas in the experiments reported herein the nymphs were born on the mutant plants and reared on these plants to adulthood (c. 16 days) during the course of which they began producing nymphs themselves. Thus,

differences in experimental procedures may account for the different outcomes. Indeed, I previously showed that the *dcl1* resistance phenotype is absent when experiments were carried out following a previously published protocol (Pegadaraju *et al.*, 2005). Our results are in agreement with those of Kuśnierczyk and coworkers (Kuśnierczyk *et al.*, 2008), who found that *B. brassicae* (cabbage aphid) is more successful on *pad3* relative to wild-type *Arabidopsis* when both plants are pre-treated with UV light to induce camalexin production. In these experiments, aged nymphs were raised on test plants for 13 days, a protocol very similar to our own assay. Furthermore, aphids produce less progeny on artificial diets containing camalexin compared to control diets, confirming that camalexin negatively impacts *M. persicae* performance. This indicates an unsuspected depth to camalexin function beyond antifungal and antibacterial defence. This work also highlights the extensive role of miRNA-mediated regulation of secondary metabolic defence pathways with relevance to resistance against an aphid pest.

Chapter 5 – Regulation of the camalexin biosynthetic pathway in *Arabidopsis*

5.1 Introduction

I previously showed that *Arabidopsis* plants deficient in miRNA processing are more resistant to infestation by *M. persicae* (Chapter 3), and that this resistance is at least partly due to these plants ability to overproduce camalexin during aphid exposure (Chapter 4). We confirmed that camalexin plays a role in aphid antibiosis as aphids fed camalexin by artificial diet produced less progeny than aphids fed on camalexin-free control diets (Chapter 4).

Production of camalexin is induced by a wide variety of plant attackers that includes bacteria, fungi, viruses, oomycetes and insects (Glawischnig, 2007). However, not all these attackers are susceptible to this phytoalexin. There is evidence that camalexin increases the permeability of fungal membranes and causes induction of genes involved in membrane stress and repair (Sellam *et al.*, 2007; Joubert *et al.*, 2011). Some necrotrophic fungi have evolved means of exporting camalexin from cells (Stefanato *et al.*, 2009), suggesting that increased virulence is attained by exporting camalexin or neutralising its effects. In contrast to the glucosinolate pathway, which can be considered a pre-formed defence, camalexin is only present in minute quantities in the absence of challenge (Glawischnig, 2007). Upon pathogen or pest perception, there is transcriptional activation of the camalexin biosynthetic genes and subsequent camalexin accumulation.

Camalexin production involves transcriptional induction of the biosynthetic genes (*PAD3*, *CYP71A13*) and is regulated by two mitogen-activated protein kinase (MAPK) cascades (Qiu *et al.*, 2008; Ren *et al.*, 2008; Mao *et al.*, 2011). The MPK4 cascade is responsive to the PAMP flg22 or the bacterial biotroph *P. syringae* (Qiu et al., 2008). The MPK3/6 pathway is activated during exposure to the fungal necrotroph *B. cinerea* (Ren et al., 2008; Mao et al., 2011). Intriguingly, both cascades converge on the WRKY transcription factor WRKY33, suggesting that this transcription factor is particularly

important in activating camalexin production. In the MPK4 pathway, MPK4 exists in a nuclear complex with mitogen-activated protein kinase 4 substrate 1 (MKS1) and WRKY33. Treatment with flagellin or inoculation with *P. syringae* results in MPK4 activation. Once activated, MPK4 phosphorylates MKS1 resulting in the release of both MKS1 and WRKY33 (Qiu *et al.*, 2008). WRKY33 is then free to activate transcription of W-box element-containing promoters which include the camalexin biosynthetic gene *PAD3* (Qiu *et al.*, 2008). In response to *B. cinerea*, it was found that activation of the MPK3/MPK6 pathway was required for camalexin accumulation (Ren *et al.*, 2008). Further work revealed that WRKY33 is a target for phosphorylation by MPK3/MPK6 and that WRKY33 binds its own promoter in a potential positive feedback regulatory loop (Mao *et al.*, 2011). Induction of camalexin production by WRKY33 following *B. cinerea* infection is independent of MPK4. Therefore perception of bacterial and fungal pathogens may trigger two distinct signalling cascades that converge on WRKY33.

It is known that many miRNAs target conserved transcription factor families. The transcription factor *WRKY33* is induced in response to several pathogens (Lippok *et al.*, 2007), and appears particularly important for camalexin production. As work described in previous chapters indicated that camalexin is important in aphid resistance, I speculated that WRKY33 may also be important in camalexin production during aphid attack.

5.2 Results

Aphid fecundity in increased on *wrky33* mutants and restored to wild-type levels on 35S:WRKY33

WRKY33 is known to positively regulate pathogen-responsive genes including *PAD3* (Petersen *et al.*, 2008; Qiu *et al.*, 2008). It is therefore important in defence against pathogens where camalexin production is an effective resistance mechanism. To test the importance of WRKY33 in *M. persicae* resistance, I tested aphid performance on both the *wrky33-1* mutant, and a transgenic line expressing the 35S:WRKY33 construct in a *wrky33-1* background (Zheng *et al.*, 2006). I found that aphid fecundity was significantly increased on the *wrky33* mutant relative to Col-0 (Fig. 5.1) (ANODE; **p<0.01, n=15*) and was returned to wild-type levels on 35S:WRKY33-expressing plants (Fig. 5.1). This data indicates that WRKY33 is important in aphid resistance in *Arabidopsis*, perhaps through its known role in regulating camalexin production by PAD3.

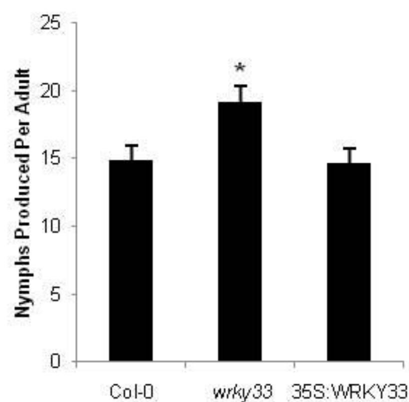

Figure 5.1 – WRKY33 is involved in aphid resistance.

Aphid performance is increased on *wrky33* relative to Col-0. Fecundity is returned to wild-type levels on plants expressing 35S:WRKY33 in a *wrky33* background. Bars represent the mean (±SE) of fifteen plants of each genotype from three experiments. Asterisk indicates differences at p<0.01 as determined by ANODE.

***WRKY33* is differentially regulated between wild-type and silencing-deficient plants during aphid infestation**

To investigate how *WRKY33* is regulated during *M. persicae* infestation, I conducted qRT-PCR experiments using RNA samples generated from mock and aphid-exposed (12h) Col-0, *dcl1* and *dcl2/3/4* plants. I found that at this timepoint, levels of *WRKY33* transcript in mock-treated samples were similar (Fig. 5.2). However, I noticed differential expression of *WRKY33* following aphid exposure. *WRKY33* is repressed slightly during aphid infestation in Col-0, but is significantly induced in both aphid-exposed *dcl1* and *dcl2/3/4* (Fig. 5.2). The fact that *WRKY33* is differentially expressed between Col-0 and *dcl1* during aphid infestation suggests that a DCL1-dependent sRNA is involved in *WRKY33* regulation. Absence of this sRNA in the *dcl1* mutant would account for increased levels of *WRKY33* mRNA. As basal levels of the transcript are similar between Col-0 and *dcl1*, this differential response must only be generated following aphid perception. Surprisingly, *WRKY33* was also induced in aphid-exposed *dcl2/3/4* plants to a level comparable to aphid-exposed *dcl1*. This was not expected, as *dcl2/3/4* mutants have a fully functional miRNA pathway and would be expected to show a response similar to Col-0. This result reveals a further level of complexity to *WRKY33* regulation during the aphid defence response, and suggests that both siRNAs and miRNAs may contribute to the regulation of this transcription factor.

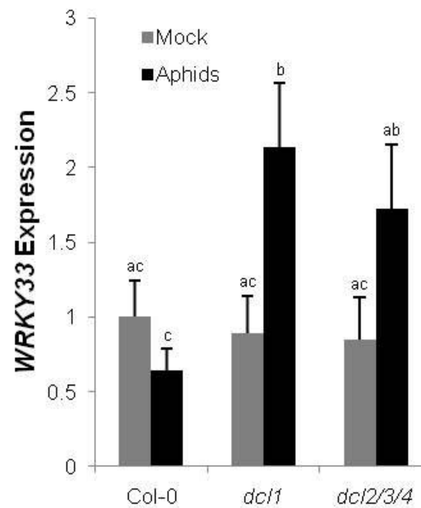

Figure 5.2 – Regulation of *WRKY33* expression in silencing-deficient Arabidopsis.

qRT-PCR analysis of *WRKY33* transcript levels in response to aphid infestation between Col-0, *dcl1* and *dcl2/3/4*. Bars represent the mean expression levels (±SE) across six biological replicates from two experiments. Letters indicate differences at $p<0.05$ as determined by t-probabilities within a GLM.

miR393 is predicted to target *WRKY33*

My results show that plants lacking functional WRKY33 are more susceptible to aphid colonisation, implying that WRKY33 is involved in aphid resistance. *WRKY33* is also differentially regulated between Col-0 and the *dcl1* and *dcl2/3/4* silencing pathway mutants during aphid exposure, suggesting that this transcription factor is under post-transcriptional regulation. To assess whether *WRKY33* mRNA is targeted by *Arabidopsis* miRNAs, I used the psRNATarget analysis server to predict WRKY33-miRNA targeting interactions. This analysis predicted that *WRKY33* mRNA is a target of miR393 and that the interaction likely results in mRNA cleavage due to base-pairing at the central 10-11 positions (Fig. 5.3). I also included the targeting events between miR393 and other experimentally validated targets for comparison (Fig. 5.3). Confidence of *WRKY33* targetting was lower

than for the other three targets (lower Expectation (E) score), however the important positions of 2-8 and 10-11 from the miRNA 5` end were perfect matches. It is also known that in animal systems, RNA secondary structure in the vicinity of a miRNA target site can have an important bearing on the success of targeting (Kertesz *et al.*, 2007). Although it is not known how important this factor is in plant systems, it is worth noting that the program predicted the *WRKY33* target site to be more accessible than for the other confirmed targets (lower Target Accessibility (UPE) score). The other targets of miR393 are genes involved in auxin perception (Navarro *et al.*, 2006). This has an impact on the allocation of resources between the camalexin and indolic glucosinolate pathways (Robert Seilaniantz *et al.*, 2011). It is therefore possible that regulation of *WRKY33* may further explain the role on miR393 in camalexin production.

<table>
<tr><th>miRNA Acc.</th><th>Target Acc.</th><th>Expectation (E)</th><th>Target Accessibility (UPE)</th><th>Alignment</th><th>Target Description</th><th>Inhibition</th><th>Multiplicity</th></tr>
<tr><td>ath-miR393a</td><td>AT3G26810.1</td><td>1.0</td><td>20.043</td><td>miRNA 22 CCUAGUUACGCUAGGGAAACCU 1
: : ::::::::::::::::::
Target 1998 GAAACAAUGCGAUCCCUUUGGA 2019</td><td>| Symbols: AFB2 | AFB2 (AUXIN SIGNALING F-BOX 2); auxin binding / ubiquitin-protein ligase | chr3:9867845-9870640 FORWARD [PFAM]</td><td>Cleavage</td><td>1</td></tr>
<tr><td>ath-miR393a</td><td>AT1G12820.1</td><td>1.0</td><td>21.316</td><td>miRNA 22 CCUAGUUACGCUAGGGAAACCU 1
: : ::::::::::::::::::
Target 1883 GAAACAAUGCGAUCCCUUUGGA 1904</td><td>| Symbols: AFB3 | AFB3 (AUXIN SIGNALING F-BOX 3); auxin binding / ubiquitin-protein ligase | chr1:4368760-4371293 REVERSE [PFAM]</td><td>Cleavage</td><td>1</td></tr>
<tr><td>ath-miR393a</td><td>AT3G62980.1</td><td>2.0</td><td>19.353</td><td>miRNA 20 UAGUUACGCUAGGGAAACCU 1
::::::::::::::::::::
Target 1712 GACAAUGCGAUCCCUUUGGA 1731</td><td>| Symbols: TIR1 | TIR1 (TRANSPORT INHIBITOR RESPONSE 1); auxin binding / protein binding / ubiquitin-protein ligase | chr3:23273116-23276375 REVERSE [PFAM]</td><td>Cleavage</td><td>1</td></tr>
<tr><td>ath-miR393a</td><td>AT2G38470.1</td><td>3.0</td><td>12.87</td><td>miRNA 20 UAGUUACGCUAGGGAAACCU 1
::::.: .:::.:::::::.
Target 960 AUCAGUCUGAUUCCUUUGGG 979</td><td>| Symbols: WRKY33, ATWRKY33 | WRKY33; transcription factor | chr2:16108361-16110766 FORWARD [PFAM] 1196-1372 PF03106.8 WRKY DNA -binding domain;</td><td>Cleavage</td><td>1</td></tr>
</table>

Figure 5.3 – The *WRKY33* transcription factor is a predicted target of miR393.

The psRNATarget program was used to assess predicted targets of *Arabidopsis* miR393 using default settings. In addition to the previously validated targets (*AFB2*, *AFB3*, *TIR1*), the transcription factor *WRKY33* was predicted to be regulated by transcript cleavage. psRNATarget is provided by the Zhao Bioinformatics Laboratory (Samuel Roberts Noble Foundation).

***WRYKY33* mRNA is cleaved at a position distant from the putative miR393 target site**

To assess whether miR393 directs cleavage of the *WRKY33* transcript I used a modified 5`RACE protocol that has previously been used to validate predicted miRNA-target interactions (Wang *et al.*, 2004). In this experiment, I detected a distinct *WRKY33* cleavage product when I used mock-infested leaf cDNA as template (Fig. 5.4a). This product was absent when aphid-infested leaf cDNA was used as template (Fig. 5.4a). As a positive control, a primer pair was used to amplify a fragment of the *PHABULOSA* transcript generated by miR165/166 family cleavage. These reactions produced a similar product irrespective of treatment (Fig. 5.4a). I subsequently cloned and sequenced both the *PHABULOSA* and *WRKY33* fragments. All *PHABULOSA* fragment clones (7/7) terminated at a position corresponding to the 10th nucleotide of miR165/166, as would be expected for canonical miRNA cleavage. All *WRKY33* fragment clones (18/18) terminated at a site ~100nt towards the 5` end of the transcript from the putative miR393 target site (Fig. 5.4b). A prediction of the secondary structure of the central portion (nts 333-1503) of the *WRKY33* transcript was made with RNAfold (Fig. 5.4c), indicating the predicted miR393 target site (red) and the termination site of the cleavage product (black arrow).

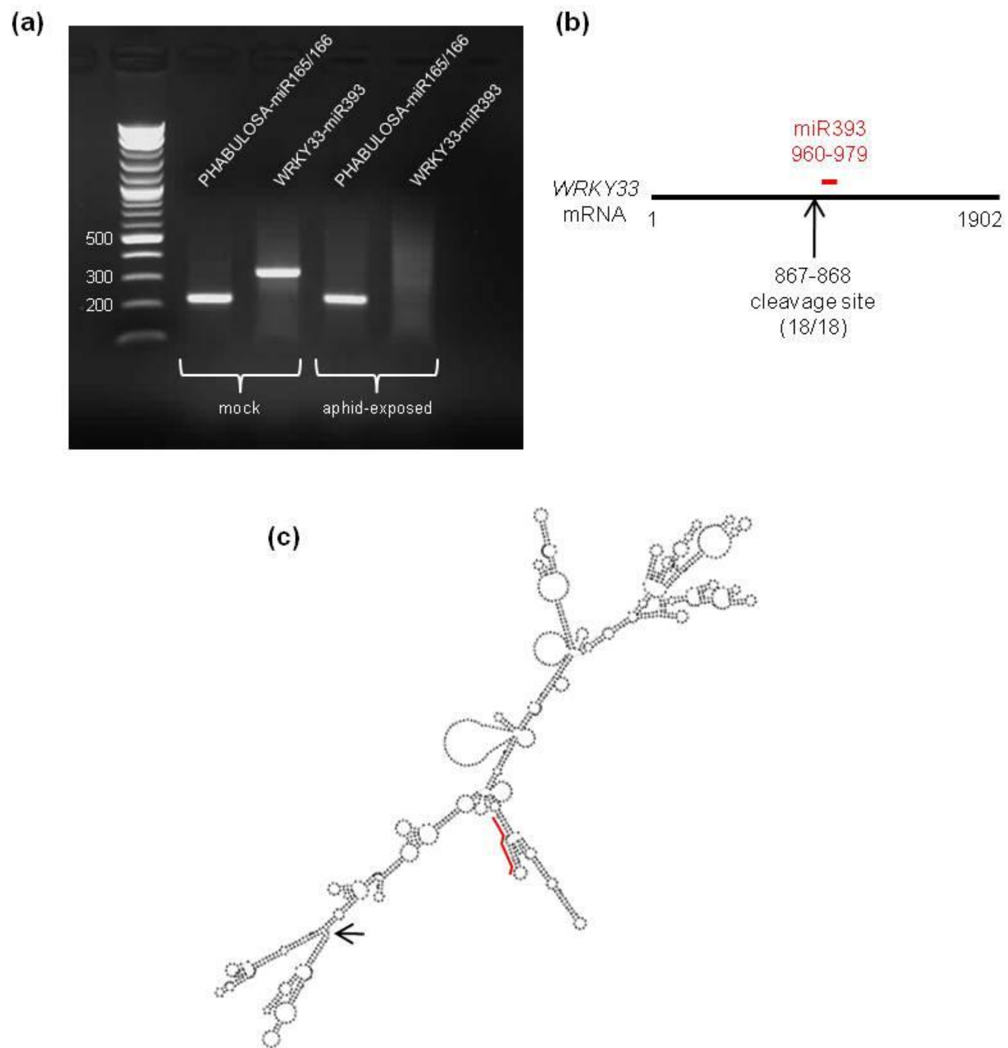

Figure 5.4 – *WRKY33* mRNA is cleaved at a site distinct from miR393 binding.

