Role of Phytoplasma Effector Proteins in Plant Development and Plant-Insect Interactions

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“I am dying by inches, from not having any body to talk to about insects...”

Sharing similar passion to Darwin, I am delighted to be part of the endeavours to understand the ecology of insect-plant-microbe interactions, encapsulated in this photograph of female *Macrosteles quadrilineatus* settling on phytoplasma-infected *Arabidopsis thaliana* plant.
Abstract

Phytoplasmas are insect-transmitted plant pathogenic bacteria that dramatically alter plant development. Phytoplasma virulence protein (effector) SAP54 mediates degradation of host MADS-box transcription factors (MTFs) via 26S proteasome shuttle protein RAD23 to abolish normal flower development and produce leaf-like flowers (phyllody). Phyllodies are common symptoms in phytoplasma-infected plants worldwide. Why do phytoplasmas degrade MTFs and induce phyllody? Are changes in host plant morphology adaptive and benefit phytoplasma spread? Because phytoplasmas rely on their insect (leafhopper) vectors for transmission from plant to plant, I hypothesized that the vegetative tissues of the leaf-like flowers render plants more attractive to the insect vectors that will aid phytoplasma dispersal in nature.

I discovered that the induction of phyllody is genetically linked with enhanced insect egg-laying preference on the infected plants that exhibit the leaf-like flower phenotype. However, SAP54 enhances insect colonisation of plants independently from floral transition and the changes in plant morphology. Interestingly, male leafhoppers are required for the preference of females to lay eggs on SAP54 plants. Moreover, SAP54 suppresses insect induced plant responses in sex-specific manner by selectively downregulating male-induced defence and secondary metabolism pathways. Furthermore, I identified four MTFs that are expressed in plant leaves and play important roles in egg-laying preferences by leafhoppers and demonstrate sex-specific regulation by SAP54.

Taken together, phytoplasma effector SAP54 enhances insect vector colonisation of plants by suppression of insect-induced plant responses independent of developmental changes. This is likely to occur by targeting MTFs – a conserved regulators of both plant development as well as plant defence against herbivorous insects. In addition to developmental changes, degradation of MTFs by SAP54 may result in modulation of male-induced plant responses to attract female insects for egg-laying and aid phytoplasma spread in nature.
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**List of abbreviations**

°C – degrees Celsius; degrees Centigrade  
ANOVA – analysis of variance  
BLAST – Basic Local Alignment Search Tool  
bp – base-pair  
Ct – cycle threshold  
DMSO – dimethyl-sulfoxide  
DNA – deoxy-ribonucleic acid  
ETI – effector triggered immunity  
G – Gravitational constant \(G=6.67408 \times 10^{-11} \text{ m}^3 \text{ kg}^{-1} \text{ s}^{-2}\)  
GFP – green fluorescent protein  
GLM – generalized linear model  
HAMP – herbivore associated molecular patterns  
HR – hypersensitive response  
M – Molar (mol/L)  
MTF – MADS-box transcription factor  
NCBI – National Centre for Biotechnology Information  
nt - nucleotide  
OD – optical density  
PAMP – pathogen associated molecular patterns  
PCR – polymerase chain reaction  
PEG – polyethylene glycol  
PMU – Potential Mobile Unit (in phytoplasma genome)  
PTI – PAMP triggered immunity  
rt-qPCR – real-time quantitative polymerase chain reaction  
RAD23 – RADIATION SENSITIVE23  
RFP – red fluorescent protein  
RGB – red green blue (colour scheme)  
RNA - ribonucleic acid  
ROS – reactive oxygen species  
SAP – secreted Aster Yellows protein  
SDS PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis  
SEM – scanning electron microscope or standard error of the mean (statistics)  
SPAD – soil plant analyses development  
SVM – sequence variable mosaics (in phytoplasma genome)  
TAIR – The Arabidopsis Information Resource  
µ – micro \(\times 10^{-6}\); µL- microliter, µg- microgram
Useful definitions

*Epiphenomenon* - a secondary phenomenon that occurs in parallel to a primary phenomenon; a side-effect of a process (trait) that has been selected for its primary cause by natural selection.

*Effector* – usually a protein or another molecule (e.g., RNA) that is produced by one organism (e.g., a pathogen) and delivered into another organism to target specific host processes; in the context of host-pathogen interactions, these are also known as *virulence factors* which promote pathogen invasion and replication (virulence) into the host.

*Fitness* – the reproductive success of an organism (Darwinian fitness); in context of a plant pathogen, it refers to the ability to replicate in the host and success of transmission to an alternative host.

*Ecological niche* – as defined by Charles Elton and Eugene Odum, is the totality of resources used by an organism in a given habitat; the niche concept, therefore, includes all activities and biotic (trophic) interactions a species has while obtaining and using the resources needed to survive and reproduce. Ecological niche of a plant pathogen would include all vector and plant species that the pathogen utilises over a gradient of environmental parameters.

*Fundamental niche* – species ecological niche in absence of competitors for the same resources or interactions that limit species access to the potentially usable resources. Plant pathogens may have the capacity to infect a greater range of plant species (*fundamental niche*) than its actual or *realised niche* because of limited dispersal by vectors or other environmental factors.

*Realised niche* – the actual utilised ecological niche in presence of competitors for the same resources or interactions that confines species to use a limited amount of theoretically utilizable resources.

*Population dynamics* – changes in the number of individuals in a population as a result of birth (or multiplication), death, immigration and emigration.

*Epidemiology* – the study of causes and drivers of changes in population dynamics of infected (diseased) organisms.
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“Complexity is the prodigy of the world. Simplicity is the sensation of the universe. Behind complexity, there is always simplicity to be revealed. Inside simplicity, there is always complexity to be discovered.”


Chapter 1

Introduction on How Plant Pathogenic Bacteria Hitch a Ride

Part of this chapter is published in:

1.1. Getting a ride goes beyond physical contact

1.1.1. Overview

Because plants are often limited in their abilities to move long distances, it is essential that plant pathogens possess strategies that allow them to move from plant to plant. In contrast to plants, plant-feeding arthropods frequently move long distances. Insects are abundant and have evolved a diverse range of plant feeding and colonization strategies that enable pathogens to adapt in various ways to utilize these herbivores as vectors for their transmission. Some pathogens use feeding wounds made by chewing insects (e.g. beetles) to enter plant tissues for further colonization. Other pathogens invade the vascular tissues for subsequent systemic spread within the plant and are transmitted by piercing-sucking insect vectors (for example, insects in order Hemiptera), which penetrate their stylets and feed in the phloem and xylem.

Many pathogens use arthropods for transmission. For example, arthropods transmit the majority of plant viruses: a single insect species can transmit over a 100 different plant viruses. In contrast, relatively few viruses use other vectors for transmission, for example, fungal pathogens and nematodes (Campbell, 1996; Brown and MacFarlane, 2001). Even for plant pathogens that use other dispersal strategies, such as water drops, arthropods may nonetheless aid their spread in nature as passive or facultative carriers.

Strategies of virus transmission by arthropods have been extensively reviewed previously (Hogenhout et al., 2008a; Blanc et al., 2011). The goal of the introduction of this PhD thesis is to provide an overview of the contribution of insects in the transmission of bacterial pathogens. To this end, I will highlight for which bacterial pathogens insect vectors have been identified and then review several alternative strategies used by these pathogens to establish a physical association with insect vectors. Then I will discuss recent evidence suggesting that the transmission goes beyond a physical association with the insect and involves active modulation of plant processes by the bacterial pathogens to promote insect herbivore attraction, colonization and bacterial transmission.

After this general introduction, I will focus on phytoplasma as a specific example of insect-transmitted plant pathogenic bacteria and will discuss their associations with plant and insect hosts and how these bacteria may have adapted to facilitate their spread in nature by their insect vectors.
1.1.2. *Insect transmission of phytopathogenic bacteria evolved multiple times.*

Insect association with plant-parasitising bacteria appears to have evolved multiple times independently (Figure 1.1A). For some bacteria insects are only one of several ways to be carried to other plants. For example, diverse strains of *Ralstonia* (formerly *Pseudomonas solanacearum*, which cause potato brown rot, bacterial wilt of tomato, tobacco, eggplant and ornamentals, and Moko disease in banana (Meng, 2013), are dispersed by several routes including water drops and insects that visit infected plants, *e.g.*, bees (*Trigona corvine* [Hymenoptera: Aphidae]), wasps (*Polybia* spp. [Hymenoptera: Vespidae]) and flies (*Drosophila* spp. [Diptera: Drosophilidae]) (Figure 1.1A,B) (Agrios, 2004). Similarly, the bacterial ooze of *Erwinia amylovora*, which causes fire blight of pears, apples and other rosaceous plants, is distributed by rain as well as via many flying and crawling insects (Nadarasah and Stavrinides, 2011).

Many other bacteria utilize insects as primary vectors and form symbiotic relationships with them. For example, *Pectobacterium carotovorum* (formerly *Erwinia carotovora pv. carotovora*), which initiates soft rot in different types of fleshy plant organs, has acquired multiple alternative insect vectors: *Delia platura* (seed corn maggot [Diptera: Anthomyiidae]), *D. florilega* (bean seed maggot), *Drosophila busckii* [Diptera: Drosophilidae] in potato, *D. radicium* (cabbage maggot) in *Brassicaceae*, *D. antiqua* (onion black fly), *Tritoxa flexa* (seedcorn maggot [Diptera: Otitidae]), *Eumerus strigatus* (onion bulb fly [Diptera: Syrphidae]) in onion and *Macronoctua onusta* (iris borer [Lepidoptera: Noctuidae]) in iris (Figure 1.1A,B) (Agrios, 2004; Nadarasah and Stavrinides, 2011). Another example is *Erwinia tracheiphila*, the causal agent of cucurbit bacterial wilt, that is transmitted by *Acalymma vittatum* (striped cucumber beetle [Coleoptera: Chrysomelidae]) and *Diabroctica undecimpunctata* (spotted cucumber beetle) (Figure 1.1A,B) as well as other insects that cause wounds, such as grasshoppers (Latin, 1995; Nadarasah and Stavrinides, 2011). *E. tracheiphila* is known to overwinter in its two beetle vectors. Likewise, *Pantoea* (formerly *Erwinia* stewartii, the causative agent of Stewart’s wilt and leaf blight of maize, depends predominantly on its vectors *Chaetocnema pulicaria* (corn flea beetle [Coleoptera: Chrysomelidae]) and *Chaetocnema denticulate* (toothed corn flea beetle) for dissemination, and uses beetles as a secondary host for overwintering inside the insect body (Elliott, 1940).
Figure 1.1. Relations between insects and plant pathogenic bacteria. (A) Phylogenetic relationship of a range of different bacteria. The Maximum Likelihood phylogenetic tree is based on 16S rRNA gene sequences. Plant pathogenic bacteria are indicated in green and numbers highlighted in orange point to their insect vectors illustrated in panel B. The GenBank accession numbers are available in materials and
methods. (B) Simplified model of phylogenetic relation of a selection of insects based on
the current literature with ▼ and ▲ indicating insect groups comprised predominantly of
phloem feeders and xylem feeders, respectively. (C) Examples of disease symptoms
cau sed by various bacterial phytopathogens with illustration of their representative insect
vectors: Top section, from left to right: Diaphorina citri, Macugonalia leucomelas,
Macrosteles quadrilineatus and Dalbulus maidis. Bottom section, from left to right: HLB-
affected leaf, showing the characteristic blotchy mottle and vein corking (indicated by
arrow); CVC-affected leaf, with bright interveinal chlorotic yellow spots (indicated by
arrow); leaf-like flower (phyllody) caused by phytoplasma (left) in comparison to a healthy
flower (right) and phytoplasma infected maize exhibiting leaf reddening. Figure published
in Orlovskis et al. (2015).

Most phloem-inhabiting bacteria solely depend on insects for transmission
to the plant host. ‘Candidatus Liberibacter asiaticus’, ‘Ca. L. africanus’ and ‘Ca.
L. americanus’, the likely causative agents of citrus greening disease
(Huanglongbing), are transmitted by psyllids, including Diaphorina citri (Asian
citrus psyllid [Hemiptera: Liviidae]) and Trioza erytreae (African citrus psyllid
[Hemiptera: Trizidae]) (Figure 1.1B,C). A relative, ‘Ca. L. solanacearum’, the
causative agent of zebra chip disease in potatoes, stunting and chlorosis in
solanaceous species and foliage discoloration in carrot, is also transmitted by
psyllids, such as Bactericera cockerelli (potato psyllid [Hemiptera: Psyllidae]) in
Solanaceae, and B. trigonica and Trioza apicalis (carrot psyllid [Hemiptera:
Trizidae]) in Apiaceae (Munyaneza et al., 2010; Nadarasah and Stavrinides,
2011).

An example of an obligate insect-borne xylem-inhabiting bacterium is
Xylella fastidiosa, which causes Pierce’s disease of grapevine, citrus variegated
chlorosis (Figure 1.1C), alfalfa dwarf disease, phony peach disease and leaf
scorch diseases in almond and various other host species (Hopkins and Purcell,
2002). The bacterium is transmitted by an entirely different group of hemipteran
insects, including the xylem-feeding sharpshooter leafhoppers [Hemiptera:
Cicadellidae: Cicadellinae], e.g. Graphocephala atropunctata, Draeculacephala
minerva, Xyphon (=Carneocephala) fulgida, Homalodisca vitripennis and
spittlebugs [Hemiptera: Cercopidae] (Figure 1.1A,B) (Janse and Obradovic,
2010; Nadarasah and Stavrinides, 2011).
Bacteria from a different clade within the Gram-negative proteobacteria, the phloem-restricted ‘Ca. Arsenophonus phytopathogenicus’ and ‘Ca. Phlomobacter fragariae’, which are sugar beet and strawberry pathogens, respectively, are also transmitted by phloem-feeding insects, although from a different group of insects within the Hemiptera, the planthoppers *Pentastiridius leporinus* [Hemiptera: Cixiidae] and *Cixius wagneri* [Hemiptera:Cixiidae], respectively (Danet *et al.*, 2003; Bressan *et al.*, 2011; Bressan, 2014).

Moreover, the more distantly related phytoplasma and spiroplasma plant pathogens of the class Mollicutes, which are cell wall-less obligate parasites and evolved from Gram-positive bacteria, are also phloem inhabitants and are transmitted by phloem-feeding piercing and sucking insects (Figure 1.1). The bacteria occur worldwide and induce leaf yellowing, stunting, decline and death in a broad range of plant species and phytoplasmas also modulate key processes in plant development through inducing, for example, shoot and root proliferation and greening of flowers (Hogenhout *et al.*, 2008; Gasparich, 2010). Phytoplasmas are transmitted by various hemipteran insect species, including leafhoppers [Hemiptera: Cicadellidae], planthoppers [Hemiptera: Fulgoroidea] and psyllids [Hemiptera: Psyllidae], whereas spiroplasmas have only leafhoppers as vectors.

Thus, plant pathogenic bacteria have adapted to employ the feeding mechanisms from a diverse range of insect species for transmission to plants, and insect transmission has evolved multiple times, particularly for phloem-inhabiting bacteria.
1.1.3. Plant pathogenic bacteria physically connect with insect guts or whole bodies.

At least three different strategies are known for bacterial plant pathogen transmission by insect vectors (Figure 1.2).

Figure 1.2. Schematic illustrations of insect transmission of phytopathogenic bacteria. (A) *Pantoea stewartii* (red flagellated structures) are associated with the alimentary tract (foregut, midgut and hindgut), including the crop and Malpighian tubules (mt) of the beetle vectors. Transmission may occur by deposition of infective feces (frass) or regurgitate on leaf surfaces where feeding takes place; once introduced into feeding wounds, *P. stewartii* spreads through intercellular spaces and xylem vessels. (B) Phytoplasmas, spiroplasmas and liberibacters are transmitted in a persistent-propagative manner. After acquisition during phloem sap ingestion in infected plants, these bacteria (red dots) invade the midgut tissue and multiply or accumulate in various internal organs of the vector, including filter chamber, visceral muscles, Malpighian tubules and the salivary gland, from where they are inoculated via saliva in the phloem of healthy plants. (C) *Xylella fastidiosa* has a non-circulative propagative relationship with sharpshooter vectors. After acquisition during xylem sap ingestion, the bacterial cells (red rods) attach to the foregut cuticle, especially in the precibarium, where they multiply and form biofilm (detailed in the box); detached bacterial cells are hypothetically inoculated via egestion in the xylem vessels of healthy plants. Longitudinal section of leaf: epidermis (ep), parenchyma (pr), phloem sieve element (ph) and xylem vessel (xl). Insect structures: foregut (fg), midgut, hindgut, filter chamber (fc), crop (cr), salivary gland (sg), Malpighian tubules (mt), esophagus (es), cibarium (cb), precibarium (pc), precibarial valve (pv). Frass (fr). Figure published in Orlovskis *et al.* (2015).

Many insect-transmitted bacterial pathogens appear to associate with the digestive tract of their vectors where they persist for several days or weeks,
possibly overwintering in the insects (Garcia-Salazar et al., 2000; Basset et al., 2003; Ammar et al., 2014). Transmission is thought to occur by regurgitates on feeding wounds and deposition of infective feces (frass) (Mitchell and Hanks, 2009; Shapiro et al., 2014) (Figure 1.2A). For some of these bacteria virulence factors associated with insect transmission have been identified. *P. carotovorum* requires at least two genes for infection of *Drosophila*, including *Erwinia virulence factor* (Evf), which improves bacterial survival in the guts of flies (Basset et al., 2003; Quevillon-Cheruel et al., 2009). In addition to the Hrc-Hrp Type III Secretion System (T3SS), which is essential for maize pathogenesis (Coplin et al., 1992), *P. stewartii* possesses a second T3SS, the *Pantoea* secretion island 2 (PSI2), which is involved in insect colonization and persistence of the bacterium in the vector (Correa et al., 2012; Ammar et al., 2014).

Phloem-inhabiting bacterial pathogens, such as *Ca. Liberibacter* spp., phytoplasmas and spiroplasmas, are transmitted in a propagative persistent manner. After ingestion, these bacteria invade the vector midgut and adjacent muscle cells, from where they reach the hemolymph and systemically colonize other insect organs, including the Malpighian tubules and salivary glands, at which point the insect vectors become competent to inoculate the bacteria into plants (Figure 1.2B) (Kwon et al., 1999; Ozbek et al., 2003; Ammar et al., 2011; Sengoda et al., 2014). The time between acquisition and inoculation is known as the latency period and can take days to months, depending on the interplay between the pathogen and the life-cycle of insect vector. In the case of ‘*Ca. P. pronorum*’ and its vector, *Cacopsylla pruni*, there is a remarkable decrease in transmission efficiency during an eight-month long winter latency period when the insect vector is on its overwintering host. *C. pruni* become efficient vector by spring time when the insect migrates to the primary host plant (*Prunus* spp.) (Thebaud et al., 2009).

The adaptation of bacteria to plant phloem and various insect tissues is associated with a dramatic change in expression of bacterial genes, including virulence factors (Toruño et al., 2010; MacLean et al., 2011; Oshima et al., 2011; Yan et al., 2013). As well, several membrane proteins that are involved in adherence of bacteria to insect cells have been identified, including the Antigenic membrane protein (Amp) of phytoplasma (Suzuki et al., 2006) and adhesion-related proteins of *Spiroplasma citri* (ScARPs) (Beven et al., 2012).
In contrast to bacteria that invade insects discussed above, the xylem-inhabiting \textit{X. fastidiosa} is foregut-borne and non-circulative in its vectors (Figure 1.2C). After acquisition, the bacterial cells attach to the cuticle in the cibarium and precibarium where they multiply and form a biofilm. \textit{X. fastidiosa} cells are detached from this site and inoculated into plants by egestion (Almeida and Purcell, 2006; Backus and Morgan, 2011). The pathogen’s ability to attach and colonize both the plant and the vector is dependent on a quorum-sensing mechanism involving a diffusible signaling factor (DFS) that is sensed by components of the regulation of pathogenicity factors (\textit{rfp}) cluster (Newman et al., 2004; Almeida et al., 2012; Baccari et al., 2014). Early adhesion and retention in the insect involve the afimbrial proteins HxfA and HxfB and the fimbrial protein FimA (Killiny and Almeida, 2014). The \textit{X. fastidiosa} extracellular polysaccharides (EPS) also plays a role in insect transmission (Killiny et al., 2013).

Thus, bacteria associate with insect vectors physically by invading the insect intestines or whole bodies or by attaching to the cuticle in the insect foregut. Moreover, this association occurs at a molecular level. This requires adaptation of bacterial pathogens to the anatomy, physiology and life cycle of their insect vectors, showing that the interactions between plant bacteria and their insect vectors go beyond physical contact.

\textbf{1.1.4. Bacteria modulate plant processes to the benefit of insect vectors}

The finding that bacteria dramatically change gene expression and produce specific virulence factors for insect invasion is suggestive of extensive co-evolution between insects and bacteria. The association with insects appears mostly beneficial for the bacteria. However, the benefit for the insects should be considered too. Indeed, \textit{P. carotovorum} induces rotting of fruit, which facilitates the life cycle of fruit flies (Basset et al., 2003). Recent findings indicate that bacterial pathogens use various strategies to promote attraction and colonization of their insect vectors (Figure 1.3).
Figure 1.3. Insect-vectored bacterial parasites modulate plant cellular and physiological processes to alter plant phenotypes and enhance insect vector colonisation and pathogen dissemination in nature. During plant-pathogen interactions, bacteria secrete extracellular virulence factors that can modulate various cellular and physiological processes in the host plant. Phytoplasma secreted effector proteins interact and destabilize conserved transcriptional regulators that have pleiotropic roles in plant development and defense responses. Liberibacter may also secrete putative effectors that target certain plant genes and interfere with plant processes. *Xylella fastidiosa* produces virulence factors that mediate bacterial spread through xylem and regulate biofilm formation. The outcome of plant-pathogen interactions at cellular level is the change of certain aspects of plant phenotype, including the development of disease symptoms. Insect responses to changes in plant phenotype can further play a significant role in plant-insect interactions and benefit pathogen transmission in nature. Modulation of the composition of organic volatile emissions and induction of foliar yellowing are implicated in attraction of insect vectors to infected plants. Suppression of plant defenses against herbivores facilitates insect feeding and egg laying, thus enhancing likelihood for pathogen acquisition. However, deceptive attraction to sub-optimal infected hosts or disruption of phloem and xylem flow by the bacterial parasite can promote insect dispersal and subsequent pathogen dissemination. Solid lines indicate direct effects. Dashed lanes signify putative effects or suggested roles of bacteria-induced plant phenotypes in plant-insect interactions. Pointed arrows indicate generation or modulation of an effect, block arrows signify complete or partial abolishment of an effect. Abbreviations: MTFs – MADS-domain transcription factors,
Pathogens promote insect attraction to infected host plants by altering the visual or olfactory cues that insects use to locate a suitable host plant. For example, ‘Ca. Phytoplasma mali’ increases production of the sesquiterpene β-caryophyllene that makes infected plants more attractive to its psyllid vector C. picta (Mayer et al., 2008a,b). Similarly, ‘Ca. Liberibacter’ induces changes in the complex blend of citrus organic volatile compounds to make plants more attractive for their psyllid vectors (Mann et al., 2012; Mas et al., 2014). ‘Ca. L. asiaticus’ induces the production of methyl-salicylate that may mimic a psyllid-derived pheromone (Mann et al., 2012). The characteristic yellowing symptoms in ‘Ca. L. asiaticus’-infected plants are associated with perturbations in phloem sugar loading, starch accumulation and phloem blockage (Kim et al., 2009; Koh et al., 2012; Martinelli et al., 2013), and modulation of foliar coloration may play a role in insect vector attraction (Figure 1.3).

Once the insect vector is attracted to an infected plant, plant parasitic bacteria may use various mechanisms to enhance insect feeding and egg laying thus promoting pathogen acquisition. Changes in solute transport in phloem or xylem, alteration of plant nutritional quality, or suppression of herbivore-induced defense responses can affect insect feeding behavior. Phytoplasma-secreted effector proteins are known to destabilize conserved transcriptional regulators, leading to changes in both plant development and defense against herbivores. ‘Ca. P. asteris’ strain Witches’ Broom protein SAP11 directly interacts with TEOSINTE BRANCHED1 (TCP) transcription factors and suppresses jasmonic acid (JA) production and salicylic acid-mediated defense responses, increasing the fecundity of aster leafhopper Macrosteles quadrilineatus, the insect vector of phytoplasma (Sugio et al., 2011a, 2014; Lu et al., 2014). Virulence factor, TENGU, was shown to affect plant development and regulation of auxin-dependent genes (Hoshi et al., 2009). Another phytoplasma effector SAP54 is known to directly destabilize plant MADS-box transcription factors, thus abolishing normal floral development as well as enhancing leafhopper colonization (MacLean et al., 2011, 2014).
Asymptomatic plants at early stage of *X. fastidiosa* infection favor insect attraction and pathogen acquisition but late infection symptoms enhance insect dispersal and pathogen dissemination (Marucci *et al.*, 2005). When reached certain density, *Xylella* quorum-sensing mechanisms can induce biofilm formation in response to certain host extracellular polysaccharides (Killiny and Almeida, 2009; Killiny *et al.*, 2013). Biofilm formation leads to xylem blockage, corking of veins and water stress symptoms, which may favor rejection of symptomatic plants by sharpshooter vectors of *X. fastidiosa*.

In summary, there is indirect and direct evidence that bacterial pathogens modulate plant processes to promote attraction and plant colonization by insect vectors.

### 1.2. *Global occurrence of phytoplasmas and their interaction with plant and insect hosts*

#### 1.2.1. Overview

Plant pathogenic bacteria possess various virulence factors (effectors) that modulate microbial recognition and induced plant defences (reviewed in Boller and He, 2009). Insect-transmitted plant pathogenic bacteria also possess virulence factors for the establishment of physical interactions with their arthropod vectors and possibly for modulation of plant pathways involved in insect attraction and defence responses to insect vectors (Orlovskis *et al.*, 2015). Furthermore, vector-borne plant pathogens may carry virulence factors that have direct effects on the insect vector, such as alterations of (feeding) behaviour and rerouting of vesicle trafficking pathways to facilitate pathogen migration through the insect vector and transmission to plants.

Phytoplasmas are globally widespread plant pathogens and require insects for their transmission from plant to plant. Different sets of effector genes are upregulated in phytoplasmas that are in the insect vectors versus those in the plant hosts (MacLean *et al.*, 2011). Currently, the functions of a few phytoplasma effectors have been characterised and these effectors were shown to alter plant development and promote insect vector colonization (Sugio *et al.*, 2011b). Research on these effectors have provided unique insights into how pathogens “manipulate” their host as “puppets”. Phytoplasma-plant-insect interactions
provides a unique model system for further translational research to better understand of how plant pathogens and insect herbivores co-evolve and find the Achilles’ heels of both bacterial pathogens and insect vectors for informing future disease management strategies. In the next sections of this introduction, I will provide a more detailed overview of what is known about phytoplasmas and their ability to modulate plant processes and insect-plant interactions that may be fundamental for their spread in nature.

1.2.2. Phytoplasmas are wall-less plant pathogens

Phytoplasmas survive only in their plant or insect hosts and cannot be cultured on artificial media in vitro. Within plants, phytoplasmas are limited to the sieve elements of phloem – tissue that transports the products of photosynthesis from mature leaves to other parts of the plant for growth or storage (Figure 1.4). Phytoplasmas are small bacteria of approximately 500 nm in diameter. They are usually spherical but may also flask-like or tubular shapes. Unlike most other bacteria, they lack rigid cell wall, envelope or secondary membrane. Instead, phytoplasmas possess a single unit membrane (Lee et al., 2000). Doi et al. (1967) observed that the ultrastructure of phytoplasmas resembles another group of pathogenic bacteria - animal and human mycoplasmas. Similarly to mycoplasmas, phytoplasma growth is constrained by tetracycline (Shikata et al., 1969), an antibiotic that targets bacterial ribosomes and inhibit protein synthesis. Because of the lack of outer cell wall, phytoplasmas are insensitive to antibiotics that inhibit cell wall synthesis. The strength and rigidity of cell membranes in many wall-less bacteria may be achieved by higher membrane sterol contents, making them more similar to the membranes of eukaryotic cells (Barton, 2005).
Figure 1.4. Transmission electron micrograph of Aster Yellows phytoplasma Witches’ Broom in an immature sieve element (se1) of an aster plant. Phytoplasma (arrow-head) are distributed throughout the cytoplasm and around a degenerated nucleus (n) of an immature sieve element. In mature sieve elements nuclei degenerate and disappear entirely. Bacteria migrate to adjacent sieve elements by passing through the pores (arrow) in sieve plates (sp). Phytoplasmas are not found in companion cells (cc). Asterisks indicate flask-shaped phytoplasmas during cell division. After budding-off the mother cell, phytoplasma increase in size and take round shape (black arrowhead). Scale bar 1µm. Image taken from Hogenhout et al. (2008b).

1.2.3. Early classification of phytoplasmas

Before the second half of the 20th century many yellowing diseases and witches’ broom symptoms in plants were believed to be caused by plant viruses instead of bacteria. However, in 1967, Doi et al. published a paper describing the agents observed in symptomatic plants as bacteria resembling the human and animal-pathogenic mycoplasmas, naming them “mycoplasma-like-organisms” (MLOs) (Doi et al. 1967; Lee et al., 2000). However, this nomenclature lacked a precise taxonomic definition. The term MLOs did not distinguish between the different physiological and structural properties of mycoplasma-like plant pathogens. For example, the causal agent of corn stunt disease is a motile, helical, filamentous mycoplasma-like-organism (Davis et al., 1972; Daniels, 1979) which is structurally distinct from the sphere-shaped bacterial agent of aster yellows disease.

In order to demonstrate that a specific microorganism is the causal agent of a particular disease, Robert Koch (1887) postulated that disease-causing organism must be cultivated in pure culture outside the host-organism, inoculated
into healthy susceptible host, and isolated again from these hosts. Because phytoplasmas cannot grow in artificial media outside host-organisms, Koch’s postulates cannot be fulfilled. These principles were important for differentiating and assigning species status to various MLOs. For example, the causal agent of corn stunt (now known as *Spiroplasma kunkelii*) can be cultured in cell-free media and transmitted to healthy maize plants by leafhoppers that were injected with *in vitro* culture of the pathogen (Chen and Granados, 1970). Inability to obtain pure phytoplasma cultures in laboratory hindered the identification and systematics of MLOs until the development of molecular classification methods.

1.2.4. Phytoplasmas belong to the class Mollicutes

Phylogenies inferred from 16S rRNA gene sequences suggest that phytoplasmas have diverged from *Acholeplasma* spp. within the class Mollicutes (Gundersen *et al*., 1994). Other previously described MLOs were found to belong to the class Mollicutes and form four major paraphyletic groups: Hominis, Pneumoniae, Spiroplasma and Anaeroplasma, the latter containing Acholeplasma and Phytoplasma (Weisburg *et al*., 1989). Phytoplasmas and spiroplasmas are the only groups of plant pathogenic bacteria in the class Mollicutes. However, plant pathogenic lifestyle is not unique to the Mollicutes, and has evolved independently in several groups of bacteria (Figure 1.1).

1.2.5. Phytoplasmas require plants and insects for survival and dissemination

Phytoplasmas infect and replicate in organisms that belong to two different kingdoms – plants and animals (insects). Their survival and dissemination depends on successful invasion and cycling between the two hosts (Figure 1.5). Phytoplasmas are transmitted from plant to plant by certain families of sap-feeding insects – leafhoppers (Cicadellidae), planthoppers (Fulgoridae) and psyllids (Psyllidae), with leafhoppers being the most common group of vectors (Lee *et al*., 2000). Leafhoppers are flying insects that belong to the order Hemiptera, suborder Auchenorrhyncha. Hemipteran insects develop from egg to adult stage via incomplete (hemimetabolous) metamorphosis. The possession of piercing-sucking mouthparts makes hemipteran insects excellent vectors of bacterial and viral plant pathogens. Stylets projecting from the insect proboscis
are well-adapted to penetrate plant tissue via apoplast (extracellular space of plant tissues), to puncture sieve elements of phloem or xylem tissue and to suck sugar-rich phloem sap or “drink” from the xylem. As a result of such feeding behaviour these insects often acquire plant bacteria, including phytoplasmas, from infected plants and transmit them to a different host. Leafhoppers are excellent flyers and frequently migrate long distances, thereby facilitating dispersal of the plant pathogens.

Figure 1.5. Phytoplasmas are transmitted from plant to plant by insect vectors. (A) Phloem-feeding insects acquire phytoplasma from infected plants during sustained phloem feeding phase. (B) Latent period is the time required for phytoplasma to replicate and systemically spread throughout the insect body. Systemic invasion of aster leafhopper *Macrosteles quadrilineatus* by Aster Yellows phytoplasma strain Witches’ Broom takes approximately 10 days. (C) Phytoplasma are injected into healthy susceptible plants together with insect saliva during plant probing. Insect stylets can reach phloem tissue and inoculate the plants with phytoplasma. (D) Phytoplasma replicates in phloem and is transported to other parts of plant following phloem movement from source to sink organs. Image taken from Sugio *et al.* (2011).
1.2.6. Aster Yellows phytoplasmas have a broad host-plant range

Molecular classification has resulted in the identification of more than a dozen different phytoplasma groups and subgroups. In spite of difficulties assigning genus and species status to phytoplasmas in the past, the Phytoplasma Working Team of the International Research Project for Comparative Mycoplasmology (IRPCM) proposed the name “Candidatus Phytoplasma” to establish a formal classification based on molecular identification (Bertaccini, 2007). Designation ‘Candidatus’ (L. Candidatus, a candidate) is given to a bacterium that is well characterised but cannot be maintained in a Bacteriology Culture Collection. A novel ‘Candidatus Phytoplasma’ species is described if its 16S rRNA gene sequence has >2.5% dissimilarity to that of any previously described ‘Candidatus Phytoplasma’ species. ‘Candidatus’ is usually abbreviated to ‘Ca.’. There are now at least 28 formally described ‘Ca. Phytoplasma’ species. Further classification of phytoplasma strains is based on restriction fragment length polymorphism analysis of 16S and 23S rRNA genes, the 16S/23S intergenic spacer region or ribosomal proteins (rp) (Lee et al., 2000; Hogenhout et al., 2008b). Alternative methods to infer phytoplasma phylogeny are based on coding sequences of prokaryotic elongation factor Tu (tuf) or secretion system genes (e.g., secA) (Makarova et al., 2012; Hodgetts et al., 2016) but these are largely congruent with the 16Sr-based phylogenies.

Aster yellows group (16SrI) is currently the largest described phytoplasma group and has a worldwide distribution (Lee et al., 2004). ‘Candidatus Phytoplasma asteris’ includes all known subgroups within 16SrI group and can infect about 200 different plant species, including many crops (Figure 1.6). The 16SrI-A, 16SrI-B, 16SrI-C phytoplasmas have broad host-plant ranges and wide geographic distributions. These phytoplasmas are mainly transmitted by the polyphagous leafhoppers Macrosteles spp., Euscelis spp., Scaphytopius and Aphrodes spp. (Lee et al., 2004). However, subgroup 16SrI-B (rp-L) also includes maize bushy stunt phytoplasma (MBSP), which only infects maize and is transmitted by a maize-specialist leafhopper Dalbulus maidis. Similarly, a 16SrI-D phytoplasma infects only two species of paulownia trees (Paulownia taiwaniana and Paulownia tomentosa) in East-Asia.
Figure 1.6. Aster yellows group phytoplasmas are generalists and infect diverse families of plant hosts. ‘Candidatus Phytoplasma asteris’ encompass all described subgroups of Aster Yellows (16SrI) group that share >97.5% similarity in 16SrRNA gene sequence. Additional biological and genetic criteria, such as the sequence variation of ribosomal protein (rp) is used to distinguish between various subgroups. Aster Yellows group phytoplasmas infect wide range of monocot and dicot herbaceous and woody plants. Adopted from Lee et al. (2004).