(a) 5`RACE indicates a *WRKY33* cleavage product of ~320nt in mock-treated cDNA sample but not aphid-exposed cDNA sample. A cleavage product of ~220nt from PHABULOSA-miR165/166 served as a positive control. **(b)** Depiction of the cleavage site and its position relative to the predicted miR393 target site. Numbers in brackets indicate that all sequenced clones terminate at the same nucleotide. **(c)** Secondary structure prediction of central portion (pos.333-1503) of *WRKY33* transcript using the RNAfold program (Institute of Theoretical Chemistry, University of Vienna). Putative miR393 binding site (red) and actual cleavage site (black arrow) are indicated.

Aphid fecundity is increased on miR393 overexpressing *Arabidopsis*

My data indicated that *WRKY33* is under post-transcriptional regulation. A computational prediction suggested that this regulation may involve miR393. However, 5`RACE revealed that *WRKY33* transcript is cleaved at a site ~100nt distant from the predicted miR393 binding site. Nonetheless, to assess whether miR393 expression level influences aphid performance, I obtained two miR393a overexpression lines (35S:miR393a lines 3 and 21) that have previously been used in performance assays of bacterial and fungal pathogens (Navarro *et al.*, 2006; Robert Seilaniantz *et al.*, 2011). I tested aphids in a performance assay using these plants and found that whilst survival was unaffected, aphid fecundity was consistently higher on 35S:miR393 (line 3) (Fig. 5.5a) (ANODE; $p=0.001$, $n=15$) compared to the Col-0 control group. Although fecundity on 35S:miR393 (line 21) was higher than on Col-0, this increase was not statistically significant. This result indicated that there is involvement of miR393 in *Arabidopsis* resistance against *M. persicae*. In the original publication of these lines, miR393 is more highly overexpressed in line 21 compared to line 3 (Navarro *et al.*, 2006). This suggested that there may also be a dose-dependent response involved in miR393-mediated aphid susceptibility. To confirm my initial result, I obtained two miR393b overexpression lines (miR393b OE lines 6 and 11) (Zhang, X *et al.*, 2011). In aphid performance assays, fecundity was significantly increased on line 11 relative to Col-0 (Fig. 5.5b) (ANODE; $p=0.05$, $n=10$). Fecundity was increased slightly on line 6 although this was not statistically significant. When taken together with the previous results using miR393a overexpressing plants, two out of four transgenic lines result in a significant increase in fecundity, indicating involvement of miR393 in aphid resistance.

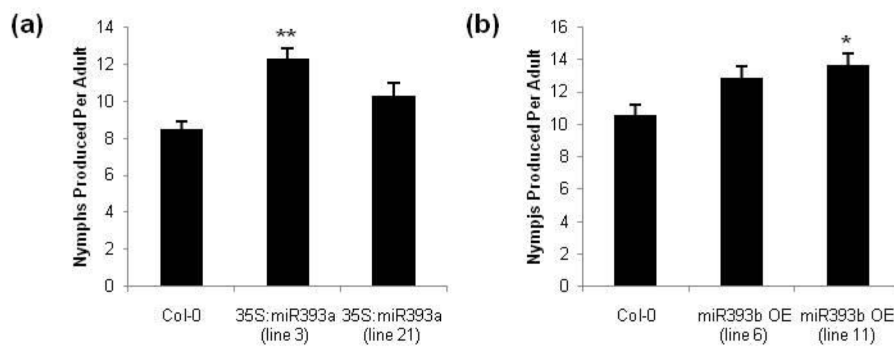

Figure 5.5 – Aphid fecundity in increased on plants overexpressing miR393.

(a) Aphid performance was significantly increased on 35S:miR393a (line 3) relative to Col-0 but is unchanged on 35S:miR393 (line 21). Bars represent the mean (±SE) of fifteen plants of each genotype from three experiments. **(b)** Fecundity was significantly increased on miR393b overexpression line 11 relative to Col-0 but was unchanged on miR393b overexpression line 6. Bars represent the mean (±SE) of ten plants of each genotype from two experiments. Asterisks indicate differences at **$p=0.001$, *$p<0.05$ as determined by ANODE.

miR393b is induced in the vasculature of aphid-exposed leaves

In order to determine whether expression of miR393 is aphid-responsive, I obtained plants expressing either the miR393a or miR393b promoter fused to a GFP reporter (miR393a-p:GFP, miR393b-p:GFP) (Navarro *et al.*, 2006). Single leaves of these plants were exposed to aphid infestation using clipcages, as previously described for qRT-PCR experiments. In this experiment, I could not observe any GFP fluorescence in the midveins of infested leaves, but I did see weak GFP fluorescence in the secondary and tertiary veins of miR393b-p:GFP leaves exposed to aphids (Fig. 5.6b-d). There was no fluorescence visible in mock-infested miR393b-p:GFP leaves (Fig. 5.6a). This provides evidence that miR393 is induced in aphid-exposed leaves, and that this induction may be confined to the vasculature system or cells with access to it. Strikingly, no fluorescence was observed in

either mock or aphid-exposed miR393a-p:GFP leaves. Therefore, aphid-induced miR393 expression therefore appears to operate solely through the miR393b locus.

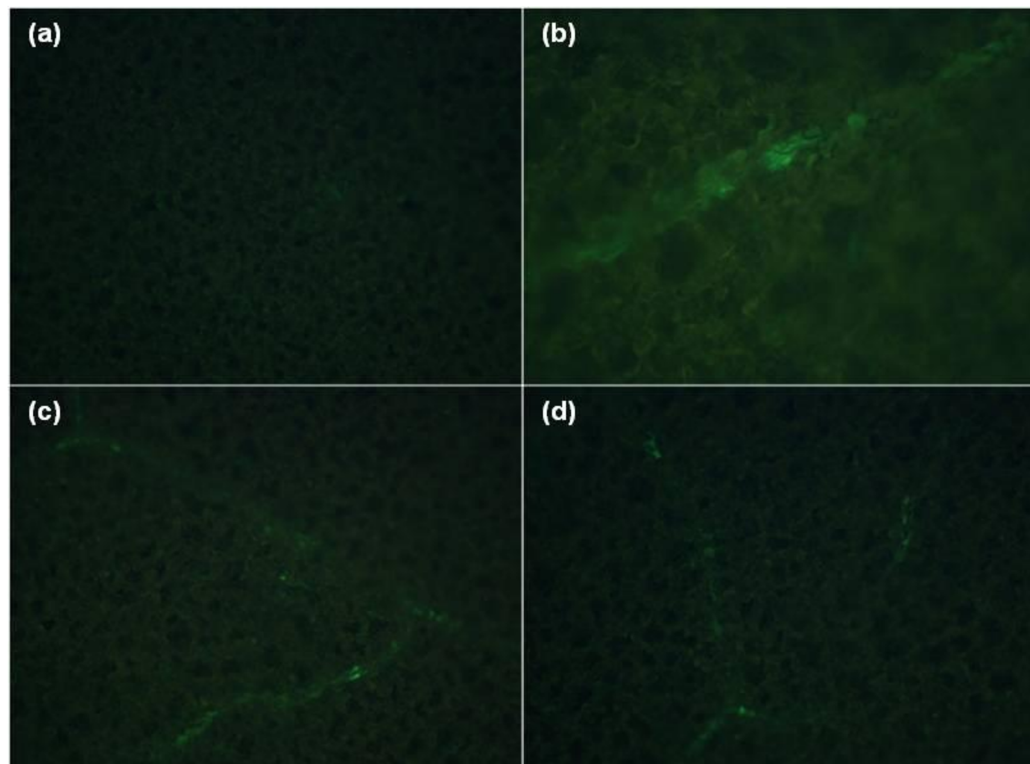

Figure 5.6 – Aphid feeding induces GFP expression in vasculature of miR393b-p:GFP expressing plants.

Transgenic *Arabidopsis* expressing either miR393a-p:GFP or miR393b-p:GFP were exposed to aphids in clipcages. (a) miR393b-p:GFP leaves exposed to empty clipcages were used as controls and we detected no GFP expression. (b-d) Faint GFP expression was observed in secondary and tertiary veins of miR393b-p:GFP leaves exposed to aphids. No GFP expression was observed in either mock or aphid-treated miR393ap:GFP leaves.

Basal *WRKY33* expression is unchanged in 35S:miR393a expressing *Arabidopsis*

I monitored the basal level of *WRKY33* transcription in Col-0 and two miR393a overexpression lines previously used in aphid performance assays (Fig 5.5a). I found that

levels of *WRKY33* transcript are similar across these lines in the absence of aphid challenge (Fig. 5.7). It is likely that there is differential regulation of *WRKY33* between these plants during aphid infestation; however time constraints did not allow for this investigation.

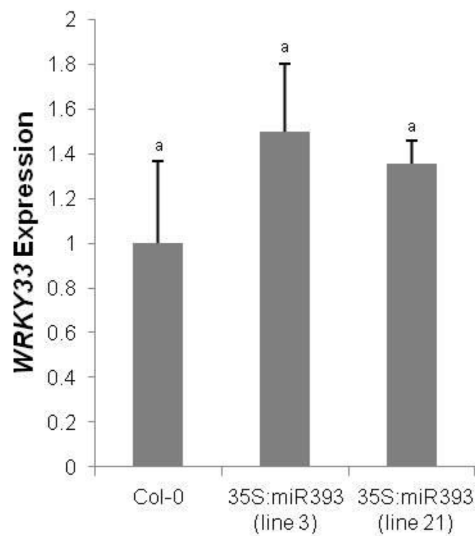

Figure 5.7 – Basal *WRKY33* expression in miR393 overexpressing plants.

qRT-PCR analysis of basal level of *WRKY33* transcript abundance in Col-0 and 35S:miR393a (lines 3 and 21). Bars represent the mean expression levels (±SE) across four biological replicates from a single experiment. Letters indicate differences at $p<0.05$ as determined by t-probabilities within a GLM.

Aphid performance is increased on the auxin mutants *arf1* and *arf9*

One mechanism by which miR393 can regulate the camalexin/glusosinolate pathways is through auxin response factors (ARFs) (Robert Seilaniantz *et al.*, 2011). ARFs are transcription factors that control induction of auxin-responsive genes following auxin treatment. ARF1 and ARF9 are of particular relevance with regard pathogen resistance. The mutants *arf1* and *arf9* are impaired to varying degrees in ability to synthesise

camalexin following *P. syringae* inoculation, and have increased basal levels of some types of glucosinolates (Robert Seilaniantz *et al.*, 2011). I decided to test the *arf1* and *arf9* mutants in an aphid performance assay to assess whether an altered camalexin/glucosinolate balance due to interference with auxin responsiveness would have an impact on the success of aphid colonisation. In these assays, aphid fecundity was increased on both *arf1* and *arf9* compared to Col-0 (Fig. 5.8) (ANODE; *$p<0.05$ **$p<0.01$, $n=10$*). This indicates that interference with auxin signalling can have knock-on effects on other defence pathways that have impact on aphid reproduction.

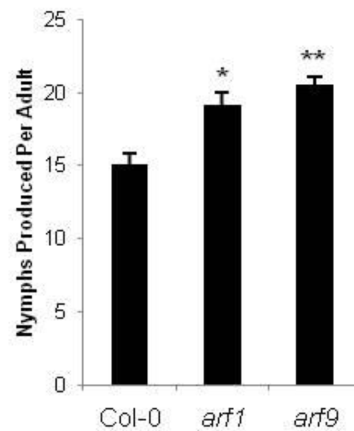

Figure 5.8 – ARF1 and ARF9 are involved in *M. persicae* resistance.

Aphid performance is similarly increased on the auxin pathway mutants *arf1* and *arf9* relative to Col-0. Bars represent the mean (±SE) of ten plants of each genotype from two experiments. Asterisks indicate difference from Col-0 at *$p<0.05$ and **$p<0.01$ as determined by ANODE.

Interaction between miR393 and *PAD3*

I previously investigated if *WRYK33* mRNA was a direct target of the miRNA miR393. Results indicated that *WRKY33* is likely a target for sRNA-mediated regulation, however this appears complex and may involve other factors besides miR393. I next wondered

whether *PAD3* may also be under some degree of sRNA-mediated regulation. I used psRNATarget to predict possible interactions between known miRNAs and *PAD3* mRNA. This analysis revealed that miR393 may also regulate *PAD3* by transcript cleavage (Fig. 5.9). To confirm this interaction, I again conducted 5`RACE using primers specific for *PAD3* transcripts. This experiment revealed that *PAD3* transcripts are cleaved to produce two major cleavage products. This only occurs during aphid infestation and not in mock-infested plants (Fig. 5.10a). Sequencing of the cleavage products revealed that they terminate at positions towards the 3` end of the *PAD3* transcript from the predicted miR393 target site (Fig. 5.10b).

miRNA Acc.	Target Acc.	Expectation (E)	Target Accessibility (UPE)	Alignment	Target Description	Inhibition	Multiplicity
ath-miR393a	PAD3	4.5	22.557	miRNA 20 UAGUUACGCUAGGGAAACCU 1 :: :.::.:::..::::: : Target 608 AUGAGUGUGAUUUCUUUGAA 627		Cleavage	1
ath-miR393b	PAD3	4.5	22.557	miRNA 20 UAGUUACGCUAGGGAAACCU 1 :: :.::.:::..::::: : Target 608 AUGAGUGUGAUUUCUUUGAA 627		Cleavage	1

Figure 5.9 – Predicted interaction between miR393 and *PAD3* mRNA.

psRNATarget prediction indicated that *PAD3* is a weak target for miR393 regulation. For this prediction, the maximum expectation threshold was set to 5.0, where 3.0 is the default. This allowed for display of miRNA-target interactions with lesser complementarity.

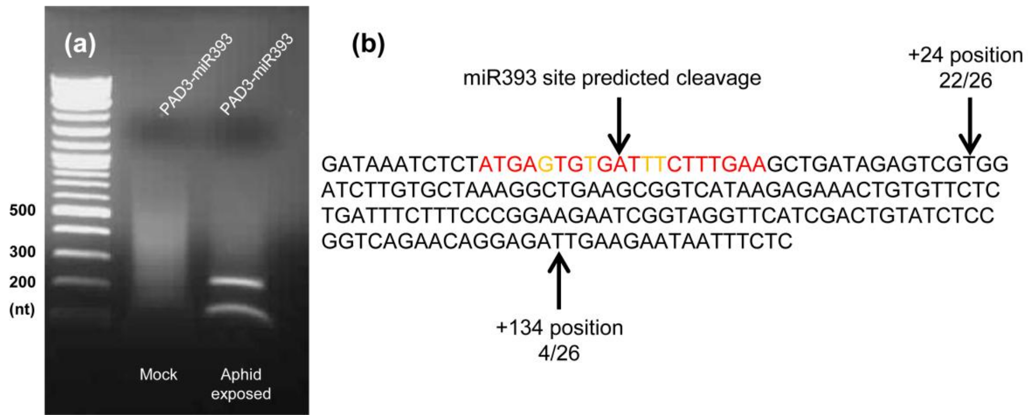

Figure 5.10 – *PAD3* mRNA is cleaved at sites distinct from miR393 binding during aphid infestation.

(a) 5`RACE indicates *PAD3* cleavage products of ~210nt and ~100nt in aphid-exposed cDNA sample but not mock-treated cDNA sample. **(b)** Depiction of the cleavage sites and their position relative to the predicted miR393 target site. +24 and +134 refer to number of nucleotides towards 3` end of *PAD3* transcript relative to predicted miR393 cleavage site. 22/26 and 4/26 refer to the number of colonies that showed termination at these positions.

5.3 Discussion

In this work, I set out to explore the regulatory mechanisms which control camalexin production in response to *M. persicae* infestation. I focussed on the WRKY33 transcription factor, as it is required for transcriptional induction of the camalexin biosynthetic machinery in responses against both bacterial and fungal pathogens. I found that WRKY33 is also involved in aphid resistance, as *M. persicae* are more fecund on *wrky33* mutants relative to Col-0. I continued to show that *WRKY33* is differentially regulated in silencing-deficient plants relative to Col-0 during aphid infestation, implying that this transcription factor is under some degree of post-transcriptional control. A prediction of miRNA-mRNA interactions suggested that *WRKY33* is a target for miR393. However, 5`RACE indicated that *WRKY33* mRNA is cleaved at a position distant from miR393 binding. In addition, miR393 was also predicted to target the camalexin biosynthetic gene *PAD3*. 5`RACE indicated that *PAD3* mRNA is cleaved at postions 3` of the predicted miR393 binding site. Nonetheless, as miR393b is induced during aphid exposure and aphids have increased fecundity on 35S:miR393 expressing plants, it suggests that miR393 plays some role in regulating camalexin production during aphid attack.

MiR393 regulates auxin perception through its targeting of the F-box gene transcripts *AFB2*, *AFB3* and *TIR1* (Navarro et al., 2006). It is also important for regulating metabolite flow through the glucosinolate and camalexin pathways during fungal and bacterial pathogenesis. It has been proposed by Robert-Seilaniantz and colleagues that this is primarily achieved through the activity of ARF1 and ARF9 (Robert Seilaniantz *et al.*, 2011). In responses to pathogens, the auxin and SA pathways act antagonistically. By interfering with auxin responses, pathogens can manipulate the extent to which SA can increase host resistance (Bari & Jones, 2009). In contrast, the role of auxin in insect resistance is not as well developed. In both previously published experiments and my own,

we find that integrity of the SA signalling pathway has little bearing on *Arabidopsis* ability to resist aphid infestation. Therefore, the role of miR393 in aphid defence seems unlikely to rely on an interaction with this signalling pathway. I did however observe increased aphid performance on the *arf1* and *arf9* mutants. Both these mutants are known to have higher basal levels of some glucosinolates, including 4-OHI3M which is thought to be involved in aphid resistance (Kim & Jander, 2007; Pfalz *et al.*, 2009; Robert Seilaniantz *et al.*, 2011). In addition, these mutants accumulate less camalexin than wild-type plants when exposed to *P. syringae* (*Pst* DC3000) (Robert Seilaniantz *et al.*, 2011). This finding is in agreement with my previous work, where I found that plants less able to synthesise camalexin were more susceptible to aphid colonisation. It seems likely that the *arf1* and *arf9* mutants are also impaired in camalexin production upon exposure to aphids, and this accounts for their relative susceptibility. In addition, plants constitutively overexpressing miR393 have increased levels of glucosinolates following flg22 treatment, but fail to accumulate camalexin after inoculation with *Pst* DC3000. If this pattern is also true during aphid exposure, it is again evidence that plants impaired in camalexin production are aphid-susceptible and that miR393 is important for production of this phytoalexin.

In addition to controlling the camalexin/glucosinolate balance through ARF1 and ARF9, I suspect that miR393 plays a more direct role through interaction with *WRKY33, PAD3* or both. Plants deficient in either siRNA or miRNA processing have altered regulation of *WRKY33* during aphid infestations compared to wild-type plants, and plants with non-functional WRKY33 are more susceptible to infestation. This indicates that WRKY33 has a role in aphid defence. However, as aphid-susceptible *dcl2/3/4* plants have a comparable *WRKY33* expression pattern to aphid-resistant *dcl1* plants, there must be other factors besides WRKY33 involved in activating camalexin production. It is known that WRKY33 is closely related to WRKY25, and that both these transcription factors have roles in salt-tolerance in a process involving abscisic acid (ABA) (Jiang & Deyholos,

2009). It is possible that the regulation of multiple functionally redundant WRKY family members by miRNAs is a major factor in determining levels of camalexin synthesis. It is also possible that WRKY33 is involved in regulating other defence pathways besides camalexin, although this was not covered by my investigation.

I found that *WRKY33* is regulated at the post-transcriptional level by the presence of a cleavage product as determined by 5`RACE, although this cleavage product was distant from the predicted miR393 target site. There are three possible explanations for this result. Firstly, *WRKY33* mRNA may be targeted by another unidentified miRNA or siRNA. Bioinformatic analysis illustrated that there is considerable homology around the cleavage site between *WRKY33* mRNA and both a known siRNA (#16276 ASRP database) and a recently identified miRNA (miR5021) (Borges *et al.*, 2011). However, I was unable to detect miR5012 in *Arabidopsis* rosette leaves. A probe to detect siRNA #16276 only detected high molecular weight RNA and not sRNA. If *WRKY33* is cleaved by another sRNA then it remains to be identified. The second possibility is that miR393 does not regulate *WRKY33* through transcript cleavage. It is possible that the reduced homology between miR393-WRKY33 relative to other known miRNA-target interactions means regulation is exclusively through translational repression. Unfortunately the time constraints on this study meant that I was unable to pursue this prospect. A third alternative is that miR393 directs an unconventional cleavage of *WRKY33* mRNA. For example, the RNA-directed DNA methylation (RdDM) pathway involves 24nt siRNAs that are able to guide DNA methylation machinery to genomic loci where there is transcription of highly repetitive elements such as transposons. In this pathway, protein complexes guided by sRNAs catalyse methylation of DNA or histones that subsequently silence expression of particular genomic loci. This illustrates the principle that sRNAs can guide catalysis of reactions distant from the sRNA-target interaction. In addition, it has recently emerged that miR393-directed cleavage products are more heterogeneous that initially thought (Si-

Ammour *et al.*, 2011). It may be that under some circumstances, miRNA-AGO1 loaded RISCs are involved in cleaving target transcripts at additional sites beyond those previously reported for miRNA-mRNA interactions. Factors such as RNA secondary structure may play a role in directing this process, which would be a novel finding in miRNA-guided gene regulation in plants. Further 5`RACE experiments detected cleavage products towards the 3` of the predicted miR393 target site on *PAD3* mRNA. This pattern is similar to cleavage products generated from the confirmed miR393 target genes *AFB2*, *AFB3* and *TIR1*. It is therefore more likely that any additional role of miR393 in camalexin production is through direct regulation of *PAD3* during aphid exposure.

To date, miR393b is unique amongst stress-responsive miRNAs as its complementary miR* strand (miR393b*) has a defensive function independent of the miR strand (Zhang, X *et al.*, 2011). It would have been interesting to assess *M. persicae* performance on plants overexpressing miR393b* to discover whether the miR* defensive function that is active against *P. syringae* is also effective against insects. MiR393b* is reported to act through AGO2 and confer defensive benefit through increased production of the antimicrobial peptide PR1. In my previous experiments, aphid fecundity was unchanged on the *ago2* and *npr1* mutants. Therefore it is unlikely that miR393b* is involved in aphid resistance.

The data presented here builds on my previous observations that camalexin is important in aphid resistance and its production is regulated by pathogen or insect-responsive miRNAs. As camalexin is mostly important for resistance against necrotrophic pathogens it raises the interesting question of how should aphid pests be compared to plant pathogens? In many of our experiments, aphid performance on defence pathway mutants mimics that described for necrotrophs. For example, Zheng *et al.* found that the fungi *B. cinerea* and *A. brassicicola* are more successful on *wrky33* mutants but that resistance is restored to wild-type levels following introducing of a 35S:WRKY33 transgene (Zheng *et*

al., 2006). We observed this same pattern following *M. persicae* infestation. This is surprising, as aphids elicit responses similar to biotophic pathogens, and their lifestyle of feeding from living tissue and dispersing when nutrient quality reduces is more reminiscent of a biotrophic lifestyle. It may be that as with differing pathovars of pathogens, aphid biotypes exist that have significantly different resistances and susceptibilities to host defence processes. The colony used in these experiments belongs to genotype O, which has become the dominant *M. persicae* lineage in the UK (B. Fenton, unpublished data). Additionally, these aphids have been maintained for several years on Chinese cabbage *(Brassica rapa* subspecies *chinensis*). Camalexin has not been detected in Chinese cabbage. However, these plants are known to contain many of the same glucosinolate biosynthetic genes found in *Arabidopsis* (Wang et al., 2011). As such, the colony may have developed significant tolerance to glucosinolates but a susceptibility to camalexin and this may explain the results described. Obtaining aphid colonies from other laboratories would allow this hypothesis to be tested.

Chapter 6 – Investigation of the *Arabidopsis* miRNA response to *M. persicae* infestation

6.1 Introduction

In recent years, it has emerged that significant portions of the genomes of eukaryotic organisms do not encode proteinaceous gene products. These non-coding regions are not inert, but in fact can be highly transcribed, producing a cornucopia of non-coding RNA (ncRNA) molecules that play significant biological roles. It has been proposed that one of these roles may be to drive the complexity that is apparent in higher organisms (Salmena *et al.*, 2011). For example, the model worm *Caenorhabditis elegans* (*C. elegans*) has a comparable number of protein-coding genes to *Homo sapiens*, yet the human genome is around thirty times larger (Salmena *et al.*, 2011). It is probable that the non-coding fractions of genomes carry considerable amounts of information that has regulatory function over the coding portion.

One of the best-studied classes of ncRNAs are microRNAs (miRNAs). MiRNAs represent the smallest functional genes known to date and are present in almost all eukaryotes (Voinnet, 2009). In plants, miRNAs are short (~21nt) RNAs transcribed from loci located mostly in the introns of coding genes or in intergenic regions. MiRNA genes are transcribed by RNA Polymerase II to produce pri-miRNAs (Lee *et al.*, 2004). Single-stranded pri-miRNAs display an imperfect fold-back structure including a characteristic stem-loop hairpin motif. Maintenance of this particular secondary structure requires activity of the RNA-binding protein DAWDLE (DDL) (Yu *et al.*, 2008). The stem may be many tens of nucleotides in length and contains some degree of mismatching. In *Arabidopsis*, miR:miR* duplexes are excised from their precursors by the endoribonuclease Dicer-like 1 (DCL1). DCL1 recognizes hairpin structures and executes two cleavage reactions to remove the loop and the 5` and 3` tails of the hairpin. This produces ~21nt miRNA duplexes that include 2nt overhangs at the 3` ends. Efficient DCL1-dependent excision of miR:miR* duplexes requires both the dsRNA-binding

protein HYPONASTIC LEAVES 1 (HYL1) and the zinc finger protein SERRATE (SE) (Dong *et al.*, 2008). At some point, the duplex unwinds into the component miR and miR* strands. The factors which determine which strand is incorporated into an RNA-induced silencing complex (RISC) are not fully understood, however this is influenced by which strand displays weakest base-pairing at the 5` end (Tomari *et al.*, 2004). It was previously thought that miR* strands were quickly degraded and had no functionality. However it has recently emerged that miR* strands may have roles in pathogen resistance independent of the miR strand (Zhang, X *et al.*, 2011).

My previous work indicated that *Arabidopsis* plants deficient in miRNA processing are more resistant to infestation by the green peach aphid *M. persicae* (chapter 3). This resistance phenotype is not present in plants deficient in other RNA silencing pathways, nor is it due to the developmental abnormalities exhibited by miRNA pathway mutants. The observed resistance negatively affected aphid reproductive capacity but had no effect on the survival of adult insects. I subsequently investigated responses of wild-type (Col-0), miRNA mutant (*dcl1*) and siRNA mutant (*dcl2/3/4*) during the early defence response to aphid infestation (chapter 4). I discovered that miRNA pathway mutants constitutively overexpress some genes involved upstream of the indole glucosinolate and camalexin defence pathways. In addition, these plants are also more able to induce transcription of genes involved in the JA and ET signalling networks and in downstream parts of the glucosinolate and camalexin defence pathways. I found that *dcl1* plants synthesise increased quantities of camalexin relative to controls during aphid infestation, and that aphids fed camalexin by artificial diet had reduced fecundity compared to camalexin-free controls. Thus, an increased quantity of camalexin may contribute to the miRNA mutant resistance phenotype.

To further understand the mechanism that allows *dcl1* plants to better induce camalexin production, I focussed on signalling mechanisms upstream of *PAD3* that might

be relevant to aphid resistance (Chapter 5). I found that aphid performance is altered on *arf1* and *arf9* mutants, which are impaired in auxin responsiveness and are less able to synthesise camalexin during *P. syringae* infection (Robert Seilaniantz *et al.*, 2011). In addition, I found that there is differential regulation of the *WRKY33* transcription factor between silencing pathway mutants and wild-type plants. Aphids were also more fecund on *wrky33* mutants, indicating that this transcription factor is involved in aphid resistance. As WRKY33 is known to bind the promoter and enhance transcription of *PAD3* (Qiu et al., 2008), it provides further insight into the mechanism of camalexin production in *Arabidopsis*.

There are ~240 known miRNAs in *Arabidopsis*, and the targets of some of these have been identified. A common trend is that miRNAs target large families of transcription factors (Sunkar & Zhu, 2004). Furthermore, the targets of some miRNAs are known to be involved in tryptophan-derived secondary metabolism. One group of miRNAs (miR160, miR167, miR390, miR393) are specifically related to auxin signalling (Zhang, W *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, W *et al.*, 2011). It has recently been reported that miR393 has a specific role in resource allocation between the indole glucosinolate and camalexin pathways (Robert Seilaniantz *et al.*, 2011).

I previously observed that Col-0, *dcl1* and *dcl2/3/4* plants respond differently to aphids, and speculated that a subset of *Arabidopsis* miRNAs orchestrate the complex and substantial transcriptional response that occurs in plants following aphid attack. In this chapter, I aim to identify both aphid-responsive miRNAs and novel miRNAs induced by aphid exposure. This would hopefully facilitate identification of specific interactions that help explain the resistance phenotype of miRNA pathway mutants.

6.2 Results

Deep sequencing of aphid-exposed *Arabidopsis* sRNA libraries

I previously identified that a transcriptional response to aphids begins in *Arabidopsis* rosette leaves following 12h infestation (chapter 4). At an earlier timepoint (6h), I could not observe any consistent changes in gene expression levels. In contrast, at later timepoints (24h, 48h) the defence response appears to be fully induced and could launch secondary or tertiary signalling events. As my objective was to identify key early changes in the sRNA transcriptome that may guide the wider transcriptional response, I chose to investigate aphid-induced changes from rosette leaves exposed to 12h aphid infestation. In these experiments, thirty *M. persicae* nymphs were contained within clipcages as previously described for generating RNA samples. Leaves exposed to empty clipcages were used as negative controls. I pooled multiple leaves subjected to the same treatment to produce three aphid-exposed and three mock-treated samples. RNA was extracted from these samples and sRNA libraries were produced in accordance with the Illumina sRNA Sample Preparation Kit. Libraries were subsequently sequenced on an Illumina Genome Analyzer.

To assess miRNA expression levels, I normalised the number of reads present in each of the libraries by rescaling the raw reads to reads per million. Read profiles were then produced for all known *Arabidopsis* miRNAs (Figs. 6.1, 6.2, 6.3). Surprisingly, we were able to detect reads matching to all known miRNAs, although the number of matches to each individual miRNA varied considerably. In general, we detected greater numbers of matches to miRNAs that were discovered first (low miRBase numbers) (Fig. 6.1) relative to more recently identified sequences (high miRBase numbers) (Figs. 6.2, 6.3). In order to categorise miRNAs that were responsive to aphid treatment, I looked for miRNAs where at

least two out of the top three number of reads were from aphid-treated libraries. In total, twenty-four miRNAs matched these criteria. Read profiles for twelve of these miRNAs are shown in Figure 6.4. Of these twelve, a smaller set of seven miRNAs were present in greater abundance in the three aphid-exposed libraries relative to the three control libraries (Fig. 6.4).

I used the same process to identify miRNAs that may be repressed during aphid infestation (Fig. 6.5). As with the set of aphid-induced miRNAs, there was considerable variation in the number or reads between libraries of the same treatment. When I looked at variation across all the libraries, eight miRNAs met the criterium where at least two out of the bottom three numbers of reads were from aphid-treated libraries (Fig. 6.5). Of this set, three miRNAs were present in lesser abundance in the three aphid-exposed libraries relative to controls.