<table>
<thead>
<tr>
<th>Phytoplasma subgroup</th>
<th>Examples of host-plants</th>
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<tbody>
<tr>
<td>16Srl-A</td>
<td>China aster, lettuce, tomato, celery, broccoli, kale, carrot, dill, parsley, potato, cabbage, onion, oat, wheat, eggplant, turnip, periwinkle, coneflower, gladiolus, erigeron, dogwood, cyclamen, chrysanthemum, daisy, dill, fennel, primula etc.</td>
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<tr>
<td>16Srl-B (rp-B)</td>
<td>hydrangea</td>
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<tr>
<td>16Srl-B (rp-K)</td>
<td>maize</td>
</tr>
<tr>
<td>16Srl-B (rp-L)</td>
<td>clover, strawberry, anemone, olive</td>
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<td>16Srl-C</td>
<td>paulownia</td>
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<tr>
<td>16Srl-D</td>
<td>blueberry</td>
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<td>16Srl-F</td>
<td>apricot</td>
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<tr>
<td>16Srl-K</td>
<td>strawberry</td>
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</table>

1.2.7. Phytoplasmas are economically important plant pathogens

Phytoplasmas cause disease in several hundred different plant species, including ornamental plants, food crops (cereals, vegetables, fruit trees), shrubs and trees worldwide (Lee et al., 2000) (Figure 1.7). Phytoplasmas infect both herbaceous dicot and monocot crops. ‘Ca. P. mali’, ‘Ca. P. pyri’, ‘Ca. P. pronorum’ cause considerable yield losses of stone fruit trees, including peach, cherry, plums, apricots as well as pears and apples (Seemüller and Schneider, 2004). ‘Ca. P. aurantifolia’ and ‘Ca. P. trifoli’ are disease agents of several legume diseases such as sesame and soybean phyllody or peanut witches’ broom, inflicting considerable yield losses in Turkey, India, Myanmar and China (Lee et al., 2000). Phytoplasmas are important pathogens of grasses (Poaceae) (Rosete and Jones, 2010). Rice (Oryza sativa) in Asia is affected by two phytoplasma disease agents causing rice yellow dwarf (RYD, ‘Ca. Phytoplasma oryzae’) and rice orange leaf (ROL). ‘Ca. Phytoplasma oryzae’ is responsible for yield losses of sorghum (Sorghum spp.) in the tropics and subtropics, especially Africa.
Phytoplasmas cause serious problems in sugarcane productions in Brazil and India, the largest sugarcane producing countries, where the crop is mostly used for biofuel production. Phytoplasmas can also infect gymnosperms, for example, pine trees in Spain, Germany and Lithuania (Valiunas et al., 2015; Schneider and Torres, 2016) and juniper in Poland (Krawczyk et al., 2016).

Although many phytoplasmas are detrimental to most plants, infection with these organisms has been practiced to increase the market value of commercially grown poinsettias (*Euphorbia pulcherrima*) (Bertaccini, 2007). Single or mixed infections with various phytoplasmas increase bushiness and branching of these ornamental plants making them more attractive to consumers.

![Figure 1.7. Phytoplasmas are economically important pathogens in agricultural regions worldwide. The occurrence of most important disease-causing phytoplasmas taken from (Foissac and Wilson, 2010).](image)

**1.2.8. Plant-phytoplasma-insect interactions play a key role in disease origin and spread**

The epidemiology of phytoplasma diseases in agricultural systems depends on the population structure of the host plants (monoculture vs. policulture crop), availability of alternative host plants (weedy plants outside the boundaries of a crop field) as well as host-plant fidelity and abundance of the insect vectors. The following examples demonstrate the agricultural relevance
and importance to understand plant-microbe-insect interactions at population level as well as the effects of changing climate and pest management practices on these pathosystems.

Lethal yellowing disease (caused by ‘Ca. Phytoplasma palmae’) of coconuts (Cocos nucifera) is a good illustration for how current crop cultivation methods affect pathogen spread in the field. Monocultures of homogeneously distributed coconut palms are prone to rapid spread of disease from a point source of infection to surrounding genotypically identical plants. Distance of spread of new infections increases with time. Current control methods rely on removal of coconut trees around the symptomatic plants to slow the spread of infection. Nevertheless, sacrificing fractions of the standing crop involves a high yield cost itself. Moreover, during the incubation period (period post infection and prior to symptom development) phytoplasmas can be transmitted as far as 100 km from the initial (primary) point of disease origin. Because coconut trees are only removed in the near vicinity of symptomatic plants, establishment of new disease foci create sources of secondary infection (Bonnot et al., 2010).

Phytoplasmas can decrease the efficiency of some cultural pest control practices. The Push-Pull Technology has been very successful at managing insect pests and parasitic weeds in Africa (Pickett et al., 2014). This method relies on intercrops that emit allelochemicals to deter pests or attract natural enemies and use of trap-plants that attract pest species away from the main crop. Napier grass (Pennisenum purpureum) is an abundant trap-plant and also the main fodder plant in Eastern Africa. Koji et al. (2012) reported recent trend in the spread of the agent of Napier stunt disease in areas where the Push-Pull Technology is applied. The disease is caused by Napier grass stunt phytoplasma (NGSP; ‘Ca. Phytoplasma oryzae’), vectored by a leafhopper Maiestas banda and several other planthopper species. They found that rainfall is a strong determinant of insect abundance in this leafhopper and planthopper vector complex. Considering the importance of insects as phytoplasma vectors, changing global and regional climate may have an effect on disease incidence and severity in P. purpureum.

Range expansion of insect vectors and availability of phytoplasma reservoirs in herbaceous weedy plants are important factors for pathogen spread to new geographical regions. Bois noir (BN) of grapevine (Vitis sp.) is the most widespread grapevine yellows disease in European and Mediterranean viticulture
regions. The causal agent of this disease, ‘Ca. Phytoplasma solani’ (16 SrXII), has two isolates, tuf-a and tuf-b, the former is associated with its wild host stinging nettle (Urtica dioica) and the latter with field bindweed (Convolvulus arvensis). The host switch of planthopper Hyalesthes obsoletus from C. arvensis to U. dioica resulted in acquisition of tuf-a phytoplasma. This led to emergence of tuf-a BN in vineyards of France and Germany where the disease had not been recorded before (Maixner, 2010). Molecular studies of population history and host-plant association (Johannesen et al., 2012; Imo et al., 2013) suggest that the introduction of tuf-a phytoplasma to vinefields was driven by vector host shifts and subsequent sympatric diversification. The spread of BN disease from Southern to Northern Germany was a result of range expansion of the vector. Mean ambient temperature can mediate latitudinal expansion of the geographical range of insect-vectors (Bale et al., 2002). This might expose insects to new host plants species and facilitate acquisition of new disease-causing agents through host switch initiating new disease cycles. It would be very difficult to predict the behaviour of such open epidemiological systems (i.e., where pathogen and vectors have multiple alternative hosts) under different global change scenarios. Flavescence dorée (FD, ‘Ca. Phytoplasma vitis’) is another grapevine yellows disease agent. Grapevine is the only host for this phytoplasma and its leafhopper vector Scaphoideus titanus making this phytoplasma-insect-plant interaction a closed epidemiological system compared to BN (Constable, 2010). Pest management practices can be more effective for closed systems where vector population growths depend on single host plant species as opposed to open systems where vectors reproduce and overwinter in wild plant refugia (which often also serve as reservoirs of the disease agents) surrounding the crop fields.

1.2.9. Phytoplasmas adapt to their plant-host and insect vector

Phytoplasma fitness depends on the success of the pathogen at the key stages of its life-cycle, as follows: 1) phytoplasmas have to be acquired by insect vectors from infected plants; 2) phytoplasmas have to propagate within plant-hosts and insect-vectors; 3) phytoplasmas have to be inoculated into healthy plants by insects to ensure continuous generations of the pathogen. Because phytoplasmas transmission depend on their plant hosts and insect vectors and interactions between insects and plants, phytoplasma fitness is likely to
dramatically increase in cases where (a) phytoplasmas do not kill their plant hosts and insect vectors too early and (b) phytoplasmas evolved mechanisms to modulate plant and insect processes to promote phytoplasma colonization and spread. I will discuss each of them separately.

Phytoplasmas infect and replicate in both insects and plants. Their survival and dissemination depends on successful invasion and cycling between these two hosts. Therefore, phytoplasmas have to adapt their own biology to maximise the success of colonisation, replication and cycling between host plants and insect vectors. During phloem-feeding insects acquire phytoplasma from infected plants (Figure 1.8A). Phytoplasmas attach to cell membrane of microvilli of insect guts and enter gut walls (Figure 1.8B). Phytoplasmas may possess similar tip structures found in spiroplasma and mycoplasma to attach to the apical plasmalemma (Hogenhout and Loria, 2008) and possess specific membrane proteins in order to be recognised and internalised by insect gut wall (Fletcher et al., 1998). Indeed, antigenic membrane proteins (AMPs) of ‘Ca. Phytoplasma asteris’, onion yellows strain M (OY-M) and chrysanthemum yellows phytoplasma (CYP), interact with cytoskeletal proteins actin and myosin as well as ATP synthase in smooth visceral muscles surrounding the insect intestine (Suzuki et al., 2006; Ishii et al., 2009; Galetto et al., 2011). These studies demonstrate that AMP-cytoskeletal complexes were formed only in insect species that are known to transmit OY-M and CYP, suggesting that bacterial AMPs have evolved to interact with specific insect proteins, determining vector specificity. Evidence that phytoplasmas adapt to their insect vectors was demonstrated by another experiment in which OY-M was transferred from plant to plant by either grafting or insect transmissions. The phytoplasma line propagated by grafting lost the ability to be insect transmitted and this was associated with lack of part of the promoter region upstream of an open reading frame (named ORF3), encoding a putative transmembrane protein, in the grafted versus the insect-transmitted OY-M. (Ishii et al., 2009). Thus, phytoplasma proteins involved in insect vector transmission appear subject to strong selection.

Upon replicating in the mid-gut epithelial cells, phytoplasmas enter the insect hemocoel and circulate to other tissue via the insect haemolymph (Figure 1.8C), finally reaching the salivary glands (Figure 1.8D) (Hogenhout et al., 2008). Phytoplasmas probably interact with proteins of the salivary gland to colonize cells and be transported to the salivary duct from where phytoplasmas enter host
plants via the saliva of the insect vector (Figure 1.8E). The way phytoplasmas colonize both hosts (insect and plant hosts) is similar to that of many insect-vectored human/animal pathogens, including the malaria parasite *Plasmodium falciparum* (Lin *et al.*, 2016).

**Figure 1.8. Phytoplasmas have to adapt to plant-host and insect-vectors to continue their life-cycle.**

**A.** Phytoplasma enters insect gut as a result of phloem feeding. Electron photograph shows AY-WB phytoplasma in the midgut epithelial cell of *M. quadrilineatus* (bl – basal lamina; mv – microvilli; pm – plasma membrane; scale bar 1 µm). **B.** Phytoplasmas are believed to share adaptations similar to that found in spiroplasmas: bacteria are internalised into plasmalemma or gut surrounding smooth muscles where it replicates. **C.** During latent period phytoplasma cross the gut barrier to enter the hemocoel and spread throughout insect body. Systemic infection of *M. quadrilineatus* takes around 10 days. **D.** Phytoplasma has to be internalised into salivary duct from hemocoel as shown in this schematic representation of such adaptation in spiroplasmas. **E.** Phytoplasma is injected into the phloem of healthy plant during insect feeding. Within 10 days post infection phytoplasma spreads throughout plant phloem and induces first disease symptoms such as stunting and increased rosette branching. Figure adapted from Fletcher *et al.* (1998) and Sugio *et al.* (2011).
In plants, phytoplasmas are phloem-limited and colonize the sieve elements. *In situ* bio-imaging studies demonstrate that mature anucleate sieve tubes contain much higher density of bacterial cells than companion cells or phloem parenchyma cells (Christensen *et al.*, 2004). Within sieve tubes phytoplasmas exhibit various morphologies, including spherical, budding (probably while dividing) (Figure 1.4) or amoeboid shapes. Phytoplasmas move to other sieve cells by passing through pores in the sieve plates (Hogenhout *et al.*, 2008). The systemic spread of phytoplasmas throughout the plant mirrors that of transport of nutrients to sink tissues, which includes rapidly growing organs, such as new shoots and roots that require sugars for growth (Bertaccini, 2007). Phytoplasma titer (bacterial cell density within phloem) is used as a proxy measure of the degree of infection and is often correlated with the level of visual disease symptoms of plants (Christensen *et al.*, 2004).

1.2.10. Phytoplasmas modulate their plant host and plant-insect interactions

Phytoplasma infections induce diverse disease symptoms in plants (Lee *et al.*, 2000), including: witches’ broom (proliferation of auxiliary shoots); virescence (greening and loss of flower pigmentation); reddening or yellowing of photosynthetic tissue; stunting (decrease in flower and leaf size, shortened internodes); phyllody (development of leaf-like structures instead of outer floral whorls); sterility of flowers (abnormal gynoecium development); indeterminate growth of floral meristem; elongation of internodes; slender shoot formation (bolting); and formation of fibrous secondary roots. In addition, phytoplasma alters plant hormone and secondary metabolite biosynthesis (Sugio *et al.*, 2011a; Lu *et al.*, 2014; Tan *et al.*, 2016). Thus, phytoplasmas appear to interfere with regulation of plant growth, development and physiology. Such effects on the plant host may be potentially beneficial to phytoplasma or its insect vectors (Figure 1.9). For example, downregulation of jasmonic acid synthesis in plants increases the fecundity of leafhopper vector of phytoplasma (Sugio *et al.*, 2011). Some of the alterations observed in infected plants are caused by phytoplasma effectors, and these will be further discussed in the next sections.
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Figure 1.9. Phytoplasmas modulate plant development and physiology which have known or may have potential effects on plant-phytoplasma and plant-insect interactions. Several phytoplasma-altered plant phenotypes are described in the literature. In addition to the known effects on plant biology, there may be other potential effects on plant immunity or nutritional quality are not yet investigated in phytoplasma infected plants. However, such effects have been reported in other plant pathogens. Together, modifications of plant biology may have numerous potential roles in plant-phytoplasma or plant-insect interactions.

1.2.11 Phytoplasma secretes effectors that move outside phloem

Many Gram-negative plant pathogenic bacteria (*Pseudomonas, Xanthomonas,Ralstonia, Erwinia*) rely on specialised needle structure (named pili), produced by the Type-III secretion system, for the delivery of effector molecules into the host cell (Cunnac et al., 2009). In contrast, phytoplasma genomes lack genes present in the Type-III or Type-IV secretory systems (Hogenhout et al., 2008). Unlike the pathogens above, phytoplasmas appear to rely predominantly on Sec-dependent secretion system (via SecA, SecE and SecY) for translocation of proteins (including the majority of candidate effectors) across the phytoplasma membrane into host cytoplasm (Kakizawa et al., 2004). Phytoplasma genomes also encode YidC (Bai et al., 2006) that function in
integration of proteins into bacterial membranes (Hennon et al., 2015). Secreted proteins often possess a N'-terminal signal peptide sequence of about 20 amino acids long that is recognized by the Sec-dependent secretion system and cleaved off during translocation across the membrane of the protein. Signal peptide sequences are conserved among diverse organisms and often consist of specific sequence of hydrophobic, polar and acidic/basic amino acids that can be searched for with prediction software, such as SignalP (Nielsen et al., 1997; Bendtsen et al., 2004). SignalP identified in about 75 proteins with signal peptides in all predicted proteins of the AY-WB genome. 19 of these had one or more predicted transmembrane regions, whereas 56 did not (Bai et al., 2009). These 56 proteins are likely to be secreted to the extracellular environment of the phytoplasmas and were named secreted AY-WB proteins (SAPs) (Bai et al., 2009).

Phytoplasma localisation has been demonstrated to be limited to the phloem tissues of infected plants via various microscopical methods such as fluorescence in situ hybridisation (Bulgari et al., 2011) or immunolabelling of anti-AMP (phytoplasma membrane protein) (Hoshi et al., 2009; Arachida et al., 2008). Work by Arashida et al. (2008) has shown phytoplasma localisation in the phloem of infected flowers from hydrangea plants. Similarly, when Arabidopsis vegetative leaves are infected with phytoplasma using infected leafhoppers, phytoplasma can be visualised in the phloem of flowers (Hoshi et al., 2009), suggesting systemic movement of phytoplasma via phloem. A common method of insect-free phytoplasma propagation in greenhouse is grafting an infected scion onto a healthy rootstock. The lateral branches developing from the original rootstock later show the characteristic symptoms of phytoplasma infection and are PCR-positive for phytoplasma of the infected scion (Hodgetts et al., 2013). Moreover, phytoplasmas can be transferred from plant to plant via parasitic plant Cuscuta spp. (dodder), which forms vascular connections between the infected donor and healthy recipient plant hosts. Similar to grafting experiments, the parasitized recipient plant develops the characteristic disease symptoms and is PCR-positive for the phytoplasma strain from the infected donor plant (Přibylová & Spak, 2013). Together, these experiments suggest systemic movement of phytoplasmas via plant phloem.

While phytoplasma is limited to the phloem, secreted phytoplasma effector proteins have been also visualised in other tissue types than phloem alone. For
example, OY secreted protein TENGU was labelled with TENGU-specific antibody in parenchyma cells, shoot apical meristems and axillary buds of OY-infected plants (Hoshi et al., 2009). AY-WB effector SAP11 has a nuclear localisation signal (NLS) required for targeting cell nuclei (Bai et al., 2009). Since phloem sieve elements are anucleate, the presence of NLS suggested potential transport and function of SAP11 beyond plant phloem. In support of this hypothesis, SAP11 was visualised in nuclei of mesophyl cells and trichomes of AY-WB-infected plants (Bai et al., 2009). Although lacking a characteristic NLS, TENGU also targets cell nuclei (Hoshi et al., 2009). Expression of AY-WB effector SAP54 under companion cell-specific AtSUC2 promoter produced leaf-like flower phenotype characteristic of infected plants and that of SAP54 overexpression lines with Cauliflower mosaic virus 35S promoter (MacLean et al., 2011). Furthermore, SAP54 was co-immunoprecipitated with GFP-tagged flower-specific MADS-box transcription factors (MTFs) from plants that were inoculated by phytoplasma prior to flowering (MacLean et al., 2014). This indicates that phytoplasma effectors have moved from the phloem of leaves where phytoplasma was initially inoculated by insects to floral organs where SAP54 targets plant proteins in floral meristems. Since the SAP54-interacting MTFs are expressed in floral organs rather than phloem tissue (Urbanus et al., 2009), this strongly suggests SAP54 movement and unloading post-phloem.

Experiments with various size GFP-fusion proteins expressed under companion cell specific AtSUC2 promoter support a model of a non-specific systemic movement of small molecules via phloem and unloading to sink tissues such as roots or reproductive organs (Stadler et al., 2005ab; Imlau et al., 2009). The relatively small size of phytoplasma secreted proteins such as TENGU (<5 kDa), SAP11 (9 kDa) or SAP54 (11 kDa) compared to GFP (~27 kDa) and the size exclusion limit of plant plasmodesmata (Stadler et al., 2005b) may explain the cell-to-cell movement of phytoplasma secreted proteins. SAPs (candidate effectors) may pass systemically in the phloem through tangential sieve plates delineating sieve tubes and through lateral plasmodesmata that connect sieve elements with companion cells and parenchyma to travel to distant sink tissues such as the shoot apical meristem (Figure 1.10) (Sugio et al., 2011b). As a result of such potential effector movement, phytoplasma effectors can be expected to act in the same tissue that may be exposed to subsequent feeding or egg-laying by the leafhopper vectors. Additionally, any effect of effectors like SAP11 and
SAP54 on tissue identity and development may also occur prior to insect colonisation.

![Figure 1.10](image-url)  
*Figure 1.10. Overview of potential systemic movement of phytoplasmas and effectors in the plant and effector unloading from the phloem. SEL=size exclusion limit of cell connections. Image taken from Sugio et al., 2011b.*

### 1.2.12. Phytoplasma effectors alter plant development and resistance to insects

One functionally characterized effector is SAP11, which was shown to interfere with various aspects of plant development as well as modulate plant-insect interactions (Sugio *et al.*, 2011a). SAP11 has a nuclear localisation signal (NLS), allowing to enter plant cell nucleus (Bai *et al.*, 2009), where the effector interacts with and destabilises TCP (TEOSINTE BRANCHEDED (TB1), CYCLOIDEA (CYC), PROLIFERATING CELL FACTORS 1 AND 2 (PCF)) transcription factors (Sugio *et al.*, 2011a, 2014). TCPs play important roles in regulating plant circadian clock, hormone pathways, mitochondrial biogenesis as well as cell differentiation and proliferation; these processes are key in gametophyte development, seed germination and patterning of vegetative and reproductive organs (Manassero *et al.*, 2013). Based on structural domains, TCPs are divided into two classes (Martin-Trillo & Cubas, 2010, Manassero *et al.*, 2013). SAP11 appears to destabilise all class II TCP transcription factors,
including CINCINNATA (CIN) and CYC/TB1 clades of TCPs (Figure 1.11). In addition, Sugio and colleagues demonstrated that SAP11 down-regulates jasmonate (JA) production in plants, resulting in enhanced fecundity of aster leafhopper *Macrostele quadrilineatus*, the principal vector of AY-WB phytoplasma. When eggs hatch, early instar leafhopper nymphs remain and feed on the plant and will acquire phytoplasmas and transmit these bacteria to other plants when they become adults. Thus, SAP11-mediated modulation of plant processes leads to an increase in the number of phytoplasma-carrier vectors thereby promoting phytoplasma spread.

Destabilisation of CYC-TCPs like BRC1 and 1 results in organ proliferation (Figure 1.11) which may benefit phytoplasmas by generating more phloem sink tissue for phytoplasma replication and, additionally attracting insect vectors (Figure 1.9). However, whether alteration of plant morphology such as organ proliferation or development of crinkled leaves are actually beneficial for phytoplasma spread in nature remains to be tested empirically.

TENGU is a candidate effector produced by Onion Yellows (OY) phytoplasma and associated with Witches’ broom and dwarfism in *Arabidopsis* (Hoshi *et al.*, 2009). TENGU inhibits auxin-related pathways. Although the interference with auxin responses may be a direct function of TENGU (independent of shoot proliferation), auxin signalling might be affected indirectly as a result of altered development of plant vegetative organs. By targeting TCP-regulated developmental processes AY-WB SAP11 might interfere with auxin pathways, too. Plant targets of TENGU have not yet been identified (Hoshi *et al.*, 2009). Similar to SAP11, TENGU was found to be localised in plant cell beyond the sieve elements of the phloem, but unlike SAP11 lacks an NLS for localization to the nucleus. Hoshi and colleagues also suggested that the Witch’s broom phenotype might augment insect-vector attraction to infected plants, thus increasing phytoplasma acquisition.
Figure 1.11. Downstream effects of the SAP11-mediated destabilization of plant TCP transcription factors. SAP11 secreted by AY-WB phytoplasma enters plant cell nuclei (upper left) and destabilizes the class II CIN-TCPs and CYC/TB1, but not the class I TCPs. CIN-TCP destabilization leads to crinkled leaves and siliques and TB1/CYC (BRC1 and BRC2 in A. thaliana) to increase in stem proliferations. CIN-TCPs are known to regulate JA production via LOX2 (lipoygenase 2) and leafhoppers lay more eggs on plants with decreased LOX2 expression and JA production, SAP11 transgenic lines and AY-WB-infected plants (Sugio et al., 2011a).
Another AY-WB effector protein, SAP54, was found to modulate flower development (MacLean et al., 2011). SAP54 induces virescence (greening of petals), phyllody (leaf-like flowers with trichomes) and indeterminate growth of inflorescence meristem in A. thaliana mirroring the phyllody symptoms of AY-WB-infected plants. Phyllodies can be observed in diverse plant species infected with distantly related phytoplasmas. Several studies found altered regulation of MADS-box transcription factors (MTFs) in phytoplasma-infected plants that are impaired in flower development (reviewed in Sugio et al., 2011b). In a yeast two-hybrid screen, SAP54 was found to interact with several MTFs, which have roles in floral transition and development of plant reproductive organs (Figure 1.12) (MacLean et al., 2014). Subsequently, it was shown that SAP54 recruits MTFs to plant 26S proteasome for degradation in RAD23-dependent manner (MacLean, 2014). Effectors of other bacterial pathogens were also shown to degrade targets via the 26S proteasome, albeit this does not involve RAD23. For example, degradation of A. thaliana immunity associated protein AtMIN7 by Pseudomonas syringae effector HopM1 depends on plant proteasome activity (Nomura et al., 2006). This indicates that plant protein targeting to proteasome for degradation may be a common mechanism of altering plant protein function by pathogen effectors.

An exhilarating scientific question is the question how degradation of MTFs, key regulators of flowering, would benefit phytoplasma or phytoplasma-vectoring insects? Hence, are SAP54-induced changes in plants adaptive and increase phytoplasma fitness? Phyllody, virescence and indeterminate growth may increase the amount of young vegetative tissue (phloem sinks) for phytoplasma replication or serve as visual cue for location of infected plants by insect vectors, thus enhancing phytoplasma acquisition (Figure 1.9). These possibilities remain to be tested empirically.
Figure 1.12. Phytoplasma effectors alter gene regulation during floral development in host plants. Transition from vegetative to floral meristems is regulated by AP1, LFY and cofactor SEP3. These genes also activate organ identity genes that give rise to sepals, petals, stamens according to ABC model. Floral meristem identity is maintained by activity of WUS. In order to terminate floral meristem growth, WUS expression is suppressed by organ identity genes like AG via activity of KNU. Stolbur, Onion Yellows (OY) and Italian clover phyllody (ICPh) phytoplasmas modify the relative quantity (up- or down-regulation) or time (misregulation) of expression of the genes involved in various stages of flower development. Adopted from (Sugio et al., 2011b).
1.2.13. Parasite effectors promote virulence and suppress plant immunity

Different groups of plant pathogenic microorganisms possess virulence factors – proteins or small non-protein molecules, known as effectors. The function of these effector molecules is primarily modulation or suppression of innate plant immune responses to evade recognition by plants and ensure successful colonisation and reproduction in the host (Boller and He, 2009; Dodds and Rathjen, 2010). Interestingly, bacteria, fungi or root nematodes are also associated with morphological changes in plant tissues, and potential effectors from these organisms are implicated in the modulation of plant development (Evangelisti et al., 2014; Le Fevre et al., 2014). Nevertheless, in many cases the fitness benefits of microbe-induced morphological changes in plants have not yet been empirically tested.

Suppression of plant defences allows successful invasion and utilisation of host resources. When microbial pathogens invade plant tissue, plants encounter conserved microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs), such as fungal chitin or bacterial flagellin, on the surface of an invading pathogen. These molecules can be recognised by specific pattern recognition receptors (PRRs) at plant cell membrane. Detection of these extracellular molecular patterns by plant receptors elicits plant immune response, known as PAMP-triggered immunity or PTI (Couto and Zipfel, 2016). PTI leads to a set of immediate plant responses, including Ca\(^{2+}\) influxes into cells, production of reactive oxygen species, callose deposition and activation of genes involved in plant defences. Plant pathogens deliver effector proteins in the host cell to suppress PAMP-triggered signalling events and PTI responses, leading to effector-triggered susceptibility (ETS). However, an effector molecule that suppresses PTI can be recognised by a specific resistance gene (R-gene) in plant, eliciting effector-triggered immunity (ETI) (Cui et al., 2015). ETI gives rise to hypersensitive response (HR) – rapid localised cell death that may restrict replication and spread of (biotrophic) pathogens. Interestingly, pathogens have evolved effectors that suppress ETI and, thus, avoid HR. The PTI, ETS and ETI together are referred as the “Zig-Zag Model” of plant immunity and often leads to an “arms-race” between plant ability to recognise the pathogen and pathogen evading the immunity (Jones and Dangl, 2006). According to this model, evasion of host defence is possible when pathogens possess effectors that suppress PTI as well as ETI and when plants lack R-genes that would recognise such effectors.
The gene-for-gene concept (Flor, 1971) predicts coevolution of R-genes and effectors involved in ETI. Therefore, effectors are expected to be selected for evading the repertoire of existing R-genes in pathogens plant-host range.

Pathogens possess a collection of effectors, many of which may promote virulence via other mechanisms than suppression of PTI or ETI. For example, *Pseudomonas syringae* (*Pto* DC3000) effectors induce extracellular accumulation of cytoplasmic proteins from host cells with a potential role to aid assimilation of host nutrients (Kaffarnik et al., 2009). Another *P. syringae* effector HopW1 targets plant actin cytoskeleton to modulate the actin-dependent processes that were shown to be required to restrict pathogen growth (Kang et al., 2014). HopAM1 interferes with abscisic acid signalling (ABA), promoting *P. syringae* virulence on *A. thaliana* under drought and salinity stress as well as enhancing ABA-mediated stomatal closure to protect bacteria from osmotic stress inside the leaf (Goel et al., 2008). *Xanthomonas spp.* secrete TAL-effectors to induce leaf canker but the exact function of canker morphology remains elusive (Pereira et al., 2014). Interestingly, phytopathogenic nematodes are also known to secrete effectors into plant to modify cell wall architecture to aid migration of infective juveniles or production of specialised (giant) feeding cells (Davis et al., 2008).

1.2.14. Putative function of other AY-WB effectors

Hitherto, no studies have tested role of phytoplasma effectors in triggering or suppressing ETI or PTI. AY-WB effectors SAP11 and SAP54 have a significant effect on host-plant development (Sugio et al., 2011b). In addition, the fecundity of insect vectors is significantly enhanced on SAP11 expressing plants (Sugio et al., 2011a). However, these two effectors do not function in isolation. The 56 candidate effector proteins are significantly up-regulated during AY-WB infection of plants or insects (MacLean et al., 2011). Moreover, different sets of AY-WB effectors are upregulated in plants and insects (Figure 1.13). Effectors SAP11 and SAP54 are up-regulated only in the plant-host, confirming that these effectors act predominantly in the plant. The function of other 54 candidate effectors in plants or insects remains to be elucidated.
1.2.15. Phytoplasma genomes have dynamic structure and suggest frequent reorganisations

To date there are only five fully assembled genomes (Oshima et al., 2004; Bai et al., 2006; Kube et al., 2008; Tran-Nguyen et al., 2008; Andersen et al., 2013) and a further nine draft genome (contig) sequences available of phytoplasmas (Saccardo et al., 2012; Chung et al., 2013; Chen et al., 2014; Kakizawa et al., 2014; Mitrović et al., 2014; Quaglino et al., 2015). These belong to 16Sr-I, -II, -III, -X and XII groups of diverse clades within the phytoplasma phylogenetic tree enabling assessment of the diversity in phytoplasma genome content and organisation. Phytoplasmas have one of the smallest genomes of all sequenced bacteria (600-960 kb) (Figure 1.13). Phytoplasmas lack genes in pathways of many amino and fatty acid as well as nucleotide synthesis. Surprisingly, glycolysis pathway or phosphotransferases that many bacteria use for phosphorylating and importing disaccharides (sucrose, trehalose) or monosaccharides (glucose, fructose) from plant or insect hosts are absent in most sequenced phytoplasma genomes. Instead, phytoplasmas have malate importers and have active enzymes to convert malate to pyruvate and perform
downstream reactions for NAD or NADP reduction and energy (ATP) generation from malate (Kube et al., 2008; Saigo et al., 2014; Siewert et al., 2014). Reduced genome size and absence of basal metabolism genes may be an adaptation to their parasitic lifestyle and reflect their dependency on their host plants and insect vectors for nutrients (Oshima et al., 2004; Bai et al., 2006).

In addition to reduced anabolic capacity, some phytoplasmas have lost DNA repair genes, such as recA (Bai et al., 2006; Chu et al., 2006), potentially allowing to accumulate mutations and evolve faster. Moreover, phytoplasma genomes are rich in repeats, which can be >20 kb (Bai et al., 2006; Jomantiene and Davis, 2006; Wei et al., 2008; Toruño et al., 2010; Andersen et al., 2013;
Chung et al., 2013; Ku et al., 2013). The repeats organize in conserved gene clusters, named Potential Mobile Units (PMUs) or sequence-variable mosaics (SVM) (Bai et al., 2006; Jomantiene and Davis, 2006). At least one PMU was shown to exist as chromosomal and extrachromosomal units in the genome of Aster Yellows phytoplasma Witches’ Broom (AY-WB) (Toruño et al., 2010). PMUs are prone to recombination and degeneration and there is evidence that the PMUs recombine and have horizontally transferred between diverged phytoplasmas (Bai et al., 2006; Hogenhout and Musić, 2010; Sugio and Hogenhout, 2012; Chung et al., 2013; Ku et al., 2013). Interestingly, AYWB PMU1 encodes several membrane-targeted proteins which may function in horizontal transfer of PMUs or interact with the plant or insect host (Toruño et al., 2010). PMUs appear to determine the genome size reduction and plasticity of the small and AT-rich phytoplasma genomes (Bai et al., 2006; Jomantiene and Davis, 2006; Hogenhout and Musić, 2009; Andersen et al., 2013). Synteny of the circular chromosomes between closely related phytoplasmas within “Ca. P. asteris” 16SrI group is low (Figure 1.15) compared to that of other closely related bacteria (Hogenhout and Musić, 2009). The majority of the metabolic genes lie in the 400 kb to 600 kb regions and the majority of the PMU-like sequences in the 150 kb to 400 kb regions of the phytoplasma chromosomes (Figure 1.14). The PMU-rich regions show large inversions and discontinuous synteny (Figure 1.15) that suggest recombination have occurred in these regions since MBSP, AY-WB and OY-M diverged from their common ancestor. Furthermore, the AY-WB and OY-M genomes also have irregular GC-skew patterns (Figure 1.14) (Oshima et al., 2004; Bai et al., 2006) that is indicative of high genomic plasticity, possibly caused by relatively recent recombination events of, for example, PMUs (Bai et al., 2006). Synteny between phytoplasmas from different 16Sr groups is very poor or absent. As well, some phytoplasmas, such apple proliferation phytoplasma “Ca. P. mali” have linear chromosomes (Kube et al., 2008).

The majority of phytoplasma virulence proteins, including SAP54 and SAP11, lie within or adjacent to PMU and PMU-like gene clusters (Figure 1.14) (Bai et al., 2009; Toruño et al., 2010). This presents an opportunity for horizontal transfer of virulence factors important for phytoplasma propagation within hosts or acquisition and transmission to new hosts. Therefore, such dynamic genome structure may enable host-switching or expansion of phytoplasma host range.
1.3. Aims, outline and outcomes of the PhD thesis

Why do microorganisms modulate development of their hosts? Phytoplasmas alter plant development by secretion of effector proteins into the host cells. This makes them an extremely interesting system to study the adaptive role of modifying host biology. Phytoplasma effector SAP54 prevents normal flower development, and, instead, induces leaf-like structures, called phyllody. Intriguingly, phyllody symptoms are common in many phytoplasma-infected plants worldwide. The aim of my thesis is to better understand the adaptive significance of SAP54-mediated degradation of MTFs generating leaf-like flowers. My hypothesis is that the vegetative tissues of the flower make the infected plant more attractive to the insect vectors that will benefit phytoplasma dispersal in nature.

To this end I first investigated how widespread and structurally similar are phyllodies induced by diverse groups of phytoplasma and effector homologs of SAP54 (Chapter 2). I discovered that the induction of phyllody is genetically linked with promotion of insect colonisation of the infected plants that exhibit the leaf-like flower phenotype.

Findings of Chapter 2 supported my hypothesis that the generation of leaf-like flowers is an adaptive manipulation of host plants by phytoplasmas in order to attract insect vectors. In Chapter 3 I directly tested this hypothesis and, surprisingly, found that SAP54 enhances leafhopper vector egg laying on plants independently from the induction of leaf-like flowers. I discuss the possibilities that the production of phyllody may be a side-effect of phytoplasma infection and
that, instead, SAP54 modulates plant vegetative organs to promote plant colonisation by insect vectors.

In Chapter 4 I explored possible roles of SAP54 in modulating visual, olfactory and other cues that leafhoppers are known to use for the selection of host plants. When testing whether insects make a choice bases on feeding (males and females) and egg laying (only females), I made the interesting discovery that male leafhoppers are required for the preference of females to lay eggs on SAP54 transgenic plants.

Next, I analysed the effect of SAP54 on male and female-induced transcriptional changes in plants (Chapter 5). I found that male and female leafhoppers elicit different plant responses. Moreover, SAP54 suppresses insect induced responses in sex-specific manner by selectively downregulating male-induced defence and secondary metabolism pathways.

Since SAP54 destabilises plant MADS-box transcription factors (MTFs), I further investigated which MTFs are expressed in leaves and are involved in insect egg laying preference (Chapter 6). I identified four MTFs that play important roles in egg-laying preferences by leafhoppers and demonstrate sex-specific regulation of these MTFs by SAP54.