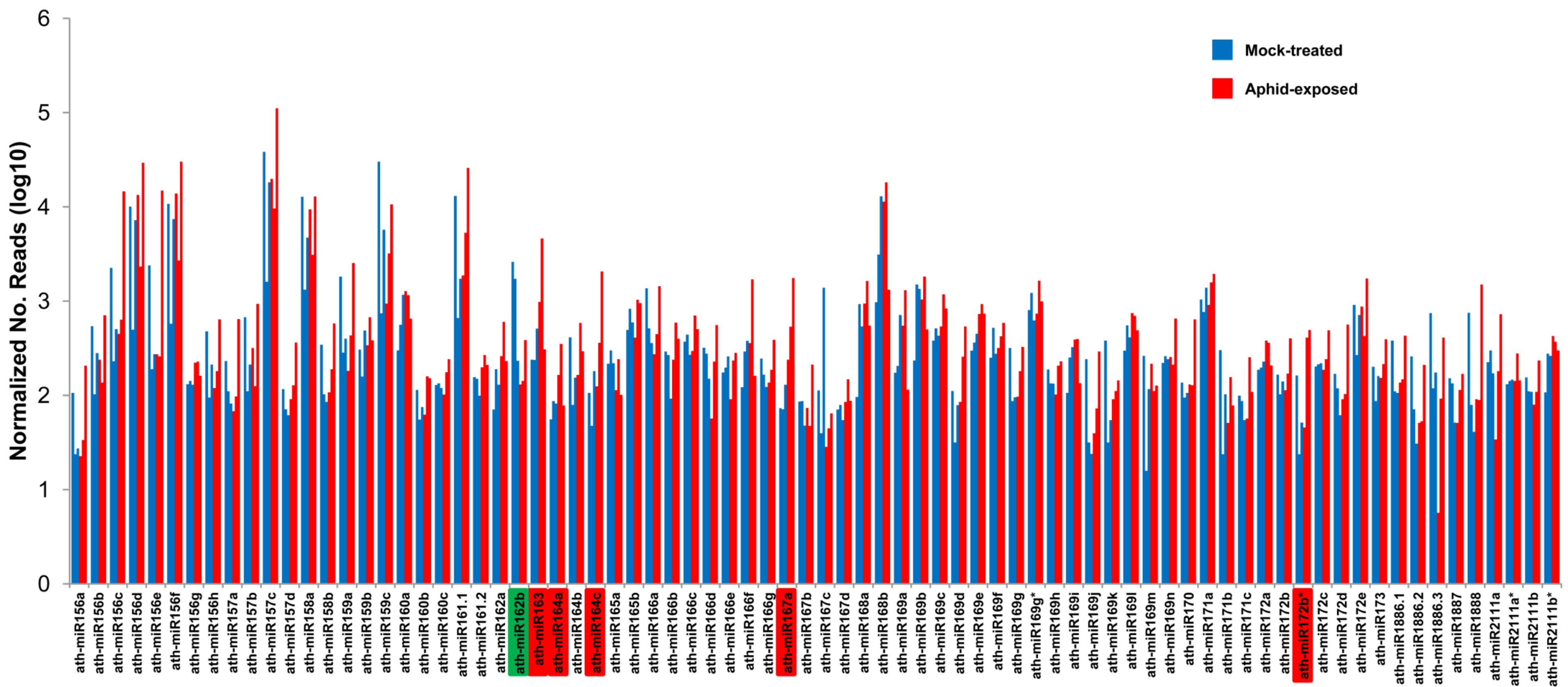

Figure 6.1 – Expression profile of *Arabidopsis* miRNAs with and without aphid treatment (part 1).
Expression profile of 1st set of 78 Arabidopsis miRNAs (miR156-miR2111) from three mock-treated and three aphid-exposed libraries. Number of raw reads were normalized to reads/million and this data converted by log10 for presentation. Individual miRNAs identified as candidates for being aphid induced (red) or aphid-repressed (green) are indicated.

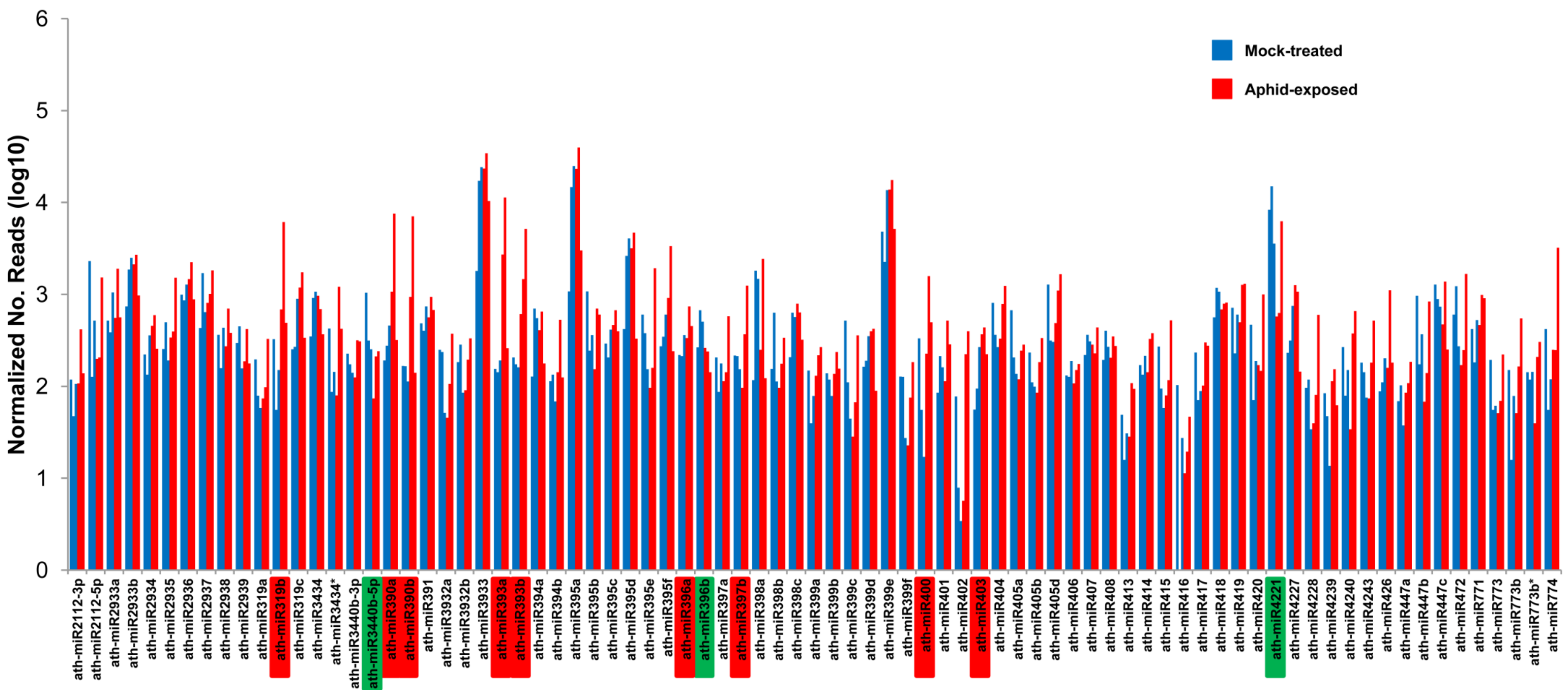

Figure 6.2 – Expression profile of *Arabidopsis* miRNAs with and without aphid treatment (part 2).
Expression profile of 2nd set of 80 Arabidopsis miRNAs (miR2112-miR774) from three mock-treated and three aphid-exposed libraries. Number of raw reads were normalized to reads/million and this data converted by log10 for presentation. Individual miRNAs identified as candidates for being aphid induced (red) or aphid-repressed (green) are indicated.

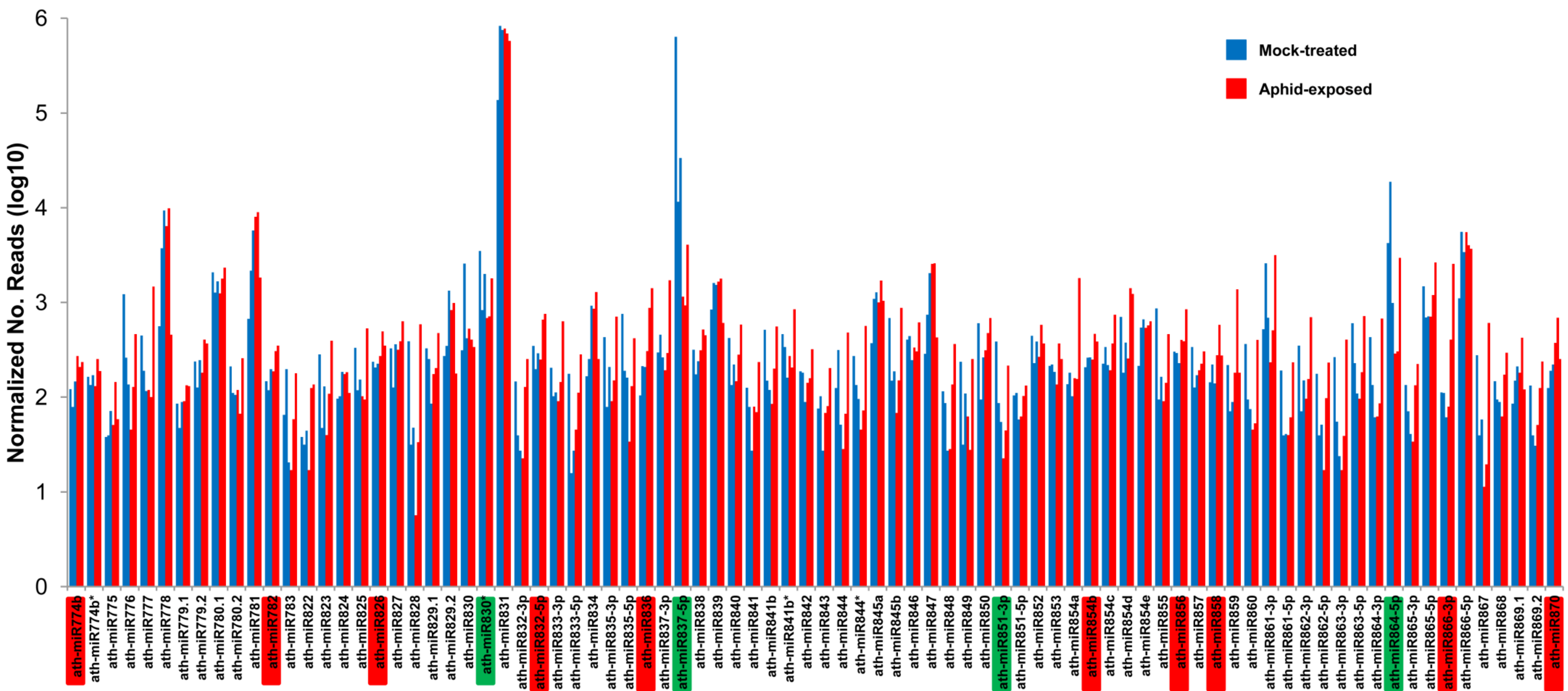

Figure 6.3 – Expression profile of *Arabidopsis* miRNAs with and without aphid treatment (part 3).
Expression profile of 3rd set of 83 Arabidopsis miRNAs (miR774b-miR870) from three mock-treated and three aphid-exposed libraries. Number of raw reads were normalized to reads/million and this data converted by log10 for presentation. Individual miRNAs identified as candidates for being aphid induced (red) or aphid-repressed (green) are indicated.

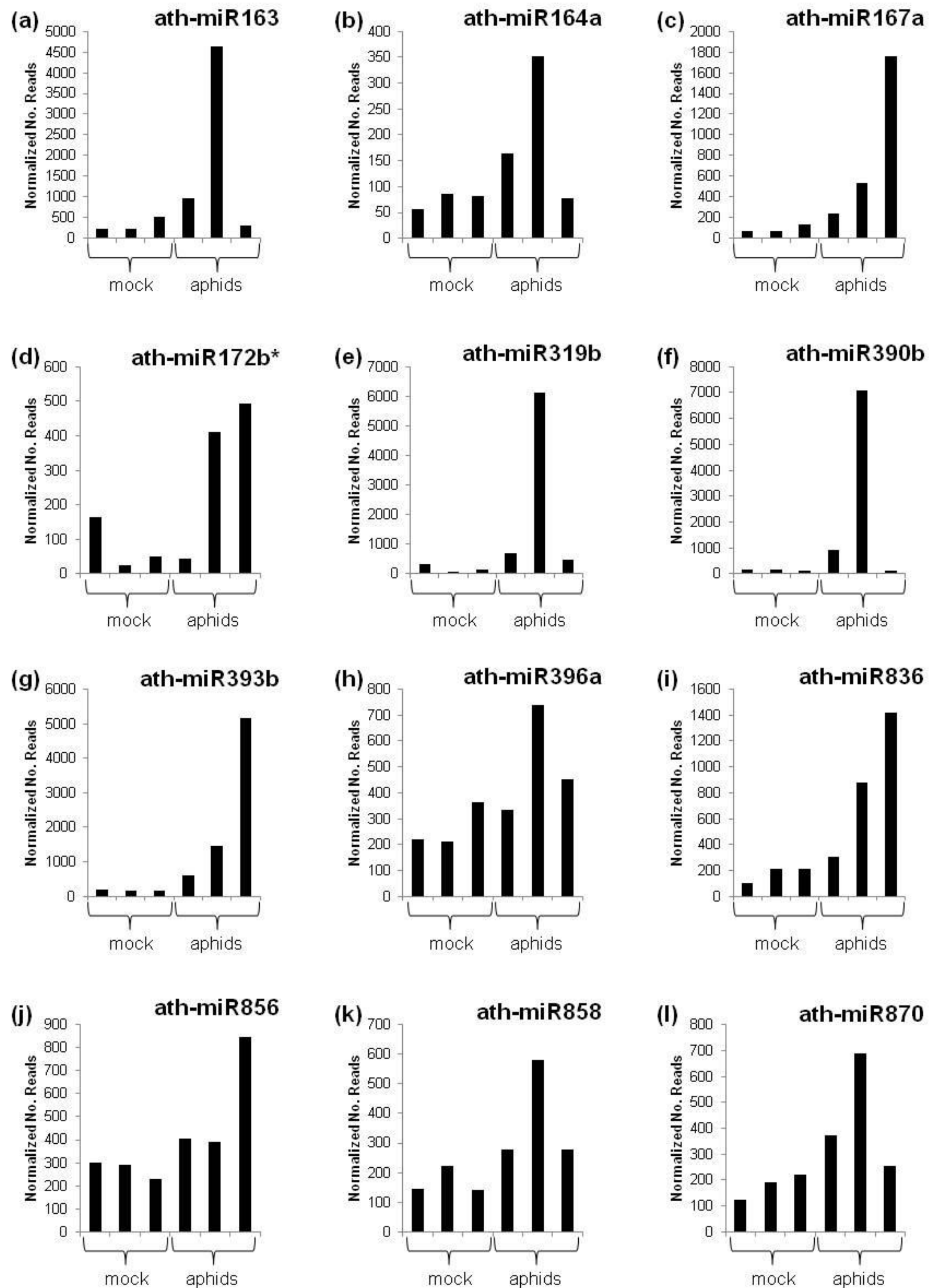

Figure 6.4 – Individual library profiles of candidate aphid-induced miRNAs determined by sRNA-seq.

Shown are the individual library profiles for 12 of 24 miRNAs that were identified by sRNA-seq as being aphid-inducible. For some of the candidate miRNAs, the normalized number of reads was heavily skewed by a single library (a,e,f,k). For other miRNAs, the normalized number of reads was consistently higher in aphid-treated libraries relative to controls (c,g,i,j,k,l). Shown are the normalized number of reads (reads/million) across the three control (mock) and three aphid-exposed (aphids) libraries.

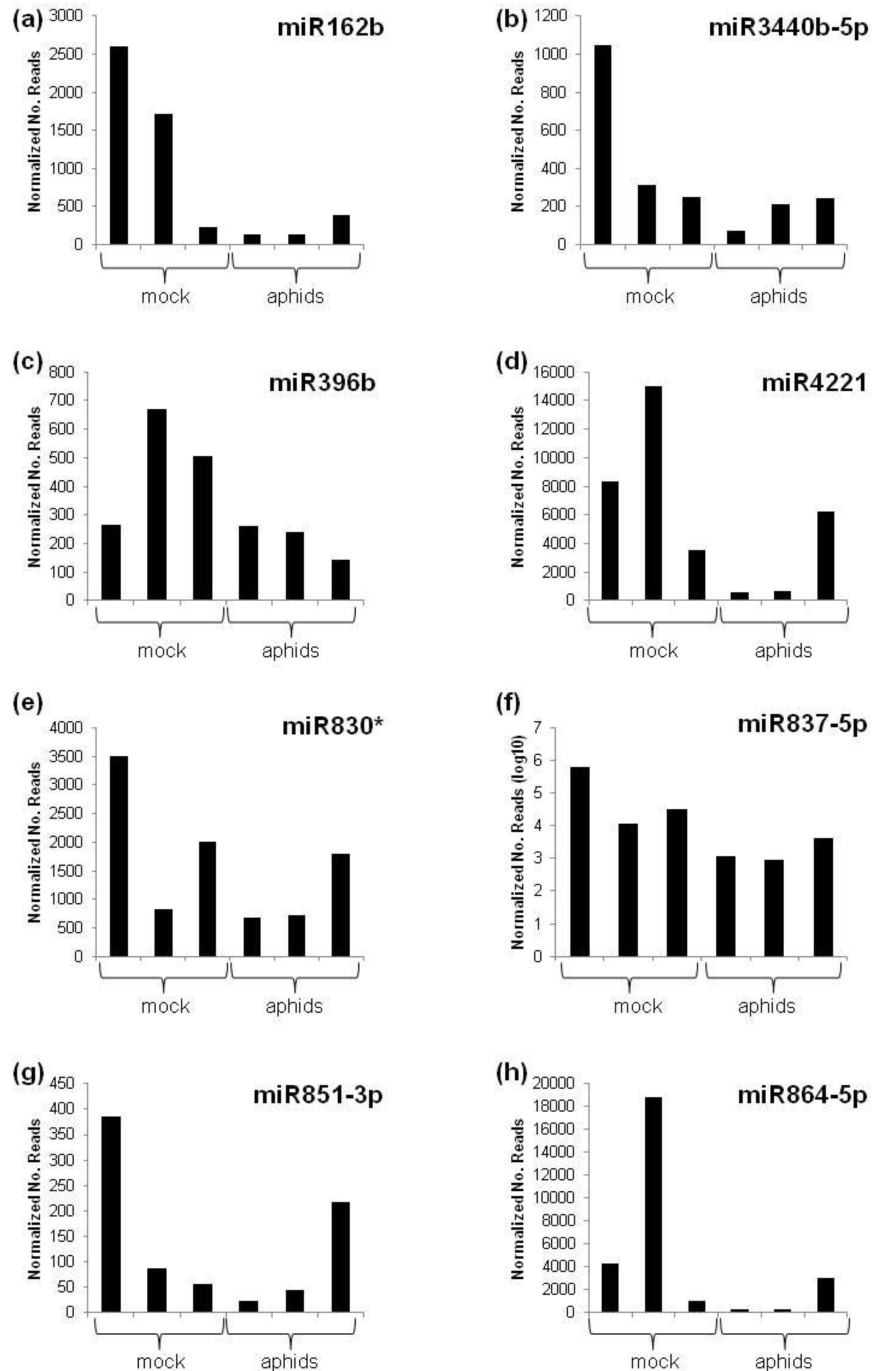

Figure 6.5 – Individual library profiles of candidate aphid-repressed miRNAs determined by sRNA-seq.

Shown are the individual library profiles for 8 miRNAs that were identified by sRNA-seq as being aphid-repressed. For some of these candidates, the normalized number of reads may be heavily influenced by a single library (b,e,g,h). For other miRNAs, the normalized number of reads was consistently lower in aphid-treated libraries relative to controls (a,c,d,f). Shown are the normalized number of reads (reads/million) across the three control (mock) and three aphid-exposed (aphids) libraries.

Northern blot validation of aphid-responsive miRNAs

Deep sequencing analysis suggested that a number of known *Arabidopsis* miRNAs are responsive to infestation by *M. persicae*. To validate these results, I selected a proportion of the miRNAs found to be variable in read number between mock and aphid treatments and assessed expression level in independent samples by small RNA northern blot (Fig. 6.6). I found that expression of many of the miRNAs tested by northern blot were more stable than data from sRNA-seq suggested. However, I did observe some variation both between mock and aphid-treated samples and between replicates. In replicate one, expression of several miRNAs (miR163, miR167, miR393) was lower in aphid-exposed leaves compared to mock-treated. A similar pattern was observed in replicate three, where expression of three miRNAs (miR167, miR390, miR393) appeared slightly lower in aphid-treated leaves. In contrast, in replicate two, expression of all miRNAs tested was higher in aphid-treated leaves. Although the pattern of expression was not consistent between replicates, miR393 appeared to be the miRNA most responsive to aphid treatment. As this miRNA has been previously implicated both in pathogen resistance and in camalexin production, this miRNA may also be important in aphid resistance.

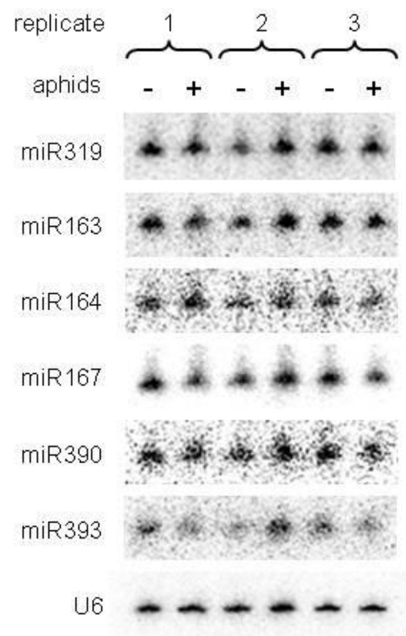

Figure 6.6 – Northern blot validation of differentially expressed miRNAs.

We were unable to confirm by northern blot the induction or repression of specific miRNAs identified by sRNA-seq. We saw that miR393 was repressed by aphid treatment in some replicates (1,3) but induced in another (2), indicating that this miRNA is aphid-responsive. This pattern was also present in a more subtle fashion for some of the other miRNAs tested (miR163, miR167, miR390). U6 was used as a loading/blotting control. 10μg RNA was loaded for each sample.

Further analysis of aphid-exposed *Arabidopsis* sRNA libraries

To reconcile the differences between the initial sRNA-seq analysis and northern blot data, I conducted a secondary bioinformatic analysis of our sRNA libraries. I suspected that the initial analysis may have been too relaxed in matching sequencing reads to known miRNAs, thus giving rise to a large number of false positive results. For the secondary analysis, we used the perl implementation of the UEA sRNA toolkit (Moxon *et al.*, 2008) (http://srna-tools.cmp.uea.ac.uk). This toolkit has been specially developed for analysis of

plant sRNA datasets and has been previously used in studies aimed at identifying stress-responsive miRNAs.

In the secondary analysis, we used the miRProf tool to assess read counts of known miRNAs across the libraries. This analysis detected much fewer miRNAs across the six libraries than our initial pipeline (Fig. 6.7). We detected 42 miRNA or miRNA* sequences that were present in at least five libraries, or in at least two libraries of one treatment but not the other (Fig. 6.7). The majority of miRNAs detected were sequences that have been known for some time, as indicated by a low miRbase number. As many of the more recently identified miRNAs (with high numbers) were identified in reproductive tissues, it is possible that many of these sequences are not found in rosette leaves at all. In addition, it is known that compared to other tissues, *Arabidopsis* rosette leaves have low miRNA content.

In general, miRProf analysis largely agreed with the northern blot experiment that had failed to validate some differentially expressed miRNAs from the primary analysis. MiRProf also revealed that a smaller number of miRNAs were aphid-responsive compared to the primary analysis (Fig. 6.7, 6.8, 6.9). I decided that similar to the primary analysis, for a miRNA to be considered aphid-induced at least two of the three libraries with the greatest number of reads must be from the aphid-exposed block. In contrast, for a miRNA to be considered aphid-repressed, at least two of the three libraries with the fewest number of reads must be from the aphid-exposed block. Using these criteria, only eight miRNAs were induced (Fig. 6.8) and a single miRNA was repressed (Fig. 6.9). Of the induced miRNAs, only two (miR163, miR393) were found in both the primary and secondary analysis. There was no correlation between repressed miRNAs between the primary and secondary analysis. Interestingly, miR319 was found to be aphid-inducible in the primary analysis, but was the sole miRNA that was aphid-repressed in the secondary analysis.

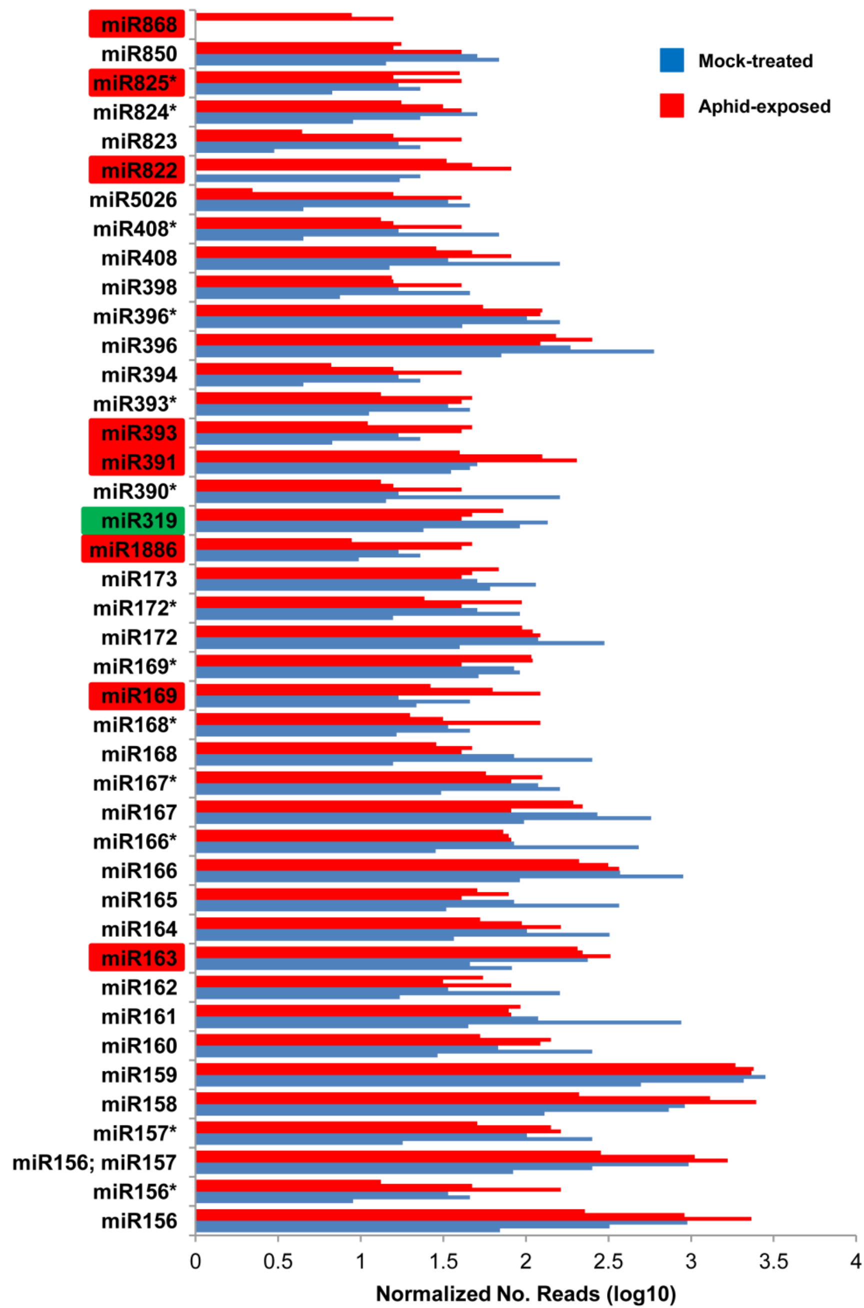

Figure 6.7 – MiRNA profiling in control and aphid-exposed libraries using miRProf.

The six libraries were re-analysed using the miRProf tool (UEA Plant sRNA toolkit). Forty-two known miRNAs were detected in at least five libraries and the normalized read counts (reads/million) (log10) are shown. In addition, included are miRNAs detected in at least two libraries of one treatment but none of the other (miR868). Individual miRNAs identified as candidates for being aphid induced (red) or aphid-repressed (green) are indicated.

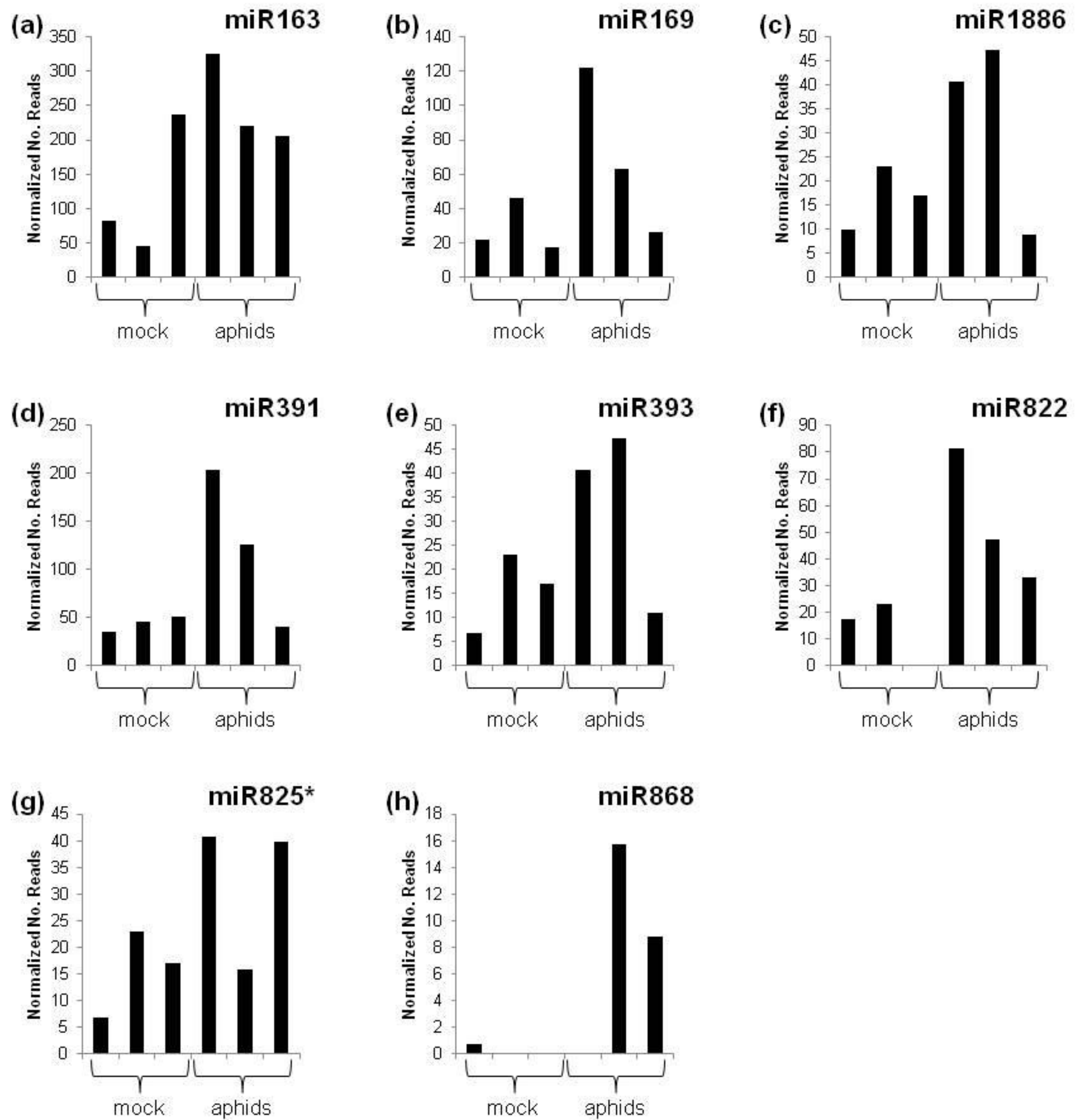

Figure 6.8 – Individual library profiles of candidate aphid-induced miRNAs determined by miRProf.

Shown are the individual library profiles for the 8 miRNAs that miRProf identified as being aphid-induced. As with the previous analysis, some profiles are skewed by a single library (a,d) whereas other profiles are consistently higher in aphid-exposed libraries relative to controls (b,c,e,f,g,h).

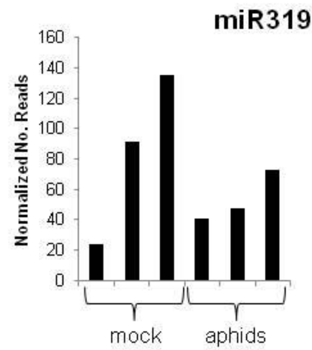

Figure 6.9 – Individual library profiles of candidate aphid-repressed miRNAs as determined by miRProf.

Shown is the individual library profile for miR319, the only miRNA identified by miRProf as being aphid-repressed. Bars represent the normalised number of reads (reads/million) across three mock-infested and three aphid-infested libraries.

Northern blot confirmation of newly identified aphid-responsive miRNAs

MiRProf analysis revealed that some miRNAs not originally identified may be aphid responsive. To assess the validity of the miRProf analysis, I again conducted sRNA northerns for some of the newly identified aphid-responsive miRNAs (Fig. 6.10). I found that miR391, identified as aphid-inducible by miRProf, was slightly induced in replicates one and two and stable in replicate three. MiR822 was very lowly expressed in all samples and appeared not be differentially expressed between treatments. I was unable to detect miR868 in any samples.

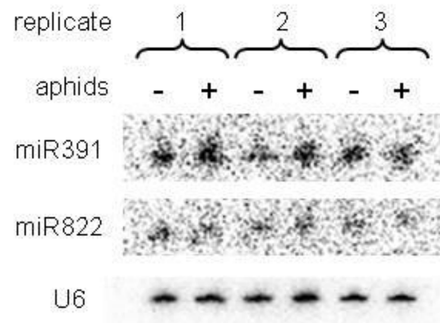

Figure 6.10 – Northern blot validation of differentially expressed miRNAs identified by miRProf.