Finally, I put my findings in the broader context of the current knowledge about plant-insect and plant-microbe interactions (Chapter 7). I highlight the novelties and advances in our current understanding how plants defend themselves against insects and how a parasite, such as phytoplasma, modulates hosts to aid their spread in nature thereby increasing its Darwinian fitness. I discuss the implications for effector roles in driving phytoplasma disease epidemiology in natural and agro-ecosystems.

Taken together, I have achieved the aim of the thesis. I found that phytoplasma effector SAP54 has pleiotropic roles in plant reproductive development and suppression of insect-induced plant resistance to egg-laying via targeting conserved groups of plant proteins with potential roles in both development and defence against herbivorous insects. I discovered that the bacterial effector protein downregulates male-induced plant responses to promote plant colonisation by female insects, thereby potentially aiding the spread of the pathogen in nature. These findings have contributed to our mechanistic understanding how plant pathogens manipulate their hosts to their advantage. This work has also proven that bacterial effector proteins can be a
useful genetic tool to better understand how plant immunity and development are regulated as an integrated system. Furthermore, I have, for the first time, demonstrated how sexual dimorphism in an important group of hemipteran insect pests is perceived by their plant hosts. Crucially, the work presented in this thesis has already resulted in several peer-reviewed publications (MacLean, Orlovskis et al., 2014, Orlovskis et al., 2015, Orlovskis et al., 2016, Orlovskis et al., 2017; see Appendices A,B,C,E) and, to my belief and determination, will serve as a source and reference for many others.
Chapter 2

Phytoplasma Effector SAP54 Hijacks Plant Reproduction by Degrading MADS-box Proteins and Promotes Insect Colonization in a RAD23-Dependent Manner

Part of this work is published in:
2.1. Introduction

Changes in plant morphology such as induction of phyllody have been widely reported in phytoplasma infected plants for several decades (Bertaccini, 2007). However, only recent advances in characterising phytoplasma effector proteins have established that phytoplasma secreted proteins are causal for changes in plant development (Sugio et al., 2011b). Understanding the molecular mechanism of phytoplasma effector SAP54 has opened new doors for testing the roles of effector function in plant reproductive development and plant-insect interactions as well as investigating the evolution of phytoplasma virulence factors and proposing the hypotheses about the adaptive significance of effector-triggered changes in the host.

Induction of phyllodies in many plant families have been associated with diverse groups of phytoplasmas. For example, ‘Ca. Phytoplasma asteris’ from 16SrI-C ribosomal subgroup has been associated with phyllody symptoms in numerous dicot plant hosts like coneflowers (Echinacea purpurea; Asteraceae), several clover species (Trifolium spp.; Fabaceae), tomato (Lycopersicon esculentum; Solanaceae) and strawberry (Fragaria x ananassa; Rosaceae) (Fránová et al., 2009; Jomantiene et al., 2011 (and references therein)). In addition to the 16SrI-C phytoplasmas above, some other members of 16SrI-C subgroup can infect but have no documented evidence of phyllody induction in other hosts such as willow (Salix spp.; Salicaceae) or lilac (Syringa vulgaris; Oleaceae) (Jomantiene et al., 2011), giving a reason to question why phyllody phenotype may not be conserved in all phytoplasmas of the same lineage. Interestingly, phyllody has been reported in related plant species that are infected by different phytoplasmas. For example, phyllody in sesame (Sesamum indicum; Fabaceae) is associated with either 16SrIX or 16SrII group phytoplasmas (Mirzaie and Rahimian, 2007; Ikten et al., 2014). Likewise, 16SrIX and 16SrI group phytoplasmas are the potential causal agents of phyllodies in Brassicaceae plants such as toria (Brassica rapa) (Azadvar et al., 2011) or rapeseed (Brassica napus) (Zwolińska et al., 2011).

Phyllody appears to be a common symptom in plants infected with diverse phytoplasma groups. If phyllody could also be induced by phytoplasmas that lack SAP54, this may suggest multiple origins (convergent evolution) of phyllody-inducing mechanism in phytoplasmas. Alternatively, single origin of phyllody-inducing SAP54 gene and horizontal gene transfer between distantly related
phytoplasmas may explain the worldwide occurrence of phyllody phenotype in plants infected by different phytoplasma groups. To distinguish between these hypotheses, a comparative genome analysis of wide variety of phytoplasma strains that induce phyllody would be required. Moreover, despite worldwide reporting of phyllody symptoms in plants infected with phytoplasmas, detailed photographic and comparative analysis of the morphology of leaf-like tissues in the related host plants or related phytoplasmas is often lacking. A significant proportion of reported phyllody symptoms is found in crop species or ornamental plants. Studies about prevalence of phyllodies in phytoplasma-infected wild species in their natural habitats are lacking, rising questions whether modern plant breeding and selection has enriched for plant genotypes that are susceptible to development of phyllody symptoms after phytoplasma infection. Only association between phyllody and phytoplasma appears in many disease reports. Nevertheless, the exact causal link of phyllodies has not been investigated due to previously limited knowledge about the effector proteins of phytoplasma and their mode of action.

It has been only recently discovered that conversion of flowers into leaves (phyllody) is induced by secreted phytoplasma effector protein SAP54 (MacLean et al., 2011). SAP54 homologs from ‘Ca. Phytoplasma asteris’ strains Witches Broom and Onion Yellows have been shown to induce phyllody by destabilising plant MADS-box transcription factors (MTFs) normally required for floral meristem identity and specification of floral organs (MacLean et al., 2014; Maejima et al., 2014). Moreover, induction of phyllody and degradation of MTFs by SAP54 is dependent on plant 26S proteasome shuttle protein RAD23 (MacLean et al., 2014). This major finding has resulted in a novel mechanistic model where SAP54 hijacks host plant ubiquitin-proteasome machinery to degrade MTFs, leading to generation of leaf-like flowers (Figure 2.1).
Figure 2.1. SAP54 induces formation of leaf-like flowers by degrading MADS-box transcriptional regulators in RAD23-dependent manner. Schematic representation of the molecular mechanism of phytoplasma effector SAP54 is based on findings in Maclean et al. (2014). (A) AY-WB phytoplasma secretes effector SAP54 within the host cells of wild-type A. thaliana Col-0 plants. The effector binds a selection of Type II MADS-box transcription factors (MTFs) and two isoforms of RADIATION SENSITIVE proteins (RAD23 C and D). This results in degradation of MTFs by plant proteasome. Since MTFs are key regulators of floral development in plants, SAP54 induces floral reversion into leaf-like structures (phyllody) and indeterminate meristem growth. (B) Presence of the interacting RAD23 isoforms C and D is crucial for SAP54-mediated degradation of MTFs and production of leaf-like flowers. In absence of RAD23 isoforms C and D, SAP54 interacts with but does not degrade MTF targets and generate normal flowers. (C) RAD23 isoforms C and D act in a redundant manner to aid MTF-degradation by SAP54. Presence of either RAD23 isoforms C or D is required for induction of phyllody.

Plant MTFs are modular proteins. SAP54 has specificity for keratin-like (K) domain of plant MTFs (Figure 2.2). The K-domain is present only in a subclass of plant MTFs - type-II MTFs - which evolved after divergence of land plants (Alvarez-Buylla et al., 2000). The K-domain is involved in protein-protein interactions with other type-II MTFs to form dimers and quartets that determine specificity for various regulatory functions (Davies et al., 1996; Yang and Jack, 2004; Smaczniak et al., 2012b). SAP54 is predicted to fold into coiled-coil helices
that mimic the structure of the K-domains of type-II MADS. Due to low sequence similarity between MTF K-domains and SAP54, this could be an example of structural convergence to selectively interact with a certain group of conserved plant targets (Rümpler et al., 2015). So far there is evidence for SAP54 interaction and destabilisation of type-II plant MTFs (MacLean et al., 2014). However, it remains to be empirically tested if SAP54 may also interact and destabilise other targets than type-II plant MTFs. Since animals also possess MTF proteins that are more closely related to type-I plant MTFs, interference with insect MTFs could result in direct effects of SAP54 on insect vectors in addition to modulation of plant reproductive development.

Figure 2.2. SAP54 interacts with Keratin-like (K) domain of type-II MADS-box transcription factors (MTFs). APETALA1 (AP1) is an example of modular type-II plant MTFs: They consist of MADS and intervening domain, Keratin-like (K) domain and variable C-terminus domain. Yeast-two-hybrid demonstrates that Aster Yellows Witches’ Broom effector SAP54 has the highest binding affinity to the K-domain of AP1 compared to MADS (+intervening) or the C-terminal domain alone. SAP54 is fused to the Binding Domain (BD) of GAL4 gene. AP1 and its individual domains are fused to the activation domain (AD) of GAL4 gene. Positive interaction between AD and BD initiates growth on selective media (-L-W-H). Whole length APETALA1 (AP1) and empty vector (EV) used as controls. Figure published in MacLean et al. (2014).
SAP54 homologs have been identified in several *Candidatus* Phytoplasma species. Furthermore, SAP54 homologs from two related ‘Ca. Phytoplasma asteris’ strains (AY-WB and OY-W) interact and destabilise similar plant MTF targets – APETALA1 and SEPELLATA family proteins (MacLean *et al.*, 2014; Maejima *et al.*, 2014, 2015). SAP54 targeted RAD23 and MTFs are conserved families of plant proteins. Although comparative analysis of MTF and RAD23 target interaction with SAP54 homologs from diverse phytoplasmas are yet lacking, the numerous reports of phyllodies in different plant species infected by diverse groups of phytoplasmas present a hypothesis that phyllody may arise from a convergent evolution or horizontal transfer of phytoplasma effectors that interact with conserved plant proteins. It is known that targeting conserved plant regulators by phytoplasma effectors can have an effect on both plant development and resistance to insects. For example, SAP11 affects plant branching as well as increases the reproduction of the insect vector of phytoplasmas (Sugio *et al.*, 2011a). It remains to be elucidated whether SAP54 interactions with MTFs and RAD23 elicit changes in plant-insect interactions in addition to alteration of plant morphology.

This chapter aims to investigate the hypothesis that plant phyllody is a convergent phenotype induced by SAP54 homologs from phylogenetically diverse phytoplasmas that interfere with similar set of plant targets. To this end I will explore morphological similarity between phyllody symptoms in various families of wild and cultivated plants and compare the floral phenotypes of several homologs of SAP54. I will further investigate the interaction specificity of SAP54 homologs with plant MTF and RAD23 targets. Finally, I investigate the potential role of RAD23-dependent SAP54 functions in plant-insect interactions.
2.2. Results

2.2.1. Phyllody is induced by diverse groups of phytoplasma in numerous plant families

How widespread are phytoplasma-induced changes in floral morphology? To better understand the prevalence of phyllody in plants and compare the morphology of leaf-like tissues induced by different phytoplasmas, I gathered photographic evidence for the occurrence of phyllody symptoms around the world and the associated phytoplasmas in different plant families. Photographs in Figure 2.3 demonstrate phyllodies that are associated with phytoplasmas of 16SrI and 16SrII groups from Europe and Asia. Partial or full reversion of flowers into leaf-like structures is a common phenotype in many phytoplasma-infected plant families (Figure 2.3). Phyllodies appear to be common in dicots, while the documentation of monocot phyllodies is lacking. Various subgroups of ‘Ca. Phytoplasma asteris’ are associated with multiple plant hosts (Lee, 2004) that exhibit phyllody symptoms (Figure 2.3). Conversion of flowers into leaf-like structures can be full (for example Figure 2.3E-f and P) or partial (Figure 2.3A, M). Interestingly, there can be a lot of variation in the exact appearance and structure of leaf-like tissue in plant population of the same genotype (Figure 2.3C) or within individual plants (Figure 2.3J and O). This suggests that there could be clonal variation in phytoplasmas infecting the same host species or mixed infections of different phytoplasmas. Alternatively, hosts may also be infected at different stages of their development, therefore, the morphological changes induced by phytoplasma infection may depend on the developmental time of plant tissue.
Figure 2.1.
Figure 2.3. Partial or full reversion of flowers into leaf-like structures is a common phenotype in many phytoplasma-infected plant families worldwide. Rapeseed (Brassica napus) has single terminal flowers arranged in a panicle inflorescence. Rapeseed infected with ‘Ca. Phytoplasma asteris’ (16SrI-B subgroup) can sometimes develop flowers with normal sepals and petals but massively enlarged carpels (arrow) (A). Infected rapeseed occasionally shows indeterminate growth where a single flower meristem (arrow) gives rise to other normal-looking flowers (B) or single normal flowers can be turned into telescopic leaf-like flowers (C). Notable is the variation in size of the leaf-like petals and swelling of carpel (arrow) in some of these flowers (C). Tale cress (Arabidopsis thaliana) is a close relative of rapeseed with single white terminal flowers (D). After infection with ‘Ca. Phytoplasma asteris’ (16SrI-A subgroup) A. thaliana develops sterile leaf-like flowers with shorter carpel and no stamens (E). Many infected A. thaliana flowers demonstrate indeterminate growth of floral meristems (F). Strawberry (Fragaria x ananassa) can be infected with ‘Ca. Phytoplasma asteris’ (16SrI various subgroups) and demonstrates the multiplication of green petals from the centre of receptacle above the normal-looking white petals (G). Chickpeas (Cicer arietinum) produce single terminal flowers with white petals (H). C. arietinum infected with 16SrI-D subgroup phytoplasma generates sterile flowers with enlarged sepals and green leaf-like petals. These often show indeterminate telescopic flowers (I). Alfa-alfa (Medicago sativa) normally produces purple flowers arranged in raceme inflorescence whereas branches of the same plant infected with 16SrII-D subgroup phytoplasma generate inflorescences consisting of leaf-like flowers that make these branches look bushier than branches with normal flowers (J). White clover (Trifolium repens) has a round umbel inflorescence consisting of single white flowers. Infection with clover phytoplasma ‘Ca. Phytoplasma asteris’ (16SrI-C subgroup) is often associated with emergence of leaf-like flowers from the base of the inflorescence (K). Infection of Petunia sp. with ‘Ca. Phytoplasma asteris’ (16SrI-A subgroup) renders normal petals (L) into more leaf-like sepals lacking the characteristic floral pigments (M). Eggplant (Solanum melongena) from 16SrII-D subgroup phytoplasma infected fields exhibits characteristics of phyllody (N). Mayweed (Tripleurospermum inodorum) has numerous yellow-petal flowers arranged in a head-type inflorescence (capitulum) (O). In habitats where ‘Ca. Phytoplasma asteris’ (16SrI-B and C subgroup) has been detected mayweed displays inflorescences containing normal or various number of leaf-like flowers (O). Coneflower (Echinacea purpurea) infected with ‘Ca. Phytoplasma asteris’ (16SrI-A subgroup) develop indeterminate leaf-like flowers instead of their normal brown disk flowers and purple ray flowers within the inflorescence (P). Pictures A-C courtesy from Agnieska Zwolińska (IORPIB, Poland); images D-F and L-M taken by Andrew Davis (JIC, UK); photos H-J and N courtesy from Ali Al-Subhi (SQUO, Oman); pictures in panel O taken
Since this represents only a limited geographical distribution and few lineages of phytoplasmas, I used a collaborative opportunity to sample wild plant species in Brazil and find novel phytoplasma groups that are associated with phyllody symptoms in South America. I photographed and collected plant material from numerous plants that demonstrated phytoplasma symptoms such as increased stem branching, phyllody or increased susceptibility by insects in the field. I amplified the phytoplasma elongation factor Tu (tuf) gene for high resolution strain level identification of phytoplasmas and detection of mixed infections (Macarova et al., 2012; details in materials & methods). In order to identify the phytoplasma, I sequenced and aligned the tuf sequences from field samples with known sequences from members of all major phytoplasma groups. Uncharacterised before, I identified phytoplasmas from groups 16SrIII and 16SrVII to be associated with phyllody phenotypes in Acteraceae and Malvaceae family plants growing in savanna (cerrado) biome in Brazil (Figure 2.4). Moreover, there is genetic variation within group 3 and 7 phytoplasmas that were identified from these phyllody exhibiting plants. These findings suggest that phyllodies are found in diverse families of dicot plants and are associated with diverse groups and strains of phytoplasmas. Due to limited number of sequenced phytoplasma genomes (see details in Section 1.2.14) and lack of known primer sequences for SAP54 in group 3 and 7 phytoplasmas, no new SAP54 homologs were identified from the Brazilian field isolates. However, further whole-genome sequencing incentives may discover novel effector homologs in these phytoplasmas.

Although the exact structure and shape of the leaf-like floral tissue vary between different plant species infected with closely related or distinct phytoplasmas, there appears to be shared similarity – greening of petals and induction of indeterminate meristematic growth (Figures 2.3 and 2.4). In order to investigate if different phytoplasmas induce distinct alterations of floral morphology, I infected pathogen-free Catharanthus roseus plants of the same developmental stage separately with ‘Ca. Phytoplasma asteris’ strain Withces’ Broom (AY-WB) of 16SrI-A subgroup and Sweet Potato Little Leaf (SPLL)
phytoplasma of 16SrII-D subgroup. Surprisingly, the leaf-like floral phenotypes generated by these two phylogenetically distinct phytoplasmas were identical (Figure 2.5), suggesting that the phytoplasma effector responsible for leaf-like flower generation, SAP54, may able to interact with similar set of plant targets. Furthermore, the phyllody phenotype induced by the same phytoplasma (AY-WB) in *A. thaliana* (Figure 2.3E) and *C. roseus* (Figure 2.5B) are structurally very similar.