MiR391 is aphid-induced in some replicates (1,2) but stable in another (3). MiR822 was stable across all samples tested. MiR868 was not detected in any samples. U6 was used as a loading/blotting control. 10µg RNA was loaded for each sample.

Identification of novel Arabidopsis miRNAs

To assess the possibility that previously unidentified miRNAs may be induced by aphid exposure, I used the miRCat tool to look for novel miRNAs that were present in the aphid-treated libraries and absent in control libraries. MiRCat found a small number of reads that were present in the aphid-exposed libraries but not in controls. However, as these reads all matched to portions of known miRNA sequences and were only found as single reads in each library, I did not classify these as novel miRNAs.

6.3 Discussion

This investigation had two primary objectives. Firstly, to identify *Arabidopsis* miRNAs that are responsive to aphid-infestation, and secondly, to identify novel miRNAs specifically induced during aphid exposure.

First analysis of the deep sequencing data suggested that a substantial number of miRNAs were differentially regulated during aphid exposure. I was unable to validate most of these changes by northern blot. However, expression of some miRNAs (miR163, miR167, miR390, miR393) was variable between treatments in several replicates. These miRNAs appeared repressed during some infestations but induced in others. This pattern was most obvious for miR393, a miRNA previously implicated in aphid resistance (Chapter 5). MiR393 is known to be induced following exposure to the PAMP flg22 or inoculation with both virulent and avirulent strains of *P. syringae* (Navarro *et al.*, 2006; Li *et al.*, 2010; Robert Seilaniantz *et al.*, 2011). The fact that it may also be responsive to *M. persicae* infestation suggests there may be considerable overlap between roles of miRNAs in response against both a bacterial biotroph and a phloem-feeding insect. This might have been expected, as both *P. syringae* and *M. persicae* elicit SA-dependent defence responses as well as camalexin production. The same miRNAs may therefore play similar roles in coordinating the responses against these two distinct plant attackers.

I previously speculated that manipulation of miR393 abundance may be important in regulating the level of camalexin production and hence aphid resistance (Chapter 5). My experiments showed that artificial overexpression of miR393 can render plants more susceptible to aphid colonisation (Chapter 5). It is therefore curious to why miR393 abundance may increase during aphid exposure. As I previously stated, this could be achieved through the action of a salivary effector introduced into the phloem stream during aphid feeding. In contrast, it may be that suppression of miR393 aids plant defence against

aphids. This may partly explain why *dcl1* plants (which lack miR393) are more resistant to aphid colonisation. My data shows that miR393 is responsive to aphid infestation, although I observed significant variation in direction of response between independent infestations. I currently cannot explain this variability, however it may be that miR393 is a focal point for both positive and negative regulatory pressures. Successful manipulation of plant processes in the majority of feeding sites may induce miR393 expression. Alternatively, successful perception of aphid feeding may activate processes to direct miR393 repression. The proportion of successful feeding sites within a colony may determine this balance.

In this chapter, miR391 is identified as an aphid-inducible miRNA worth future investigation. MiR391 was identified as aphid-inducible both by miRProf analysis of our sequencing data and by northern blot. MiR391 is less well-studied relative to miR393, and has to date only been implicated in the response to phosphate starvation (Lundmark *et al.*, 2010). Predicted targets of mR391 are mostly genes of unknown function, and no predictions have been validated experimentally. It would be interesting to determine how important the interaction between miR391 and its predicted target transcripts are with respect to aphid resistance. It may be that this miRNA executes functions that are highly specific to defence against aphids, but have no involvement in defence against other pathogens studies to date.

On the occasions where northern analysis confirmed miRNA induction, the differences between mock and aphid-treated samples was often subtle. My previous work showed that changes induced by aphid feeding may occur in a highly localised fashion, such as the phloem cells (Chapters 4 and 5). These changes may often only occur in a small number of cells around each feeding site. The experiments described here assess abundance of mature miRNA sequences at the whole leaf level. It is possible that there are significant alternations in the miRNA profile of cells that are either in close proximity or

able to perceive aphid feeding. However, these changes may be masked by stable expression in the majority of cells in the leaf where aphid challenge is not perceived.

From experiments described here, the majority of *Arabidopsis* miRNAs appear not to be transcriptionally responsive to aphid challenge. However, the absolute abundance of a miRNA gives little indication to whether it is being incorporated into an active RISC. Assessment of this could be achieved by high throughput sequencing after crosslinking immunoprecipitation (HITS-CLIP) to assess the AGO1-bound sRNAs in mock-treated and aphid exposed samples. This would uncover any preferential loading of miRNAs into RISCs in response to aphid treatment. However, this experiment would also have the background problem described above if conducted at the whole leaf level. A protocol would have to be devised to enrich for cells responding to aphid challenge. One solution might be to feed aphids on a reporter line expressing GFP under an aphid-inducible promoter. Plant tissue could then be digested to isolate protoplasts, and GFP-expressing cells recovered using a cell sorter. This would give the most accurate portrayal of both miRNAs and mRNAs that are differentially regulated during aphid infestation.

It has been proposed that in eukaryotic systems, many different RNA species may communicate to regulate activity of one another. This theory, termed the competing endogenous RNA (ceRNA) hypothesis involves miRNAs, coding-gene mRNAs and long non-coding RNAs (lncRNAs) including pseudogenes (Salmena *et al.*, 2011). In animals, many coding-gene mRNAs contain multiple miRNA target sites. Similar miRNA target sites are also present in some lncRNAs. It has been proposed that coding mRNAs and lncRNAs may compete for a limited pool of miRNAs. By this mechanism, altered transcription of lncRNAs could influence the expression of coding-genes in a miRNA-dependent manner but without the requirement for altered miRNA gene expression. The ceRNA hypothesis is largely focussed on gene regulation in animals; in particular with how disruption of miRNA function by other non-coding RNAs plays a role in cancer

development. However, there are parallels with gene regulation in plants. There is currently one described example of a naturally occurring *Arabidopsis* lncRNA that can influence coding gene expression through competition for a miRNA (Franco-Zorrilla *et al.*, 2007; Ebert & Sharp, 2010). In plants, this process has been termed target mimicry, and has been subsequently developed as a tool for miRNA gene knockdown (Todesco *et al.*, 2010; Ivashuta *et al.*, 2011).

In addition to sequencing six *Arabidopsis* sRNA libraries, I sequenced a library of sRNAs from the aphids that comprised all infestations. I had two main objectives for sequencing this library. The first was to assess whether sRNAs from wild-type *Arabidopsis* could be detected in aphids. Other investigations have described gene knockdown in several orders of insect after feeding on transgenic plants producing siRNAs targeting insect transcripts (Baum *et al.*, 2007; Frizzi & Huang, 2010; Zha *et al.*, 2011). In addition, it has recently emerged that intact plant miRNAs can transit the digestive system of mammals, and may have regulatory function over mammalian gene transcripts (Zhang, L *et al.*, 2011). I speculated that plants may employ such a mechanism to defend themselves from phloem-feeding insects they encounter in nature. Secondly, this library and the recently available genome scaffold for *M. persicae* would have facilitated sRNA identification for this aphid species. There are currently only miRNA sequences available for one aphid species, *Acyrthosiphon pisum (A. pisum)* (pea aphid), which is a specialist of legumes. It would have been interesting to compare sRNA profiles between *A. pisum* and *M. persicae*, one a specialist aphid and the other a generalist. This would offer insight into roles that sRNAs may have in other important aspects of aphid biology, such as determining host range. Unfortunately, time constraints meant I have been unable to pursue this investigation.

Chapter 7 – General Discussion

7.1 Summary of research

It was uncovered that *M. persicae* produces less progeny on *Arabidopsis* mutants impaired in miRNA processing (Chapter 3). Upon aphid exposure, these mutants have higher expression of the camalexin biosynthetic gene *PAD3* and accumulate more camalexin than wild-type plants (Chapter 4). *M. persicae* produces more progeny on *pad3* and other mutants that are unable to produce camalexin (Chapter 4). Moreover, these aphids are less fecund on artificial diets containing camalexin than on camalexin-free diets (Chapter 4). These findings indicate that (i) the miRNA pathway is involved in the negative regulation of camalexin biosynthesis upon aphid attack, (ii) camalexin may contribute to aphid resistance of the *Arabidopsis* miRNA pathway mutants, (iii) and camalexin negatively impacts aphid fertility but not survival.

The contribution of miR393 to *Arabidopsis* aphid resistance was investigated (Chapter 5), because it was previously shown that miR393 is involved in the *Arabidopsis* defense response to *P. syringae* and the PAMP flg22 (Navarro *et al.*, 2006; Li *et al.*, 2010) and co-workers in the Hogenhout laboratory have demonstrated that components of the flg22-mediated PTI pathway play a role in aphid resistance (Bos et al., 2010; D. Prince, unpublished results). Furthermore, miR393 negatively regulates the auxin receptor genes *TIR1, AFB2* and *AFB3* thereby preventing activation of auxin response factors ARF1 and ARF9 with the resulting effect of promoting glucosinolate production over camalexin (Robert Seilaniantz *et al.*, 2011) (Fig. 7.1). Aphid feeding appears to induce phloem-specific expression of miR393b, but not miR393a (Chapter 5). Additionally, *M. persicae* are more fecund on 35S:miR393 lines and on *Arabidopsis arf1* and *arf9* mutants (Chapter 5). These results suggest that miR393 is a negative regulator of the *Arabidopsis* defense response to *M. persicae* attack. This is consistent with the findings described in chapter 3 that miRNA pathway mutants are more resistant to *M. persicae*.

It was also investigated if the WRKY33 transcription factor, which directly regulates the expression of *PAD3*, is negatively regulated by miRNAs (Chapter 5). Bioinformatic predictions indicated *WRKY33* mRNA is a putative target of miR393. Aphids produced more progeny on the *Arabidopsis wrky33* mutant, but not on the 35S:WRKY33 line (Chapter 5). Moreover, upon aphid exposure, *WRKY33* expression is upregulated in *dcl1* mutants but not in wild type plants. 5`RACE experiments on cleaved *WRKY33* transcripts in mock-inoculated *Arabidopsis* did provide preliminary evidence for degradation of *WRKY33* transcripts by (an) unknown miRNA(s) but no evidence for the involvement of miR393 (Chapter 5). Indeed, *WRKY33* expression is not constitutively downregulated in (non-aphid-exposed) 35S:miR393 lines (Chapter 5). However, it is still possible that miR393 inhibits translation of *WRKY33* mRNA in aphid-exposed plants, and this needs further investigation. These results are nonetheless consistent with findings described in chapter 3 that miRNA pathways mutants are more resistant to *M. persicae*, and those in chapter 4 that the camalexin pathway is involved in *Arabidopsis* defense response to aphid attack.

To discover *Arabidopsis* miRNAs that are responsive to *M. persicae* infestation, I deep-sequenced three aphid-exposed *Arabidopsis* sRNA libraries and three aphid-free control libraries (Chapter 6). Whilst the expression of the majority of host miRNAs was unchanged during aphid infestation, the expression of nine miRNAs, including miR393, were altered in the aphid-treated versus mock-treated samples. However, subsequent northern blot hybridisation experiments revealed that miR393 expression is induced during some infestations but repressed in others, suggesting that it may be temporally and spatially regulated during aphid infestation. Thus, it is likely that miRNAs play a role in plant defence regulation during aphid infestation.

In conclusion, data presented in all four experimental chapters provide independent evidence that the *Arabidopsis* miRNA pathway negatively regulates plant defence

signalling during aphid infestation. In the absence of miRNA processing, plants become more resistant to *M. persicae*. In addition, it was found that specific miRNAs are upregulated during aphid attack and this may downregulate the production of camalexin, which can be toxic to the aphid. Because it is not in the plants best interest to increase expression of miRNAs that downregulate plant defense, the possibility that aphids play an active role in upregulating miRNAs directly or indirectly should be considered. This is the first report of miRNA involvement in regulating plant resistance against a phloem-feeding insect pest.

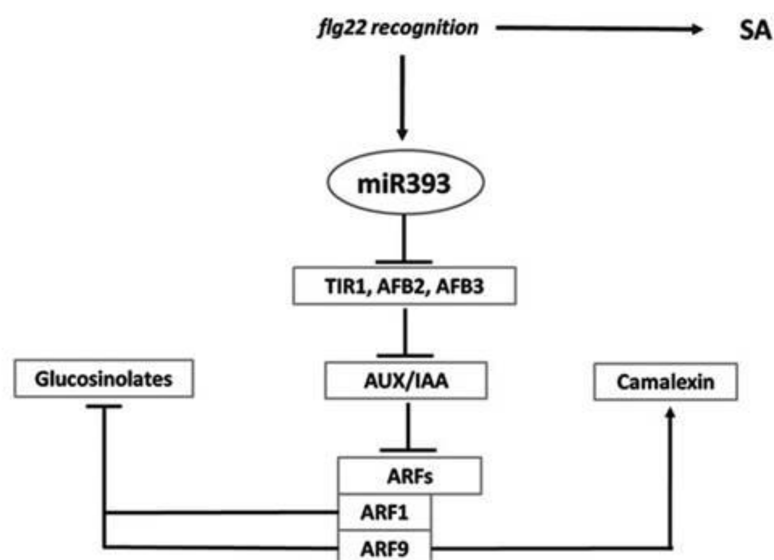

Figure 7.1 – Current model for miR393 involvement in glucosinolate/camalexin pathways.

MiR393 is induced by PAMP (flg22) treatment or by expression of a 35S:miR393 construct. The microRNA targets three auxin receptors (*TIR1*, *AFB2*, *AFB3*) reducing plant sensitivity to auxin and preventing activation of ARF1 and ARF9. As ARF9 is a positive regulator of camalexin production, and both ARF1 and ARF9 are negative regulators of glucosinolate production, this has the effect of channelling resources away from camalexin towards glucosinolates. Image adapted from (Robert Seilaniantz *et al.*, 2011).

7.2 Camalexin is toxic to a number of plant attackers

Camalexin production is induced by a wide variety of plant attackers including bacteria, fungi, viruses, insects and oomycetes (Glawischnig, 2007). However, the proportion of these colonisers that are susceptible to camalexin is much narrower. The bacterial biotroph *P. syringae* reproduces less rapidly at camalexin concentrations of 500μM (Rogers *et al.*, 1996), however it is debatable whether camalexin accumulates to this concentration in vivo (Glawischnig, 2007). The concentration required to retard growth of the fungal necrotroph *A. brassicae* is ten times lower (Pedras *et al.*, 1998), and more likely represents a concentration that occurs in the leaves of infected plants. In my experiments, I observed that a camalexin concentration of 62.5μM was sufficient to reduce aphid fecundity when aphids were raised on artificial diets (Chapter 4). This level is comparable to experiments conducted with *A. brassicae*. It is possible that aphid performance is reduced at lower concentrations; however this was not tested in my experiments. Using a PAD3p::GUS reporter line, I observed that *PAD3* induction is highly localised to the site of penetration by aphid stylets (Chapter 4). The concentration that occurs around feeding sites is not known, however it is fair to presume that it is significantly higher than the level measured at the whole leaf or whole plant level. It is known that following inoculation with the fungal necrotroph *B. cinerea*, levels of camalexin at the infection front can be nearly twenty times higher than in tissues 1cm away from the infection (Kliebenstein *et al.*, 2005). It is therefore possible that the concentrations fed in artificial diet assays are comparable to the quantities of camalexin found at aphid feeding sites. Furthermore, due to technical limitations, we had to cap the duration of the artificial diet assay to two days. This is short in comparison to our plant assays where aphids are raised for 14-16 days. It may be that longer exposures to lower quantities of camalexin result in similar outcomes compared with the acute exposure given artificially.

When I assessed aphid performance on plants of varying ability to produce camalexin, I found significant variation in results. Aphids were more successful on plants unable to synthesise camalexin (*pad3*, *cyp79b2/cyp79b3*) or on plants with varying deficiencies in camalexin production (*arf1*, *arf9*, *wrky33*, 35S:miR393a, 35S:miR393b). In contrast, I observed decreased aphid performance on miRNA pathway mutants, including *dcl1* plants, which I found synthesises greater quantities of camalexin than wild-type plants. To more precisely assess the role of camalexin in the miRNA mutant resistance phenotype, aphid performance could be assessed on a *dcl1/pad3* double mutant relative to *dcl1*. I would expect that aphid fecundity on *dcl1/pad3* would be partially returned to wild-type levels. Work is continuing towards generating the *dcl1/pad3* double mutant for this experiment. In addition, it would be interesting to assess fecundity on either a double miR393 mutant or a miR393 knockdown. I expect a reduction in aphid fecundity on either of these lines relative to wild-type plants. Comparing aphid fecundity on the miR393 mutant/knockdown and *dcl1* plants would indicate what proportion of the *dcl1* resistance phenotype is dependent on miR393 alone. A final experiment may be to generate a silent mutation of the putative miR393 target site in *PAD3* to produce a miR393-resistant *PAD3* transcript. This experiment would indicate the extent of this specific miRNA-mRNA interaction in aphid resistance, because in the scenario that miR393 directs degradation or translational repression of *PAD3* transcripts, plants expressing a miR393-resistant *PAD3* would be better able to induce camalexin production (during aphid exposure) than plants expressing a miR393-susceptible *PAD3*. These plants would be more resistant to *M. persicae* as well.