Taken together, given that the leaf-like floral phenotypes are present in diverse groups of plants and potentially induced by different phytoplasmas, phytoplasma effectors may be adapted to interact with the such a set of plant targets to produce similar leaf-like structures in many different host plants.
Figure 2.4.
Figure 2.4. Wild plants infected with group 3 and 7 phytoplasmas demonstrate changes in plant morphology and resistance to insects. Wild plants of *Sida* spp. (Malvaceae), *Conyza bonariensis* (Asteraceae), *Sonchus oleraceus* (Asteraceae), *Crotalaria incana* (Crotalarieae) and *Euphorbia heterophylla* (Euphorbiaceae) were collected in tropical savanna biome (Cerrado) in Goias State, central Brazil, and analyzed for phytoplasma infection. All collected plant samples were tested with general phytoplasma primers (details in Materials and Methods). Phytoplasma-positive samples were further amplified for *tuf* gene, cloned and transformed into *E.coli* to detect potential infection with multiple phytoplasma strains. Ten different *E.coli* colonies with *tuf* gene clones from each plant sample were sequenced for the *tuf* gene. Obtained sequences were aligned with reference sequences from diverse phytoplasma groups and Maximum likelihood tree (bootstrap 1000) constructed using MEGA. All plant hosts sampled were infected with single phytoplasma strain, grouping within either group 3 (red) or group 7 (yellow) phytoplasmas. Numbers on plant photographs correspond to the sequenced phytoplasma isolates on the phylogenetic tree. Infected plants demonstrated diverse symptoms: leaf proliferation (72, 69), phyllody (48, 50, 51) or indeterminate floral growth (46,49), reddening (66), leaf yellowing (52, 55, 63) or no characteristic symptoms (47,62, 60). Infected *Sida* plants had gall-like leaf structures and leaf crinkling but no obvious oviposition sites of galling insects. *Sonchus oleraceus* (60) demonstrated very high aphid colonisation compared to non-infected conspecifics few decimeters away. I would like to acknowledge Joao Roberto Spotti-Lopes (ESALQ, Sao Paolo) and Julio Barbosa (State University of Ponta Grossa, Parana) for assistance during field sampling and host plant identification.
Figure 2.5. Infection with different phytoplasmas generates similar leaf-like flower phenotypes in host plant. Non-infected Madagascar periwinkle (Catharanthus roseus; Apocynaceae) has single terminate flowers with reduced sepals and five large petals (A). Upon infection with ‘Ca. Phytoplasma asteris’ strain Withces’ Broom (16SrI-A subgroup) (B) or Sweet Potato Little Leaf phytoplasma (16SrI-D subgroup) (C) plants develop sterile leaf-like flowers with characteristic greening of petals. At later stages of infection both phytoplasmas induce smaller-sized leaf-like flowers as shown in picture C. Photographs by Zigmunds Orlovskis and Andrew Davis (JIC, UK). Bars approximately 1 cm.
2.2.2. Diverse phytoplasmas have SAP54 homologs that induce phyllody

Since leaf-like flower phenotype is induced by phytoplasma effector SAP54 (MacLean et al., 2011), I looked for homologs of SAP54 from publicly available data. Reciprocal BLAST search in GenBank revealed several sequences homologous to SAP54 from AY-WB phytoplasma from diverse groups of phytoplasma (Figure 2.6A). SAP54 sequence contains several leucine-rich motifs throughout the peptide length, indicating their potential conserved role in protein-protein interactions with the plant targets of SAP54. Phylogenetically distinct phytoplasmas from various 16Sr (sub-)groups have been identified in the same or closely related plant species (Lee, 2004). Infection of the same host (i.e., sympatric phytoplasmas in the same ecological niche) would, therefore, present opportunities for convergent evolution or horizontal gene transfer of virulence genes that essential for phytoplasma fitness. I wanted to investigate whether the phylogeny of SAP54 homologs is similar to the species-level relationship between the phytoplasma groups where the SAP54 homologs have been found. I compared the phylogenetic relationship of SAP54 and a housekeeping gene (elongation factor Tu), which has previously used in phytoplasma classification and is congruent with 16S ribosomal DNA-based identification (Macarova et al., 2012). Interestingly, the effector and species-level phylogenies are not corresponding (Figure 2.6B,C). Given that the phytoplasmas investigated here are generalists and can infect multiple plants hosts (Lee et al., 2004; Bertaccini and Duduck, 2009), these data suggest that SAP54 has evolved independently from the rest of phytoplasma genome either by adapting to the MTF repertoire of its respective host plant range in each phytoplasma clade separately or horizontal transfer of SAP54 between phylogenetically diverse phytoplasmas that infect the same host plant species.
Figure 2.6

A

B

C

Protein Conservation

Consensus sequence

0.1

0.06

16Sr Candidatus species group

I P.aurantifolia II P. solani III P. pruni IV P. asteris V P. trifolii VI P. pseudolabrum VII P. pseudoflava VIII P. pseudoflava IX P. phoenicium X P. pseudolabrum Y P. pseudolabrum Z P. pseudolabrum
Figure 2.6. SAP54 homologs from diverse phytoplasmas show convergence of the effector sequence. (A) Alignment of SAP54 protein demonstrates conserved motifs of over the entire length of the effector protein. N'-terminal and C'-terminal ends of the peptide appear to be leucine-rich among SAP54 homologs. Alignment does not include the signal peptide. SAP54 sequences from diverse members of phytoplasma 16Sr groups I, II, III, VI, IX and XII demonstrate convergence of the effector sequence (B) which is not congruent with species-level phylogeny that is based on diversification of phytoplasma elongation factor Tu (tuf) (C). Alignments made using ClustalW. Phylogenetic trees constructed using Maximum Likelihood method. Numbers at the nodes indicate bootstrap (1000) support. Strain identity and GenBank accession numbers for phytoplasma sequences are given in materials and methods (Table 8.1).

In order to determine whether SAP54 homologs from phylogenetically distinct phytoplasmas can induce phyllody, transgenic A. thaliana plants that ectopically express three different SAP54 homologs were generated. Interestingly, SAP54 homologs from 16Sr group I, II and XII can induce sterile leaf-like flowers with characteristic greening of petals and indeterminate growth (Figure 2.7). SAP54 homologs from Aster Yellows Witches' Broom phytoplasma (AY-WB) (16SrI-A subgroup) and Peanut Witches' Broom phytoplasma (PnWB) (16SrII group) generate very similar morphology of leaf-like flowers. However, sometimes SAP54 homolog from PnWB generates inflorescence that resembles cauliflower-like florets. SAP54 homolog from Stolbur (16SrXII) group phytoplasma causes elongation and swelling of carpels that has often been observed in rapeseed fields infected with 16SrI-B subgroup phytoplasma (Figure 2.3A-C). Otherwise the morphological changes in leaf-like flowers induced by Stolbur and AY-WB homologs of SAP54 are very similar.
2.2.3. SAP54 homologs show conserved interaction with type-2 MTFs and RAD23 proteins

Since different phytoplasma lineages have SAP54 homologs that show sequence similarities independent from phylogenetic relationships among phytoplasmas, and the SAP54 homologs can induce leaf-like flowers, I hypothesise that SAP54 homologs have evolved to interact with similar set of plant targets that are required for induction of phyllody in different host species that these phytoplasmas infect. It was previously demonstrated that SAP54 interacts with type-II plant MTFs and RAD23 proteins (MacLean et al., 2014). I, therefore, analysed the protein-protein interactions between a subset of A.
thaliana MTFs and RAD23 proteins using yeast-two-hybrid approach. Surprisingly, SAP54 homologs from AY-WB, PnWB and Stolbur phytoplasmas demonstrate similar specificity to RAD23 protein isoforms C and D as well as SOC1 and SEPELLATA family MTF proteins (Table 2.1; Appendix C), suggesting that the SAP54 interactions with RAD23 and MTFs could be conserved in many phytoplasmas.

Table 2.1. SAP54 homologs from AY-WB, PnWB and Stolbur phytoplasmas demonstrate conserved protein-protein interactions with plant MTFs and RAD23 proteins. SAP54 homologs were cloned as prey in yeast expression vector containing the Binding Domain of GAL4 gene. Plant MTF and RAD23 target proteins were cloned as bait in yeast expression vector containing the Binding Domain of GAL4 gene. Upon interaction of the pray and the bait proteins, expression of marker genes allows yeast growth on selective media. Positive interactions indicated by yeast growth on –L-W-H 5 mM 3-AT SD media are depicted “+” and shaded green; no interactions indicated by no yeast growth are depicted as “-”. SEP1-3 are SEPELLATA family MTF proteins; SOC1= SUPPRESSOR OF CONSTANS1; FLC= FLOCERING LOCUS C; RAD23= RADIATION INSENSITIVE23 (isoforms A,B,C,D); ev= empty vector not containing SAP54, MTFs or RAD23. Any differences in SAP54 homolog interaction with plant targets are shaded red. Yeast images for the full dataset are included in Appendix C.

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<th>BD-SAP54 Interaction</th>
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So far yeast-two-hybrid analysis have identified type-II plant MTFs as potential interactors of SAP54 (MacLean et al., 2014). For this reason only members of type-II have been tested for degradation by SAP54 in planta assays. In order to investigate whether SAP54 may targets other plant MTFs than type-II plant MTFs, I cloned members of all subclasses (Mα, Mβ, Mγ; Smaczniak et al., 2012) of A.thaliana type-I MTFs to investigate the possible degradation by SAP54. I found that SAP54 only partially degrades type-I plant MTFs in transient Nicotiana benthamiana expression assays (Figure 2.8). Given that the levels of type-II MTFs were much reduced or absent in plants producing SAP54 in an analogous experiment (MacLean et al., 2014; Fig.2) compared to type-I MTFS (Figure 2.8), the slight reduction of type-I MTF levels may be non-specific due to interference with protein translation or proteasome function in presence of SAP54 or occurrence of type-I MTFs in higher order protein complexes that may be indirectly perturbed by SAP54. In planta pull-down assays have shown that SAP54 specifically interacts with members of type-II MTFs but not type-I MTFs (MacLean et al., 2014).

![Figure 2.8. Aster Yellow Witches' Broom effector SAP54 partially destabilises type-I MTFs AGAMOUS-like 50 (AtAGL50), AGAMOUS-like 62 (AtAGL62), AGAMOUS-like 80 (AtAGL80). Nicotiana benthamiana leaves were infiltrated with A. tumefaciens cultures containing either flag-tagged SAP54 or Red Fluorescent Protein (RFP) control together with myc-tagged type-I plant MTF. Leaves were harvested 3 days after agro-infiltration to assess the protein levels using Western blotting with antibodies for flag- or myc-tags. In presence of both SAP54, there the amount of MTFs is slightly reduced compared to co-expression with RFP control. Well loading control stained for RuBISCO with Ponceau stain on the SAP54 blot.](image-url)
Taken together, these data indicate that SAP54 interactions with type-II plant MTFs and RAD23 are specific and conserved across diverse phytoplasma groups. Targeting type-II MTFs by SAP54 from AY-WB phytoplasma results in degradation of type-II MTFs in proteasome-dependent manner (MacLean et al., 2014). It remains to be tested whether SAP54 homologs from other phytoplasmas also degrade its MTF interactors.

2.2.4. SAP54 promotes insect colonisation in a RAD23 dependent manner

It has been demonstrated earlier that SAP54 requires RAD23 proteins in order to destabilise several type-II plant MTFs and induce leaf-like flowers (MacLean et al., 2014). It is also known that single phytoplasma effectors, such as SAP11, can play a role in modulating both plant development as well as plant-insect interactions (Sugio et al., 2011a). Since we know only about SAP54 effects of floral development, I wanted to use the knowledge about the mechanisms of SAP54 activity to investigate whether SAP54 could have potential effects on plant colonisation by the insect vector of phytoplasma. I designed an assay to measure insect choice to reproduce on two alternative host plants. I placed \textit{rad23bd} and \textit{rad23bcd} (Vierstra, 2009) mutants in a choice arena opposite each other (see materials & methods Figure 8.1) and released a mixed population of phytoplasma-free male and female aster leafhopper \textit{Macrosteles quadrilineatus} – the principal vector of AY-WB phytoplasma. After 5 days I removed the adults and isolated the plants to later count the leafhopper progeny on each plant. Leafhoppers did not show any preference to reproduce on phytoplasma-free \textit{rad23bd} or \textit{rad23bcd} plants (Figure 2.9). However, when \textit{rad23bd} or \textit{rad23bcd} plants are infected with AY-WB phytoplasma, \textit{M. quadrilineatus} demonstrates significant preference to reproduce on \textit{rad23bd} mutant plants (Figure 2.9). Given that only \textit{rad23bd} demonstrate leaf-like flower phenotype in presence of phytoplasma, these results suggest that phytoplasma effector SAP54 may alter plant floral development and promotes plant colonisation by its insect vector in RAD23-dependent manner.
Figure 2.9. Aster leafhopper *Macrosteles quadrilineatus* demonstrates oviposition preference for *rad23bd* plants with leaf-like flowers. In dual-choice tests *M. quadrilineatus* produces significantly more progeny on AY-WB infected *rad23bd* plants with leaf-like floral phenotype compared to *rad23bcd* plants with normal floral phenotype ($t_5=4.7; p=0.042$). Insects do not show any preference for non-infected *rad23bd* or *rad23bcd* plants ($t_5=0.45; p=0.694$). The graph represents the percentage of *M. quadrilineatus* nymphs found on each test plant within a single choice cage. Data analysed using paired t-test. Bars on the graph are 1 standard error of the mean. White scale bars are approximately 1 cm.

### 2.3. Discussion

It was most intriguing to find that the induction of leaf-like flowers and enhancement of insect reproduction on infected plants are genetically linked via 26S proteasome cargo protein RAD23 (Figure 2.9). Given our current mechanistic understanding (Figure 2.1), these data suggest that degradation of MTFs may be causal for induction of phyllody and the attraction of insects to the infected host plants. Furthermore, this supports the hypothesis that generation of leaf-like flowers may be an adaptive phenotype of phytoplasmas to attract insect vectors and enhance their spread in nature (Figure 2.10). However, alternative explanations such as flower or MTF independent attraction of insect vectors are plausible and will require to be tested (Figure 2.10).
Figure 2.2 Graphical representation of alternative hypotheses for SAP54-mediated promotion of insect attraction to phytoplasma infected plants. 

Hypothesis 1 (red arrows): SAP54 destabilises MTFs to induce leaf-like flowers in RAD23-dependent manner. The leaf-like flowers are required for attraction of insect vectors to infected plants. 

Hypothesis 2 (purple arrows): SAP54 destabilises MTFs in RAD23-dependent manner to induce changes in plants that required for attraction of insect vectors. The leaf-like flowers are side-effect of MTF degradation. 

Hypothesis 3 (black arrows): SAP54 attracts insect in RAD23 dependent manner but independently from degradation of MTFs or induction of leaf-like flowers.

Given the first hypothesis, it is reasonable to speculate that induction of phyllody is under natural selection to promote acquisition by insect vectors. This is supported by the observations that plant phyllody phenotype is associated with diverse groups of phytoplasmas and shares similar features of indeterminate floral growth, greening of petals and absence of stamens and carpels (Figures 2.3-2.5). Moreover, SAP54 homologs from different phytoplasmas induce similar morphological changes in plants – indeterminate growth of floral meristems and complete reversion of floral organs into leaf-like structures (Figure 2.7). Nevertheless, there is variability in the phyllody phenotype which may originate from promiscuous interaction with multiple MTF targets from the same plant (variation in leaf-like floral phenotype by PnWB homolog of SAP54 in A. thaliana, Figure 2.7). Alternatively variation in the leaf-like flower phenotype such as seen in infected rapeseed fields (Figure 2.3A-C) may originate from plants being infected at different stages of their development or from variation in plant genotype, or clonal variation of SAP54 alleles in the field. Nevertheless, variation in the exact appearance in phyllody may be host-specific. For example, C. roseus
plants infected with the AY-WB and SPLL phytoplasmas induce phyllodies that are visually indistinguishable (Figure 2.5) despite that the A.thaliana transformed with SAP54 homologs from AY-WB and SPLL (SPLL SAP54 shares 100% nucleotide similarity with PnWB SAP54 homolog) may sometimes demonstrate different floral phenotypes (Figure 2.7).

Given that SAP54 sequences do not necessarily share sequence similarity between closely related phytoplasmas (Figure 2.6B-C) and that effectors are located on potential mobile genetic elements (Ku et al., 2013), there is a possibility that SAP54 effector may be exchanged by horizontal gene transfer between different sympatric phytoplasma groups infecting the same host species. Nevertheless, the sequence similarity between SAP54 from ‘Ca. P. phoenicium’ in herbaceous Helmanthoteca echioides (Asteraceae) from Italy and ‘Ca. P. asteris’ from Brassica napus (Brassicaceae) field in Poland (Figure 2.6B-C) suggests that perhaps in some cases SAP54 may demonstrate convergent sequence evolution than horizontal gene transfer due to large geographical distances that separates them and different host plants they infect. Alternatively, of course, horizontal gene transfer may have occurred in the ancestral phytoplasma, and SAP54 could have been under strong purifying selection in two sister lineages.

The conservation of certain amino acid residues within the secondary folded structure of SAP54 may be more important rather than conservation of the entire SAP54 peptide. For example, SAP54 homologs from ‘Ca. P. asteris’ (AY-WB and BPIP) and ‘Ca. P. solani’ (Stolbur) share little sequence similarity (Figure 2.6A). Nevertheless, these SAP54 homologs demonstrate similar interaction with plant MTFs and Rad23 proteins (Table 2.1) as well as similar phenotypes when SAP54 homolog from ‘Ca. P. solani’ is compared to ‘Ca. P. asteris’ homolog of SAP54 in Figure 2.7 or compared to ‘Ca. P. asteris’ infected rapeseed in Figure 2.3C. Alignment of SAP54 homologs indicates conservation of certain leucine-rich motifs at the N’-terminal and C’-terminal end of SAP54 protein (Figure 2.6A). These may be key for folding into the coiled-coil helical structure of the SAP54 protein (Rümpler et al., 2015). Therefore, conservation of structural similarity in the effector folding may be key to interactions with similar set of MTF targets from different plant families.

SAP54 is significantly upregulated in the plant host (MacLean et al., 2011) and appears to have evolved specificity for type-II plant MTF, resulting in specific
degradation of this clade of plant proteins (MacLean et al., 2014). I confirmed that SAP54 is not likely to target type-I MTFs \textit{in planta} (Figure 2.8). Hence, the affinity for K-domain, may determine target specificity (Figure 2.2). Although the duplication event that gave rise to type-I and type-II MTFs is believed to precede divergence of plants and animals (Alvarez-Buylla et al., 2000), the K-domain is present only in type-II plant MTFs and absent in other eukaryotes (Ng and Yanofsky, 2001). Thus, targeting the K-domain would avoid interfering with insect MTFs and compromise the fitness of the insect vector. Selective interaction with type-II plant MTFs means that SAP54 targets a structurally conserved class of plant proteins with conserved functions in plant reproductive development (Theissen et al., 2000; Ng and Yanofsky, 2001). Type-I plant MTFs are believed to evolve new functions faster than type-II plant MTFs (Nam et al., 2004). In contrast, recruitment of type-II MTFs as homeotic genes regulating reproductive development is believed to occur before the divergence of ferns and seed plants (Munster et al., 1997) and maintained similar structural and functional complexity after divergence of angiosperms and gymnosperms (Becker et al., 2000). Similarly, RAD23 proteins are structurally and functionally conserved across eukaryotes (Schauber et al., 1998; Vierstra, 2009). Therefore, by interacting with conserved MTF and RAD23 proteins, SAP54 is likely to target a similar developmental processes in diverse groups of flowering plants (angiosperms), which may enable this plant pathogen to infect multiple hosts and, thus, occupy a broad ecological niche.
“I cannot give any scientist of any age better advice than this: the intensity of the conviction that a hypothesis is true has no bearing on whether it is true or not.”