Camalexin is known to inhibit growth of several necrotrophic fungal pathogens. My data shows that it is also effective at impairing the reproductive development of a hemipteran insect. In addition, camalexin exhibits anti-proliferative and pro-apoptotic properties against some human cancer cell lines (Mezencev *et al.*, 2003; Mezencev *et al.*,

2011). Thus, camalexin may target dividing cells. Finally, there is evidence that camalexin disrupts cell membranes (Joubert *et al.*, 2011). Treatment of the fungus *A. brassicicola* with 125µM camalexin shows hugely increased fungal membrane permeability and induction of genes involved in membrane stress and repair compared to camalexin-free controls (Sellam *et al.*, 2007; Joubert *et al.*, 2011). Pathogens have evolved mechanisms to limit camalexin-induced damage. For example, a pathogenic strain of *B. cinerea* expresses an ABC transporter to pump camalexin out of cells (Stefanato *et al.*, 2009), indicating that camalexin is taken up by cells and may perturb intracellular processes.

Camalexin inhibits aphid fertility but not adult survival. Given that camalexin has anti-proliferative and pro-apoptotic properties, it is plausible that camalexin inhibits growth of aphid embryos. Another possibility is that camalexin negatively affects cells in the aphid digestive tract that are probably exposed to the highest concentrations of camalexin. As these cells are involved in obtaining nutrients from the diet, reduced functionality may result in nutrient deficiencies, which could impact embryo development. Given that camalexin also has anti-microbial properties (it inhibits *P. syringae* proliferation for instance), another possibility is that camalexin reduces aphid fecundity by reducing the number of primary intracellular endosymbionts *Buchnera aphidicola* (*B. aphidicola*), which produce essential amino acids required for growth and reproduction of aphids. Indeed, aphids cured of their symbionts through antibiotic treatment produce considerably fewer offspring than untreated aphids (Prosser & Douglas, 1991). However, an experiment described in chapter 4 indicated that *B. aphidicola* numbers were similar in aphids raised on plants producing zero, low and high levels of camalexin. There was also no difference in *B. aphidicola* numbers of aphids raised on camalexin spiked- or camalexin-free artificial diets. Thus, camalexin does not appear to affect *B. aphidicola* population growth. Nonetheless, it remains possible that camalexin can disrupt other functions in the symbiotic relationship. Camalexin could reduce the efficiency with which *B. aphidicola*

can synthesise the essential amino acids required by aphids or interfere with the maternal transmission of symbionts to aphid offspring.

7.3 MiRNAs target multiple gene transcripts in plants

The mechanisms of gene regulation that are guided by small RNAs (sRNAs) have been the focus of extensive investigations both in plants and animals. MiRNA-target pairing is known to be heavily influenced by matches in the seed region, at positions 2-8 nucleotides from the miRNA 5` end. Complementarity at positions 10-11 is crucial for determining whether regulation occurs by transcript cleavage or translational repression. It is generally accepted that miRNA-transcript interactions in plants display greater complementarity than those present in animals (Voinnet, 2009). However it is becoming clear that plant miRNAs also regulate target transcripts displaying a lower degree of sequence similarity.

It has emerged that miR393 targets multiple transcripts, including *TIR1*, *AFB2* and *AFB3* and possibly others. Additionally, miR393-directed cleavage products are more heterogeneous than might be expected from a miRNA that targets multiple transcripts with near-perfect complementarity. Significant quantities of cleavage products are detected in the region ~100-200-nt towards the target 3` end of the canonical cleavage site on *TIR1*, *AFB2* and *AFB3* transcripts (Si-Ammour *et al.*, 2011). With this finding, it seems reasonable to consider *PAD3* as a legitimate target for miR393-mediated regulation. In my experiments with aphid-treated *Arabidopsis*, I find two distinct *PAD3* cleavage products 24nt and 134nt towards the 3` end of the transcript from the putative miR393 target site. These products are absent in mock-treated plants, probably because *PAD3* is so lowly expressed that interactions between this transcript and miR393 are uncommon. Therfore it

is likely that the miR393-*PAD3* interaction has specific roles in stress responses where production of camalexin is important.

MiRNA duplexes are composed of two strands that have 2nt overhangs at the 3` ends. MiRNAs function through the miR strand, that is complementary to the target, whilst the miR* strand, which has the same sequence as the target and is generated during miRNA maturation, was previously thought to be junk RNA holding no significant biological function. However, miR393 appears unique in that miR393b* has a role in a defence processes independent from miR393 and which operates through an AGO2-catalysed pathway. Whilst there is evidence that miR393b and not miR393a is upregulated in the phloem during aphid feeding (Chapter 5), aphid fecundity and survival are not affected on the *ago2* mutant (Chapter 3) indicating that miR393b* likely does not have a role in plant defense to aphids. It remains to be seen whether other miR* sequences are active in this pathway and have roles in plant defence.

7.4 MiRNAs as negative regulators of defence

In previous studies, miR393 emerged as a key regulator of defence against biotrophic and necrotrophic pathogens through its role in regulating auxin receptor genes (Navarro *et al.*, 2006), which are involved in resource allocation into camalexin versus glucosinolate production pathways (Robert Seilaniantz *et al.*, 2011) (Figure 7.1). MiR393 is a negative regulator of camalexin production and its regulation appears to be altered during some aphid infestations. Additionally, I found that miRNAs, including perhaps miR393, may have distinct roles in negatively controlling camalexin production through the targeting of *PAD3* transcripts. Thus, whilst camalexin production appears to be induced upon aphid

attack, it is subsequently suppressed because of the induction of miRNAs. There is an example in the literature of an *Arabidopsis* miRNA (miR164) that functions as a brake of developmental signalling with important consequences for the timing of leaf senescence (Kim *et al.*, 2009). Perhaps miR393 is involved in a balancing act between limiting pathogen colonisation and ensuring continued growth? This could involve fine-tuning camalexin production to a level where pathogen or pest performance is sufficiently limited but with minimal impact on rate of plant growth. It is also possible that miR393 is induced to prevent defence hyper-activation and to prevent quantities of camalexin accumulating that are toxic to host cells.

In addition to miR393, there are other miRNAs that are involved in *Arabidopsis* defence to insect herbivores. The miRNA miR163 is highly expressed in *A. thaliana* but silent in the close relative *Arabidopsis arenosa (A. arenosa)* (Ng et al., 2011). MiR163 is known to target transcripts of farnesoic acid methyltransferase (FAMT), an enzyme that converts farnesoic acid (FA) to methyl farnesoate (MeFA). MeFA is an unepoxidized analogue of insect juvenile hormone III (Shinoda & Itoyama, 2003), which has previously been implicated in plant defence, since its presence negatively impacts the growth and development of some insect herbivores. FAMT is induced in both *A. thaliana* and *A. arenosa* by insect damage and the fungal elicitor alamethicin, whilst miR163 is only induced *A. thaliana* and not in *A. arenosa* (Ng *et al.*, 2011). Thus, *A. arenosa* produces more FAMT because of the absence of miR163 during insect attack, whilst FAMT production is negatively regulated by miR163 in *A. thaliana*. This difference may have an evolutionary advantage as *A. arenosa* is an outcrossing plant with a long vegetative stage compared to the inbreeding *A. thaliana* and is therefore more likely to encounter insect herbivores or pathogens that would benefit from a more aggressive defence response (Ng *et al.*, 2011). Interestingly, my sRNA-seq data suggested that miR163 is induced in whole leaves following aphid exposure although this was not confirmed by northern blot

hybridisation experiments (Chapter 6). Nonetheless, if MeFA negatively impacts the growth and development of aphids, the absence of miR163-mediated negative regulation of FAMT in *A. thaliana* miRNA pathway mutants is likely to contribute to the aphid-resistant phenotype of these plants. To test this possibility, we obtained *A. thaliana mir163* mutants but aphid performance assays on these plants have not yet been conducted.

Differential expression/presence of miRNAs may not vary only among plant species, but also between ecotypes within a species. It is known that there are significant differences in siRNA profiles between the closely related *A. thaliana* Col-0 and Ler ecotypes (Zhai *et al.*, 2008). These differences are known to have impact on the *FLC* locus and control of vernalization and flowering time (Zhai *et al.*, 2008). It is less clear how miRNA profiles differ between ecotypes. However, differences in either miRNA expression or targeting preferences may contribute to the ability of *Arabidopsis* ecotypes to respond to the environment. For example, all *Arabidopsis* accessions studied to date are able to produce camalexin, but there are considerable differences in the quantities synthesised in response to various treatments. Some ecotypes can be considered high camalexin producers under certain stresses, but may be low producers under an alternate stress (Glawischnig, 2007). It seems unlikely that these differences are caused by disparity in the efficiencies of the camalexin biosynthetic enzymes. Therefore, there may be differences in elicitor recognition and signal transduction mechanisms and variation in miRNA-mediated regulation represents one possible mechanism by which signal transduction may differ between *Arabidopsis* ecotypes.

MiRNA-mediated regulation during environmental responses may also be involved in the priming phenomenon, where it has been observed that plants exposed to either chemical treatment or pathogen challenge are better able to resist future assaults (Conrath *et al.*, 2006). Induction of both defence pathway transcripts and their negative regulators could enable plants to launch specific defence responses upon stress or pathogen/pest

attack. Thus, primed plants may have increased presence of both defence gene transcripts and counter-defensive miRNAs. Once these plants perceive stress or pathogens/pests above a certain threshold, a change in miRNA levels would allow more rapid adjustments of the proteinaceous components of the defence pathway and a better adapted immune response to the particular stress/pathogen/pest.

7.5 Pathogens and pests manipulate plant silencing pathways

Many plant viruses encode suppressor molecules that usurp antiviral RNA silencing and interfere with endogenous silencing processes guided by miRNAs (Ding & Voinnet, 2007). Additionally, some effector proteins from virulent strains of *P. syringae* are known to interfere with aspects of both siRNA- and miRNA-mediated gene silencing (Navarro *et al.*, 2008). This is additional evidence of the possibility discussed above that plants keep tight control over induction of defence pathways through the regulatory action of some miRNAs. Similarly to *P. syringae* and viruses, it is plausible that aphids modulate host-silencing processes to enhance colonisation. For instance, aphids may induce or suppress specific miRNAs that modulate the plant defence response to the aphid benefit. Aphid saliva, which is released into the cytoplasm of plant cells during stylet probing and the phloem stream during feeding, may contain proteins that alter the expression of miRNAs directly or indirectly. Specifically, one or more of these aphid proteins could interfere with miR393-specific regulation. The role of miR393 in glucosinolate/camalexin production during PAMP treatment and *P. syringae* infection has been previously described. In this model, miR393 regulates auxin responsiveness through targeting of the auxin receptor transcripts *TIR1*, *AFB2* and *AFB3* (Fig. 7.1). Similarly, an aphid protein that promotes

miR393 transcription, processing or activity, could shift balance of metabolic flow towards glucosinolates (many of which have no effect on aphid performance) and away from camalexin, which is deleterious to aphid fecundity (Chapter 4). The work described in this thesis also uncovers a role of the WRKY33 transcription factor in aphid resistance (Chapter 5). Thus, it is possible that miR393, or other currently unidentified sRNAs, target *WRKY33* mRNA to further repress camalexin accumulation.

Pathogen attack results in the induction of *WRKY33* and camalexin production. These are also induced by PAMPs such as flg22. Aphids do not induce *WRKY33* in wild type *Arabidopsis* (Figure 5.7a). However, aphids do induce *WRKY33* in a *dcl1* mutant (Figure 5.7a) suggesting that during aphid attack, *WRKY33* may be negatively regulated by miRNAs. WRKY33 directly regulates *PAD3* expression and camalexin production, which is toxic to the aphid. Hence, it seems to be to the benefit of the aphid but not the plant to limit *WRKY33* expression. It is interesting that the biotrophic pathogen *P. syringae* induces *WRKY33*, but unlike aphids it benefits from the upregulation as basal resistance of plants to *P. syringae* DC3000 is compromised on *WRKY33* overexpression lines (Zheng *et al.*, 2006). To this end, this bacterium produces effectors that downregulate certain miRNAs, including miR393, to promote disease and also grows better on miRNA-deficient mutants (Navarro *et al.*, 2008) and is restricted in growth on miR393 overexpression lines (Navarro *et al.*, 2006). In contrast, aphids are more fecund on miRNA-deficient mutants, do not upregulate *WRKY33* in wild-type plants, and do better on both *wrky33* mutants and miR393 overexpression lines. The *wrky33* plants are also more susceptible to necrotrophic fungi, but unlike aphids, these pathogens induce *WRKY33* (Zheng et al., 2006). Because WRKYs are predicted to regulate miRNA expression (Zhou *et al.*, 2008; Pandey & Somssich, 2009) it remains to be determined if the upregulation of specific miRNAs during aphid attack is a self-regulatory process (as discussed previously) or if aphid effectors upregulate specific miRNAs, including miR393, that reduces camalexin production via

direct negative regulation of *PAD3*, and more indirectly via the auxin perception pathway. However, the latter seems most plausible.

Figure 7.2 illustrates a model of how miRNA-mediated regulation of plant defence during aphid attack might operate. In this model, it is assumed aphids suppress PTI-related pathways, evidence for which has accumulated during the past few years in the Hogenhout lab (Bos *et al.*, 2010) (D. Prince, unpublished work; this thesis). In this model, aphid feeding triggers a basal defence response in plant cells (Fig. 7.2a,b). This response may be triggered by perception of either host DAMPs produced during puncturing of the phloem by aphid stylets, or aphid PAMPs present on the surface of the aphid stylets and saliva. DAMPs or PAMPs are recognised by a currently unknown pattern-recognition receptor (PRR) and may involve the action of the leucine-rich repeat receptor-like kinase (LRR-RLK) BAK1 (Fig. 7.2b). This triggers mitogen-activated protein kinase (MAPK) cascades (Fig. 7.2c) that ultimately result in transcriptional reprogramming via pathogen-responsive transcription factors. Indeed, it is known that WRKY33 is released from its suppressor MKS1 by an MPK4-dependent phosphorylation event during *P. syringae* infection or flg22 treatment (Qiu *et al.*, 2008), and is activated during *B. cinerea* infection in a distinct process that appears to operate through MPK3/6 (Ren *et al.*, 2008; Mao *et al.*, 2011). Free WRKY33 is able to transcriptionally activate *PAD3* (Fig. 7.2d). Because the data presented in chapter 5 suggest that miR393b is upregulated in the plant phloem under aphid attack, an aphid effector (injected into plant cells during aphid probing and feeding (Fig 7.2g) may upregulate miR393b expression, thereby reducing camalexin production either directly by reducing the accumulation of *PAD3* (Fig. 7.2e) (in a scenario where miR393 targets *PAD3* transcripts) or indirectly via downregulating the auxin receptors and channelling resources away from camalexin production (Fig. 7.1; not illustrated in Fig. 7.2). Such a putative effector would likely need access to companion cells from the phloem stream to be functional (Fig. 7.2g), and could act via two broad mechanisms. Firstly, by

promoting transcription of miRNA loci, perhaps by interaction with the Pol II transcriptional apparatus (Fig. 7.2h), or secondly, by direct interaction with miRNA-AGO complexes, either by modifying AGO loading preferences, or enhancing interactions between RISCs and their mRNA targets (Fig. 7.2i). Either mechanism would result in cleavage or translational repression of *PAD3* mRNAs (Fig. 7.2e) with reduced accumulation of this gene product and reduced production of camalexin (Fig. 7.2f).

The model in figure 7.3 illustrates how disabling the miRNA pathway will render plants more resistant to aphids. In these miRNA pathway mutants, PAMP or DAMP perception and downstream signalling is likely to be similar to that in susceptible Col-0. However, in *dcl1* plants, mature miRNAs are not excised from their precursors (Fig. 7.3a). Therefore effector action has little impact on post-transcriptional regulation (Fig. 7.3a,b), allowing increased accumulation of *PAD3* mRNA (Fig. 7.3c). As WRKY33 is known to operate a positively regulating feedback loop (Mao *et al.*, 2011), *WRYK33* transcripts accumulate resulting in more WRKY33, which positively regulates *PAD3* expression and downstream camalexin production (Fig. 7.3d).

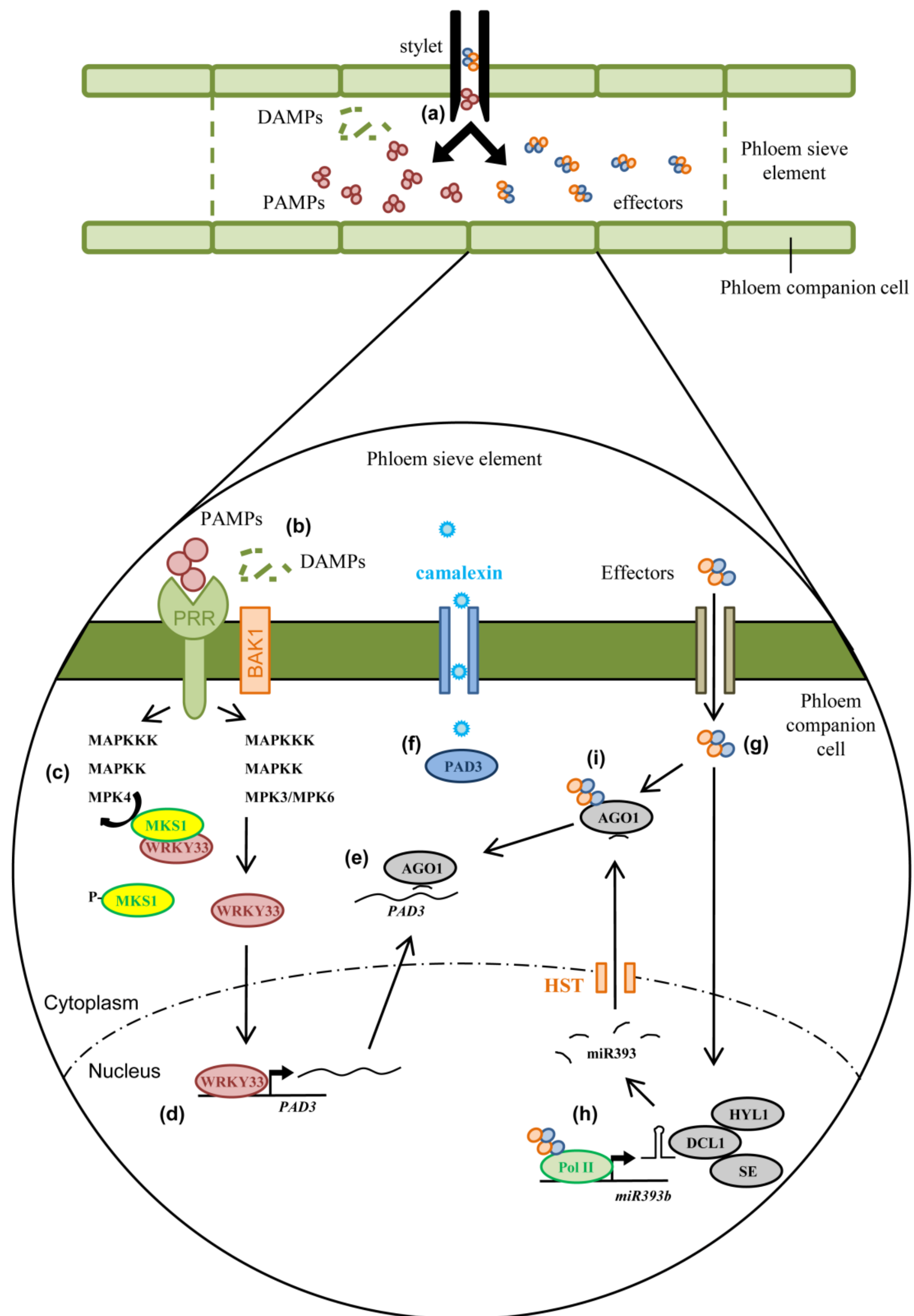

Figure 7.2 – Model of Col-0 response to M. persicae infestation.

(a) Puncturing of the phloem by aphid stylets generates Damage-Associated Molecular Patterns (DAMPs) and subsequent salivation introduces Pathogen-Associated Molecular Patterns (PAMPs) and effectors into phloem stream. **(b)** Detection of DAMPs or PAMPs by an unknown Pattern Recognition Receptor (PRR), perhaps associated with BAK1 triggers a host defence response. **(c)** Activation of MAP-kinase cascades trigger activation of WRKY33. **(d)** WRKY33 induces defence-related genes including PAD3. **(e)** Transcripts are subject to post-transcriptional regulation in cytoplasm, leading to a modest increase in PAD3 and small

amounts of camalexin production **(f)**. Additionally, salivary effectors may penetrate companion cells **(g)** to interact with components of the host miRNA pathway. This could increase *miRNA* gene transcription **(h)** or improve or modify targeting efficiency **(i)** leading to a dampened defence response.

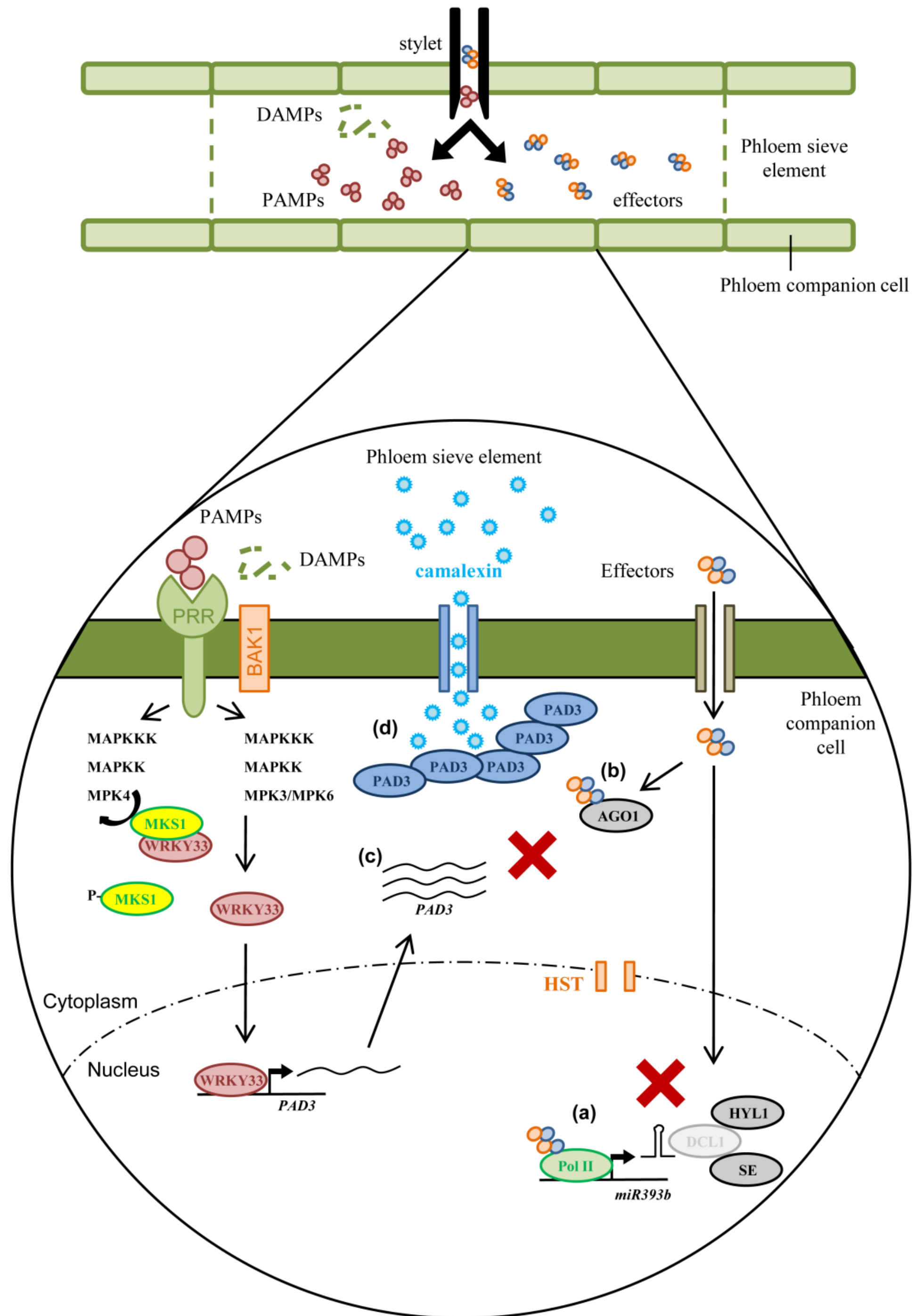

Figure 7.3 – Model of miRNA mutant resistance to *M. persicae* infestation.

In response to DAMPs or PAMPs, miRNA mutants generate a similar response to Col-0 (Fig. 7.2) with the following exceptions. **(a,b)** A *dcl1* mutant is unable to produce mature miRNAs, rendering effector manipulation of this pathway redundant. Removal of post-transcriptional regulation allows increased accumulation of *PAD3* mRNA **(c)**. This results in greatly increased amounts of PAD3 and camalexin production **(d)**.

In my experiments using PADp:GUS reporter lines (Chapter 4), I noticed that there was considerable variation in the levels of GUS staining around *M. persicae* feeding sites. Sequencing data indicated that at the whole leaf level, miR393 is responsive to aphid exposure (Chapter 6). A further possibility is that miR393 may be exposed to both positive and negative regulatory pressures during aphid infestation. As high levels of miR393 expression are deleterious to plant defence against aphids, detection of aphid feeding (perhaps by aphid PAMP perception) may trigger events to reduce miR393 expression or activity (Fig. 7.4). An aphid effector capable of inducing miR393 expression or activity may counteract this process (Fig. 7.4). This push and pull on miR393 expression may account for the variable miR393 expression measured by northern blot (Chapter 6) or variable levels of GUS staining around aphid feeding sites (Chapter 4).

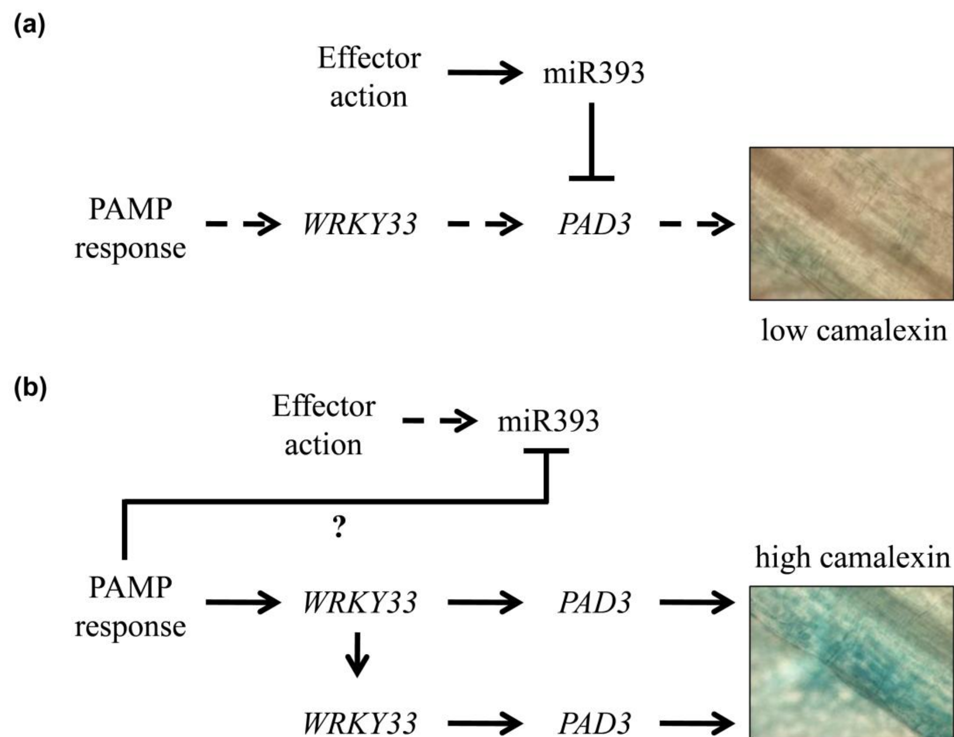

Figure 7.4 – miR393 as a node for determining levels of camalexin production.

(a) Elicitation of a PAMP response is weaker than effector action on miR393, resulting in either increased miR393 transcription or improved targetting efficiency. MiR393 suppresses camalexin production by targeting *PAD3*. **(b)** The PAMP response is stronger than effector action and suppresses miR393 transcription or activity by an unknown mechanism. This results in primary camalexin production through WRKY33-PAD3. WRKY33 self-activation reinforces the strength of this response. Solid arrows represent strong signalling, broken arrows represent weak signalling.

7.6 Aphid crop protection strategies

Currently, most aphid-control measures involve the spraying of chemical insecticides. However, this strategy is limited in its effectiveness. It may not be economically viable to spray crops when the yield losses imposed by aphids are lower than the cost of spraying (Dedryver *et al.*, 2010). In addition, as aphids are important primarily in virus transmission, spraying will have little benefit to plants that have already acquired a virus.

Finally, some aphid species (including *M. persicae*) are notorious for having developed resistance to multiple classes of chemical insecticide (Nauen & Denholm, 2005; Puinean *et al.*, 2010).

Another strategy to limit agricultural losses is the use of resistant plant varieties. This has been employed historically in the production of aphid-resistant apple varieties (Dedryver *et al.*, 2010). More recently, attempts at introducing aphid resistance into lettuce, barley and wheat have been met with varying degrees of success. One particularly successful example has been the use of the *Vat* gene to confer resistance against the melon aphid (*Aphis gossypii*). This durable R gene has been effective at controlling aphid infestations in France against asexual melon aphid populations (Dogimont *et al.*, 2010).

The use of aphid-resistant transgenic plants is another strategy that is currently being trialled. One strategy takes advantage of the aphid alarm signal (*E*)-β-farnesene (*E*βf). When aphids are attacked by predators, they release a sticky substance of which *E*βf is the primary constituent. Exposure to *E*βf causes other aphids nearby to drop off the host plant or to disperse to distant tissues. *Arabidopsis* does not naturally produce *E*βf, as it lacks *E*βf synthase, the enzyme for *E*βf production. However it does produce related sesquiterpenes from a common precursor. Transgenic *Arabidopsis* engineered to produce the pheromone synthesise sesquiterpene profiles enriched for *E*βf that elicit potent alarm and repellent responses in *M. persicae.* The pheromone also functions to attract aphid enemies (Beale *et al.*, 2006; de Vos *et al.*, 2010; Yu *et al.*, 2011). Therefore, transgenic production of *E*βf appears to protect plants by increasing rates of parasitism on aphid colonies.

To date, camalexin has only been detected in a small number of closely related cruciferous plants. However, as the precursors of camalexin are involved in auxin signalling, they are likely present in a significant number of plant species. It may be

possible to introduce the genes responsible for camalexin production (*CYP71A13*, *PAD3*) into other naturally non-producing species. As it is likely that aphids do not encounter high quantities of camalexin frequently in nature, this may hold potential as a crop protection strategy. However, introducing the camalexin biosynthetic machinery into foreign hosts may have unforeseen consequences. The channelling of large amounts of resources to phytoalexin production at the expense of auxin signalling may cause undesirable side-effects. Knowledge of tryptophan metabolism in each host would allow an assessment of this likelihood to be made. Additionally, whilst camalexin may be an effective defence against generalist aphids such as *M. persicae*, it is likely that brassica specialists have a higher tolerance to this plant compound. It might therefore be interesting to examine pathways involved in regulating basal defence responses which would be more effective against a number of aphid species.

7.7 Antagonising the antagonist

Perhaps a better strategy may be to target the braking action of miRNAs on metabolite production. This could be achieved in two ways. Firstly, it may be desirable to reduce miR393 activity upon aphid infestation. This could be achieved by target mimicry, where plants would be engineered to express a miR393 "sponge" or antagonist RNA. This principle has been successfully demonstrated in plants as a means of quenching miRNA activity (Todesco *et al.*, 2010). Although miR393 overexpressors or knockdowns do not exhibit significant developmental phenotypes relative to other miRNAs, there are miR393-associated phenotypes (Zhang, X *et al.*, 2011). This is likely due to miR393 involvement in auxin sensitivity. This could be overcome by expressing the construct under a specifically

aphid-inducible promoter. An example of this would be *At5g62360* (a pectin methylesterase inhibitor), a gene not induced by several plant pathogens but strongly induced by *M. persicae* feeding (de Vos *et al.*, 2005). Upon aphid perception, induction of the miR393 antagonist would quench available miR393, removing the braking action of miR393 on camalexin production. This would also only be active in cells able to perceive aphid feeding. By tailoring this response to be highly localised this would hopefully prevent unwanted side effects on other pathways, or a loss of plant productivity due to over-allocation of resources towards defence. Alternatively, emerging technologies such as transcription activator-like (TAL) effectors make introduction of single base mutations into genes of interest easily achievable. In *Arabidopsis*, this could be used to modify *WRKY33* mRNA to resist binding of miR393 or other miRNAs. This may eliminate the brake effect that miRNAs have on camalexin production. However, unless this strategy was also targeted to cells directly involved in aphid response, there may again be unforeseen side-effects of disrupting a miRNA-mRNA interaction at the whole organism level.

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