Sir Peter B. Medawar (1979), “Advice to a Young Scientist”, p 39

Chapter 3
Phytoplasma Effector SAP54 Mediates Insect Vector Attraction to Host Plants Independently of Developmental Changes

Part of this work is published in:

3.1. Introduction

Phyllody is common in many phytoplasma infected plants and induced by phytoplasma effector SAP54 (MacLean et al., 2011). Moreover, several homologs of SAP54 have been identified in phylogenetically distinct phytoplasmas, and, when ectopically expressed in Arabidopsis, can induce phyllody (Chapter 2). The induction of phyllody and attraction of phytoplasma insect vectors appears to be mechanistically linked via 26S proteasome cargo protein RAD23, given that RAD23 is required for generation of phyllody and plant colonisation by insect vector of phytoplasma (MacLean et al., 2014). This presents a hypothesis that phytoplasma may induce changes in plant morphology in order to attract insect vectors of phytoplasma. Thus, phyllody may be an adaptive manipulation of host plant by the parasitic phytoplasmas to enhance the spread of the pathogen in nature.

Nature is full with examples of alteration of host development, behaviour and inter-specific interactions by parasites (Figure 3.1). In many cases host modification is detrimental to the host but beneficial to the parasite by enhancing their survival, reproduction or transmission as part of the parasitic life-cycle. For example, the severe limb malformations of frogs by trematodes of the genus Ribeiroia (Figure 3.1B) is thought to increase predation of the locomotion-impaired frogs by birds, which are essential for spread of the trematodes in the environment (Johnson et al., 2004). In addition to developmental alteration of hosts by parasites, modulation of host behaviour is implicated in spread of parasites, too. Rodents infected with Toxoplasma gondii change their behaviour increasing the likelihood of predation by cats (Figure 3.1C), which are the definitive hosts for T. gondii (Berdoy et al., 2000). Likewise, nematomorph worms coerce their cricket hosts to orientate towards water bodies and perform a “suicidal death jump” (Figure 3.1D) to release the parasite in water where it can complete its life cycle (Thomas et al., 2002; Biron et al., 2005a,b).
Figure 3.1. Parasites alter host development and behaviour. (A) Phytoplasma effector SAP54 changes flower development and induces formation of sterile photosynthetic leaf-like flowers (bottom image) instead of normal flowers (top image). Additionally, phytoplasma vectoring insects can be found on infected leaf-like structures. Photo of a healthy and AY-WB infected Catharanthus roseus flower with aster leafhopper Macrostelea quadrilineata by Andrew Davis, JIC. (B) Trematode parasite Ribeirioa ondatrae alters amphibian limb development (www.hcn.org/blogs/goat/amphibian-alterations). (C) Unicellular apicomplexan parasite Toxoplasma gondii can infect rodent brains and alter their vigilance behaviour to encounter its natural feline predators. Picture taken from www.wallpaperspicturesphotos.blogspot.co.uk/ and www.istockphoto.com. (D) Parasitic nematomorph worms such as Spinochordodes tellinii infect cricket host to coerce the insect for a suicidal entering of water where the parasite exits the insect body. Picture from www.en.wikipedia.org/?title=Nematomorpha. (E) Fungi of genus Ophicordiceps develop spore-containing fruiting body from infect tropical ants like this Dinomyrmex gigas (https://www.flickr.com/photos/orionmystery/).

Such host manipulations by parasites are often viewed as an extended phenotype of parasitic gene(s), where parasite genome is responsible for alteration of phenotype of another organism (the host) as an adaptation to increase parasite fitness (Dawkins, 1982; Hughes, 2014). However, the molecular or physiological mechanisms underpinning host manipulations by
parasitic organisms are largely unknown limiting our ability to investigate if the modulations are adaptive or neutral (Poulin, 1995, 2013; Thomas et al., 2005). Only a few parasite genes that orchestrate dramatic changes in host phenotype and behaviour have been identified so far (Hoover et al., 2011; Sugio et al., 2011; MacLean et al., 2014). If host manipulation is an adaptation of a parasite, natural selection has to act on parasitic genes that encode this phenotype (Dawkins, 1990, 2004). Alternatively, changes in host biology may be just a side-effect of parasitic infection that does not contribute to parasite reproduction, survival or spread in nature and, therefore, are not an extended phenotype (Dawkins, 2004). It is a challenging task to demonstrate whether a particular manipulation of host organism is adaptive. For example, a fungal parasite of genus Ophiocordyceps infects Camponotus ants and coerces the insect host to perform the “dead-grip” in a micro-habitat (Figure 3.1E) with the right humidity and temperature suitable for the development of fungal fruiting body and dispersal of the spores (Andersen et al., 2009). Outside this manipulative zone the fungus is not able to reproduce. Since the reproductive fitness of the fungus depends on the exact details of manipulation of the ant, the fungal genes responsible for this manipulation should be under natural selection. In many other host-parasite systems, however, parasites induce multiple changes in the host simultaneously. For example, the freshwater shrimp Gammarus pulex, infected with acanthocephalan parasite Pomphorhynchus laevis, demonstrates altered coloration as well as positive phototactism and immune depression (Thomas et al., 2010), rising questions which of these traits, if any, are adaptive and causal for enhanced trophic transmission of parasite to fish predators. It is difficult to unambiguously test the adaptive role of parasite induced changes in their hosts without a mechanistic understanding about these modifications and experimental tools to manipulate or isolate individual parasite altered traits (Cézilly and Perrot-Minnot, 2010).

Mechanistic understanding about the generation of leaf-like flowers in phytoplasma-infected plants allows, for the first time, to directly test the adaptive function of the changes in host morphology by a single parasite gene. Understanding the functions of host manipulation by parasite effector (virulence) genes has significant implications for understanding the epidemics of vector-borne plant diseases (Lefèvre and Thomas, 2008) and modelling disease spread in natural and agro-ecosystems.
This chapter aims to test the hypothesis that induction of leaf-like flowers by phytoplasma effector SAP54 is an adaptive manipulation of the plant host by phytoplasma to attract insect vectors. A series of experiments resulted in a breakthrough finding that refutes this hypothesis and demonstrates that leafhoppers make a choice to colonise SAP54 plants independently from morphological changes in the host plant. Additional adaptive roles of phyllody are investigated and discussed.

### 3.2. Results

**3.2.1. Leafhoppers prefer rosette leaves over normal or leaf-like floral structures**

Given the finding that leafhopper *M. quadrilineatus* preferentially colonises phytoplasma infected *rad23bd* mutants with leaf-like flowers (MacLean et al., 2014), I wanted to further examine if the leafhoppers are attracted by leaf-like flowers or repelled by wild-type flowers. I observed the behaviour and residency preference of 10 male and 10 female *M. quadrilineatus* on infected *rad23* mutants that had either leaf-like flowers or normal floral phenotype (Figure 3.2A). *M. quadrilineatus* was not repelled by the normal flowers of infected *rad23bcd* plants. Both male and female leafhoppers were found to land and explore the inflorescence, possibly feed on flower pedicel and inflorescence stems and probe the petals or carpels. Similarly, leafhoppers were found in leaf-like flowers of infected *rad23bd* mutant plants, where they feed on the leaf-like petals and pedicel of telescopic flowers. These observations suggest that the insects may not prefer the leaf-like flowers over the wild-type ones.

Next, I quantified the distribution of *M. quadrilineatus* adults between rosette leaves and floral tissue of the phytoplasma infected *rad23* mutants over 5 day period. Interestingly, most leafhoppers preferred to reside on the rosette leaves rather than floral stems and flowers of *rad23bd* plants with leaf-like flowers or *rad23bcd* plants with normal-looking flowers (Figure 3.2B). Moreover, the leaf-like and normal flowers attract approximately equal proportion of the insects. These data suggest that the flowers may not be required for leafhopper attraction.
Figure 3.2. Leafhoppers demonstrate similar distribution on plants with leaf-like and wild-type flowers. (A) *M. quadrilineatus* leafhoppers were photographed whilst residing and feeding on all parts of infected *Arabidopsis thaliana rad23BD* and *rad23BCD* plants, including rosette leaves and petioles, stems, cauline leaves and flowers. Insects were found on carpels, sepals, petals and pedicels of wild-type-looking *A. thaliana* flowers as well as leaf-like flowers. White scale bars on each picture are 1 cm. (B) Number of insects found on rosettes or floral tissue is plotted as percentage of the total number of insects on wild-type plants and plants with leaf-like floral phenotype. Bars
represent standard error of the mean of 8 independent replicate cages. *M. quadrilineatus* has significant residency preference for rosette leaves compared to other floral structures both on AY-WB infected *rad23BCD* mutant plants with leaf-like (L) flowers and AY-WB infected *rad23BD* mutant plants with wild-type flowers (GLM with time as covariate; \( F_{1,137}=1797.78; \, P \leq 0.001 \)). There is no difference between insect residency on wild-type and leaf-like flowers during the five-day leafhopper choice experiment (GLM with time as covariate; \( F_{1,67}=0.19; \, P=0.666 \)).

### 3.2.2. Leafhoppers prefer SAP54 plants for oviposition independently from induction of leaf-like flowers

Given that leafhoppers demonstrate oviposition preference for infected *rad23bd* plants with leaf-like flowers (Chapter 2) but flowers themselves do not appear to trigger leafhopper attraction (Figure 3.2), I further investigated whether leafhoppers prefer to reside and lay eggs on transgenic plants expressing phytoplasma effector SAP54. 10 male and 10 female *M. quadrilineatus* adults were given a choice between 35S:GFP-SAP54 plants with leaf-like flowers and 35S:GFP plants with normal flowers for 5 days. Insect residency and egg-laying was recorded. *M. quadrilineatus* adults spent more time on GFP-SAP54 transgenic plants with leaf-like flowers versus GFP transgenic plants with wild-type flowers (Figure 3.3), and these insects also produced more progeny on GFP-SAP54 transgenic plants with leaf-like flowers (Figure 3.4A). However, when insects were not given a choice between host plants, by caging the leafhoppers on either GFP-SAP54 transgenic plants with leaf-like flowers or control GFP transgenic plants with wild type flowers, no increase in nymph production was observed (Figure 3.5). Thus, greater number of *M. quadrilineatus* progeny on SAP54 plants may result from preferential orientation and egg-laying choice on SAP54 plants with leaf-like flowers rather than increase in leafhopper reproductive efficiency *per se*. 
Figure 3.3. Aster leafhopper *Macrosteles quadrilineatus* demonstrates greater residency preference for SAP54 expressing plants with leaf like-flowers. More insects were found on SAP54 plants over the entire 5 day choice period (GLM with time as covariate; $F_{1,57}=22.14; P \leq 0.001$). Picture scale bar is 1 mm. Bars in the graph are one standard error of the mean.

Figure 3.4.
Figure 3.4. Flowers and transition from floral to vegetative phase are not required for SAP54-mediated enhancement of insect colonization. (A) *M. quadrilineatus* produces more nymphs on 35S:GFP-SAP54 transgenic *A. thaliana* (Col-0) plants with leaf-like flowers than on 35S:GFP (Col-0) control plants with wild type flowers (p≤0.001). (B) Removal of flowers and floral stems does not affect *M. quadrilineatus* colonization preference of 35S:GFP-SAP54 transgenic *A. thaliana* (Col-0). (C) Leafhoppers also prefer GFP-SAP54 transgenic plants prior to transition from vegetative to floral growth. (D) *M. quadrilineatus* lays more eggs on single rosette leaves of GFP-SAP54 transgenic plants. Bars shown are one standard error of the mean. Each experiment was replicated in six independent choice cages. Significant difference in leafhopper reproductive preference are indicated as follows *** p≤0.001; ** p≤0.025 (paired t-test). All experiments were repeated three times with similar results.

Figure 3.5. *M. quadrilineatus* leafhoppers produced similar nymph numbers when confined to 35S:GFP-SAP54 or 35S:GFP plants. Leafhoppers were released on whole plants, which were caged inside a plastic tube as shown at left and right (picture scale bars are 5 cm). The middle graphs show mean numbers of leafhopper nymphs produced in these cages of three independent experiments (paired t-test; n=6; p=0.773). Bars shown are one standard error of the mean.
To analyse the impact of leaf-like flowers on leafhopper preference further, I removed both the leaf-like and wild-type flowers from 35S:GFP-SAP54 and 35S:GFP plants in the insect choice experiments and found that the leafhoppers still preferred the GFP-SAP54 plants (Figure 3.4B), suggesting that the presence of leaf-like flowers is not required for leafhopper preference for SAP54 plants. A. thaliana plants used in insect choice tests so far were grown at long days to induce bolting and flowering. Therefore, I conducted choice tests on A. thaliana plants grown at short days before flowering. Again, M. quadrilineatus produced more nymphs on GFP-SAP54 versus GFP (control) plants (Figure 3.4C), suggesting that insect preference for the GFP-SAP54 plants does not involve physiological and developmental transformations that occur during floral transition. To confirm this finding, leafhoppers were also given a choice between single leaves of GFP-SAP54 and GFP plants grown at short days. Importantly, leafhoppers preferred to lay eggs onto single leaves of GFP-SAP54 plants (Figure 3.4D), suggesting that leafhoppers are attracted solely to the leaves of GFP-SAP54 plants. Taken together, these data demonstrate that leaf-like flowers are not required for host plant selection by the leafhopper vector, and that SAP54 modulates processes in leaves to promote leafhopper attraction.

### 3.2.3. Phyllody is not required for leafhopper oviposition choice

The above experiments provide evidence that leaf-like flowers are not required for insect vector preference. Nonetheless, these flowers could contribute to the insect preference. To test this, I conducted choice experiments with A. thaliana lines displaying leaf-like flowers, including MTF mutant lines ap1 (Mandel et al., 1992) and lfy (Weigel et al., 1992) and the 35S:SVP transgenic line (Gregis et al., 2013). All these lines produce flowers that share leaf-like structures reminiscent to those of phytoplasma-infected and GFP-SAP54 transgenic plants (MacLean et al., 2011). I found that leafhoppers produce similar numbers of progeny on both plants indicating no colonization preference for either wild type or mutant plants with leaf-like floral phenotypes (Figure 3.6). These data are in agreement with insect preference for rosette leaves rather than floral stems or flowers (Figure 3.2). Thus, the leaf-like flowers are neither required nor involved in the host preference of M. quadrilineatus, the insect vector of phytoplasmas.
Figure 3.6. Aster leafhopper *Macrosteles quadrilineatus* has similar oviposition preference for plants with normal and leaf-like flower phenotype. *M. quadrilineatus* did not show a preference for colonization of Col-0 wild-type versus Col-0 *apetala1* (*ap1-*12) (p=0.835), Col-0 versus Col-0 *leafy* (*lfy-*1) (p=0.985) and Col-0 versus 35S:SVP (Col-0) (p=0.960). Choice experiments were conducted with whole plants retaining both vegetative and floral organs. Data shown as percentage of *M. quadrilineatus* nymphs found on each test plant per total number of nymphs within a single choice cage (bars are standard error of the mean). Data were analysed by paired t-tests. All experiments were repeated three times with similar results. Picture scale bar is approximately 1 mm.

### 3.2.4. SAP54 plants do not show increased longevity

Although leaf-like flowers (phyllody) may not be contributing to leafhopper oviposition preference for SAP54 plants, abolishment of flowering and fruit production may delay plant senescence and increase the longevity of the plant host and thus enhance the possibility of phytoplasma acquisition by its insect vector. To test this hypothesis, I compared the time of bolting, flowering and senescence of 35S:GFP-SAP54 and 35S:GFP transgenic plants under different photoperiods. Plants were grown side by side and photographed weekly until complete senescence. Surprisingly, 35S:GFP-SAP54 required similar time till the complete senescence (death) of the plant compared to 35S:GFP and wild-type Col-0 plants (Figure 3.7). Moreover, 35S:GFP-SAP54 plants demonstrated...
significantly earlier bolting as well as slightly earlier onset of senescence compared to controls. This suggest that abolishment of plant reproduction by generation of sterile leaf-like flowers in SAP54 expressing plants may not contribute to leafhopper attraction by increase in plant survival.

Figure 3.8. SAP54 plants show earlier bolting but similar longevity compared to control and wild-type plants. A. thaliana (Col-0) wild type and A. thaliana (Col-0) 35S:GFP-SAP54 and 35S:GFP transgenic plants were grown under short day (8h/16h day/night) and long day (14h/10h day/night) photoperiods at 22°C, 48% humidity without prior vernalisation until complete death of the plants. Average time (weeks) until the first appearance of 1 cm bolts at the centre of rosette (bolting), the first appearance of yellowing stems and leaves (senescence) and complete yellowing of the entire plant (death) was recorded. 35S:GFP-SAP54 showed significantly earlier (1.5 weeks) bolting compared to 35S:GFP and wild-type Col-0 plants under short day photoperiod (ANOVA; \( F_{2,15}=13.33; p<0.001; \) Tukey pairwise comparison). Bars are one standard error from the mean. Experiment was performed using 6 plants and repeated twice with identical results.
Photographs were taken to assess whether, in addition to plant longevity, plants with leaf-like flower phenotype showed any differences in plant architecture or biomass. At short day photoperiod 35S:GFP-SAP54 plants demonstrate very similar above-ground biomass compared to 35S:GFP plants from start of bolting until senescence (Figure 3.8A). However, 35S:GFP-SAP54 plants show slightly more branching and greater shoot biomass after bolting at long-day photoperiod (Figure 3.8B). Taken together, these data suggest that under certain environmental conditions phyllody may play potential additional roles in plant-insect interactions independently from adult leafhopper attraction. This remains to be explored and tested empirically.
Figure 3.8. SAP54 plants display increased areal shoot biomass compared to control plants under long day photoperiod. Representative photographs of 35S:GFP-SAP54 and 35S:GFP transgenic plants were grown under short day (8h/16h day/night) (A) and long day (14h/10h day/night) (B) photoperiods for the experiment in Figure 3.6. Numbers indicate the age (weeks) after the plant after sowing. Scale insets = 20 cm.
Figure 3.8. (continued)
3.3.5. Phyllody does not increase phytoplasma titer

Although adult leafhoppers are mainly attracted to rosette leaves of SAP54 (Figure 3.4) and phyllody is not likely to contribute to this attraction (Figure 3.6) or longevity of the plant (Figure 3.7), higher pathogen titer in the leaf-like flowers compared to normal flowers may facilitate phytoplasma acquisition by leafhopper nymphs that have hatched on rosettes but move to upper parts as late instars. To test this hypothesis, I infected rad23bcd and rad23bd plants with AY-WB phytoplasma and measured the amount of phytoplasma DNA relative to plant DNA as a measure of phytoplasma replication (bacterial titer) in rosette leaves and inflorescence (Figure 3.9).

![Figure 3.9](image)

**Figure 3.9. AY-WB infected rad23bcd plants and rad23bd plants have similar phytoplasma titer.** 3-weeks old plants were inoculated with AY-WB phytoplasma by adding 5 infected male *M. quadrilineatus* on each plant for 5 days. 21 days post inoculation rosette leaves and floral tissue were harvested for qPCR of phytoplasma DNA and plant DNA as a reference. Data show the amount of phytoplasma 16S rRNA gene copies relative to plant actin DNA. Infected rad23bd plants developed leaf-like flowers but infected rad23bcd – normal flowers (picture insects; scale=1mm). Leaf-like flowers contained the same amount of phytoplasma DNA compared to normal flowers (t2,4 = 0.24; p=0.815). There is no difference in phytoplasma titer between rosettes of infected rad23bcd and rad23bd plants (t2,4= 1.24; p= 0.256). Flowers contain slightly but not significantly less phytoplasma compared to rosette tissue in both rad23bd (t2,4=3.02; p=0.039) or rad23bcd plants (t2, 4= 1.78; p=0.15). Data analysed using two-tailed t-test;
experiment included 4 replicate plants and was repeated 2 times with similar results. Bar represents 1 standard error of the mean.

Interestingly, there was no difference in phytoplasma titer of infected flowers or rosettes between rad23bcd or rad23bd plants, suggesting that leaf-like flowers *per se* do not enhance phytoplasma replication within the plant. In addition, pathogen titer in rosette leaves is not RAD23 dependent.

### 3.3. Discussion

Hitherto, direct analyses of the adaptive significance of parasite extended phenotypes have been limited because many parasites (such as phytoplasma) are not amenable to genetic manipulation and parasite genetic factors that induce the dramatic host alterations are often unknown. Given that leafhoppers feed and lay eggs mostly on vegetative tissues, including stems and leaves (Weintraub and Beanland, 2006), and that the plant 26S proteasome cargo protein RAD23 is required for both the induction of leaf-like flowers and insect vector attraction (MacLean *et al.*, 2014), I hypothesized that leafhoppers may be attracted to leaf-like flowers of phytoplasma-infected and SAP54 transgenic plants. However, this study has shown that leaf-like flowers are not required nor are involved in attraction of the phytoplasma insect vectors (Figures 3.4 and 3.6). Moreover, leafhoppers preferred plant vegetative tissues above reproductive organs (Figure 3.2). Thus, leaf-like flowers do not promote leafhopper colonization, even though these two phenotypes are genetically connected via SAP54 interaction with the 26S proteasome cargo protein RAD23 (Figure 3.10).
Figure 3.10. Phytoplasma effector SAP54 mediates insect vector attraction to host plants independently of the presence of leaf-like flowers. The phytoplasma virulence proteins (effector) SAP54 interacts with specific MADS-box transcription factors (MTFs) and degrades these via the 26S proteasome leading to the development of leaf-like flowers that resemble those of phytoplasma-infected plants (MacLean et al., 2011; MacLean et al., 2014). The SAP54-mediated degradation of MTFs is dependent on SAP54 interaction with the 26S proteasome shuttle factor RAD23 (MacLean et al., 2014). Leafhoppers prefer to lay eggs on SAP54 transgenic lines and phytoplasma-infected plants and this preference is also dependent on RAD23 (MacLean et al., 2014). Nonetheless, leaf-like flowers are not required for the leafhopper egg-laying preference. Whether MTFs that are degraded by SAP54 regulate other cellular processes, such as plant defence responses to insect pests, remains to be investigated.

In order to test the role of phyllody in plant colonization by insects I removed the phyllody phenotype from potential additional effects of SAP54 on insect vector (Figure 3.4) as well as generated phyllody de novo independently from SAP54 (Figure 3.6). This holistic approach allowed to functionally uncouple the multiple simultaneous changes in host plants in presence of phytoplasma or its effector SAP54 alone. In order to test the functions of parasite altered host phenotypes, other studies have used pharmacological approaches to mimic or
interfere with parasite induced phenotypes (Perrot-Minnot et al., 2012; Hojo et al., 2015). This has provided correlative evidence for involvement of certain neurotransmitters such as dopamine or serotonin in altered host behaviours (Cézilly and Perrot-Minnot, 2010). Elucidating the cause-effect relationship between certain manipulations of host biology and their function often requires mechanistic understanding about parasite genes and their host targets. By employing such knowledge from previous studies (MacLean et al., 2014), I managed to demonstrate that phyllody phenotype is not required in the plant colonization by leafhopper vector *M. quadrilineatus*. Although, without empirical testing, I cannot exclude that other species of phytoplasma vectors may be attracted to phyllody, in the light of experimental evidence from this work I further discuss the role of phyllody as adaptive manipulation or side-effect of phytoplasma infection.

Phyllody-inducing ‘*Ca. Phytoplasma asteris*’ phytoplasmas, such as AY-WB, often infect annual plants (Lee et al., 2004), which die upon flowering and seed production. Phytoplasmas are dependent on insect vectors for spread before plants die (Weintraub and Beanland, 2006). Hence, phytoplasmas that produce effectors, such as SAP54, which attract insect vectors, are likely to spread faster than phytoplasmas that do not produce such effectors. Similarly, increase in plant survival would benefit acquisition of generalist phytoplasmas and transmission to alternative host species even when healthy conspecifics of the original host have died. Given that leaf-like flowers neither increase plant longevity (Figure 3.7) nor affect leafhopper oviposition preference (Figures 3.4 and 3.6), phyllody does not appear to have any benefits for attraction of adult leafhoppers. The possible increase in shoot biomass under long day photoperiod (Figure 3.8B) may result from the indeterminate growth of leaf-like flowers on SAP54 plants. However, given the overall preference of leafhoppers for rosette leaves compared to the leaf-like flowers (Figure 3.2) together with oviposition preference for SAP54 rosette leaves independently from bolting and generation of leaf-like flowers (Figure 3.4), the apparent increase in areal biomass above the rosette is not likely to contribute to leafhopper reproductive preference for SAP54 expressing or phytoplasma infected plants. Furthermore, similar phytoplasma titer in normal and leaf-like inflorescences (Figure 3.9) indicates that adult insects that preferentially reside and lay eggs on plants with phyllody may not acquire phytoplasma with higher efficiency than from infected plants with no phyllody.
Experimental evidence thus far strongly suggests that phyllody itself or the associated traits such as phytoplasma titer in leaf-like tissue is not likely to be an adaptive manipulation of the host plant by phytoplasma to enhance pathogen acquisition or colonisation by adult leafhoppers. However, phyllody may have additional functions, for example, affect pathogen spread by enhancing or hindering the transmission of phytoplasma from infected to healthy plants by the first generation progeny of adult leafhoppers on SAP54 or infected plants with leaf-like flowers. According to the Deceptive Host Hypothesis, plant pathogens may initially enhance vector attraction to virus or bacteria infected plants but subsequently alters the quality of the host plant to facilitate the spread of pathogen-carrying vectors to healthy, yet uninfected, host plants (Mauck et al., 2010; Mann et al., 2012). While there are more leafhopper nymphs to potentially acquire phytoplasma on SAP54 expressing plants, it remains to be investigated whether SAP54 induced phyllody contributes to the further transmission of phytoplasma to healthy plants by affecting nymph survival or how soon nymphs leave the infected plants. Although the phyllody may not be an adaptive manipulation to attract adult leafhoppers for reproduction on the host plant, there is a possibility that increased biomass on plants with indeterminate floral growth could potentially support larger number of leafhopper progeny. The no-choice experiment assessed if the total leafhopper egg production and nymph survival was dependent on SAP54 or SAP54-induced leaf-like flowers (Figure 3.6). In this experiment SAP54 plants developed leaf-like flowers by the time nymphs had hatched from the rosette leaves. Since there was no significant increase in nymph number on SAP54 plants (Figure 3.6), the potential benefits of larger shoot biomass on nymph survival are unlikely. Nevertheless, nymph survival could benefit from increase in plant biomass when the number of insect nymphs on the control plant without leaf-like flowers approaches or exceeds the carrying capacity of a single plant. Additional experiments with increased number of nymphs per plant would be necessary to test this hypothesis. Contrary to the 35S:GFP-SAP54 plants, phytoplasma infected plants are stunted and do not appear to have greater biomass compared to healthy plants (Sugio et al., 2011). For this reason, increase in biomass on infected plant that produces leaf-like flowers could support high number of nymphs compared to infected plant with no leaf-like flowers. Furthermore, it remains to be elucidated whether increase in
biomass would arrest the developing nymphs longer on the infected plant or encourage to find an alternative (non-infected) host.

Conversion of flowers into leaves is different from other cases where insect-vectored bacterial or fungal pathogens alter plant floral development (Mescher, 2012). For example, fungi in the genera *Puccinia* and *Uromycetes* alter floral architecture of infected plants to mimic other sympatric species and attract pollinating insects (Ngugi and Scherm, 2006). The rust fungus *Puccinia monoica* induces pseudoflowers in its plant host *Boechera stricta* that mimic co-occurring buttercup flowers providing olfactory cues and nectar awards to entice pollinating insects which can transfer fungal spores from the opposite mating types (Roy, 1993; Roy and Raguso, 1997). This fungus induces major transcriptome programming of its host plant, likely through the production of multiple effectors (Cano et al., 2013). Similarly, fungus *Molinia vaccinii-corymbosi* induces floral mimicry in vegetative tissues to attract pollinating insects that transfer their ascospores to plant reproductive organs (BATRA and BATRA, 1985). In contrast to the examples above, phytoplasmas rely on phloem-feeding insects for dispersal rather than pollinating insects. And the induction of leaf-like flowers does not contribute to the host selection by phytoplasma vectors.

It is possible that the induction of phyllody is a side-effect of SAP54-mediated modulation of a processes involved in insect attraction. Likewise, limb malformations in *R. ondatrae* infected amphibians may not play a direct role in amphibian predation by herons and may not significantly affect the life-cycle of the trematode, and therefore remains a subject of empirical testing (Figure 3.11). *Ribeiroia*-mediated limb malformations in amphibians may be a “side-product” of infection itself or indirectly derive from the activity of certain parasite effectors. Moreover, environmental pollutants are also implicated in development of limb abnormalities, thus providing alternative explanations for the origin of complex developmental phenotypes observed in the field (Skelly et al., 2007).
Figure 3.11. Morphological changes induced by parasites may not be always required for transmission and spread of parasites. Experimental evidence suggests that phytoplasma induced developmental changes in flowers are not required for the enhancement of plant colonisation by insect vectors. Insect preference for oviposition on SAP54 expressing plants is mediated through different mechanisms. Likewise, the abnormal development of amphibian limbs may be a consequence of environmental factors or presence of the trematode parasite but not necessarily a requirement for enhanced amphibian predation by the avian host of the trematode.

Complementary to adaptionist view held by Dawkins (Dawkins, 1982), the activity of parasite genes in the host may lead to emergence of non-adaptive secondary structures which derive as a result of genetic and developmental correlations together with selected features. In their seminal paper Gould and Lewontin (1979) used the example of the Spandrels of San Marco to illustrate how existence of certain intuitively adaptive features of an organism (like the spandrel shape to accomodate iconography of the cathedral) are influenced by evolutionary constraints (spandrel shape is architectural by-product of the dome structure) and urged to consider the non-adaptive hypothesis in explaining complex phenotypes or behaviours in nature (alongside adaptive explanations). For example, there are several adaptive explanations for insect-induced galls on plant leaves (Stone and Schönrogge, 2003). However, the variation and convergent evolution of several insect gall features – air spaces, sticky outer surface or spines – in cynipid wasps are difficult to conceive as necessary
adaptations for insect survival inside the gall (Stone and Cook, 1998). These may arise as by-products from the parasite interfering with conserved developmental programmes. A recent study by Perrot-Minnot et al. (2012) recreated the decreased photophobic effect of acanthocephalan infected amphipods by injecting them with serotonin and demonstrated no increase in predation by fish. These findings revisited the paradigm that all parasite-altered complex host behaviours should be regarded as parasite adaptation. Furthermore, it is important to approximate more natural experimental conditions when testing the adaptive significance of altered host phenotypes. For example, based on many model system (non-human) studies, it has been suggested that Plasmodium-infected mosquito vectors are more responsive to human cues due to manipulation by the malarial parasite. Contrary to this hypothesis, Vantaux et al. (2015) demonstrated that mosquitoes do not show altered long range or short range locomotory behaviour and preference for human odour by using host-vector-parasite system that has coevolved in nature.

According to the principle of Darwinian pluralism where adaptation and selection are separable, features like phyllody in phytoplasma-infected plants may have been selected together with primary adaptive role of SAP54 in enhancing insect colonisation and may, therefore, be a secondary epiphenomenon of the activity of parasite gene that may have the potential to eventually acquire novel functions. In a similar way like the human culture is an emergent property of human genes responsible for learning but not necessarily a Darwinian adaptation selected on the basis of genetic variation (Dawkins, 2004). The non-adaptive explanation and the adaptionist view are equally instrumental in understanding the evolution of parasite-altered host phenotypes and the mechanistic insight is key to uncouple the two.

Finally, I want to discuss how developmental side-effects such as leaf like flowers may originate. In this chapter I demonstrated that insect attraction and induction of phyllody are functionally un-coupled. However, Chapter 2 showed that these phenotypes are genetically linked. SAP54 induces leaf-like flowers by mediating degradation of MTFs via interaction with RAD23 (MacLean et al., 2014). MTFs are regulatory hubs for a plethora of physiological processes in plants, including plant immunity (comparable to animal HOX genes); several MTFs appear to (in)directly regulate cytokinin and jasmonic acid (JA) synthesis and response genes (Gregis et al., 2013), which affect plant-insect interactions
(Schafer et al., 2011; Erb et al., 2012; Giron et al., 2013; Naessens et al., 2015), such as that of the AY-WB leafhopper vector *M. quadrilineatus* (Sugio et al., 2011). In addition, MTFs regulate age-related resistance responses to pests (Wilson et al., 2013). Therefore, SAP54-mediated degradation of MTFs may modulate plant responses to leafhoppers as well as alter of floral meristem identity and floral organ architecture later in development. Findings of this chapter lead to hypothesis that certain MTFs may be expressed in plant vegetative tissues and have yet uncharacterised regulatory roles in plant-insect interactions. This hypothesis will be further explored in Chapter 6. Pleiotropic effects of transcription factors are known in animal systems, too. For example, Toll-like receptors are known to play roles both in development and defences against pathogens. In *Drosophila melanogaster* Toll\(^\text{10b}\) controls development of dorsal axis and is linked to fungal defences. Knock-out of this gene results in both developmental defects and impaired resistance to fungal antagonists (Lemaitre B et al., 1996; Artero et al., 2003).

Targeting conserved plant proteins, such as MTFs, by phytoplasma effector may enable the phytoplasma parasites to infect a broad range of plant species. The 26S proteasome shuttle proteins RAD23 are also conserved among plant species (Vierstra, 2009). Compatibility of phytoplasmas with multiple plant species is likely essential given that AY-WB phytoplasma and related parasites are transmitted by polyphagous insect species, such as leafhoppers of the genus *Macrosteles* (Lee et al., 2004; Weintraub and Beanland, 2006). Because these insects readily feed on many plant species, phytoplasmas will increase their fitness if they can modulate these plants to increase attraction and colonization of insect vectors. In agreement with this, SAP54 homologs are found in diverse phyllody-inducing phytoplasmas that infect a wide range of plant species (Bertaccini, 2007; Sugio et al., 2011; Maejima et al., 2014). Thus, generalist parasites, especially those dependent on alternative hosts for transmission, could gain fitness benefits via interfering with conserved host processes.
“The experiment should be set up to open as many windows as possible on the unforeseen.”


**Chapter 4**

**Males Are Required for the Attraction and Increased Reproduction of Female Leafhoppers on *SAP54-Expressing Plants***

4.1. Introduction

The phytoplasma effector SAP54 induces the formation of leaf-like flowers (MacLean et al., 2014). In Chapter 3 I demonstrated that leaf-like flowers are not required for the attraction of the phytoplasma leafhopper vector Macrosteles quadrilineatus and that this leafhopper prefers to lay eggs on single leaves of SAP54 expressing plants (Orlovskis and Hogenhout, 2016). This indicates that SAP54 modulates leaves in a way that attracts the phytoplasma insect vector for egg-laying. So far I studied only the production of M. quadrilineatus eggs and nymphs as a measure of leafhopper attraction to plants. However, the cues that are involved in leafhopper attraction to SAP54 transgenic plants and selection of the host plant for feeding and egg-laying have not yet been investigated. Plant visual appearance and volatile compounds can act at a distance to orientate and attract insects to a host plant prior to direct contact with the leaf surface (Rid et al., 2016; Todd et al., 1990a,b). Furthermore, mechanical barriers such as epidermal trichomes or thickness and composition of the leaf cuticle can determine insect choice to stay on the leaf and start feeding or laying eggs (Serrano et al., 2014). Plant nutritional quality and constitutive levels of defence chemicals in healthy or infected plant may further influence insect arrestment on a plant and duration of feeding or egg-laying (Mann et al., 2012). Therefore, SAP54-mediated enhancement of leafhopper reproduction may be achieved via cues perceived by leafhoppers at a distance prior to direct contact with host plant or cues that act after direct contact between an insect and a plant. For example, insect herbivory can induce leaf volatiles that subsequently determine the host plant choice of the insects that have not yet come in direct contact with the insect-infested plant (Allmann et al., 2013; Mumm & Dicke, 2010). Mating behaviour can also affect the attraction of male or female leafhoppers to a plant (Heady et al., 1986). Therefore, sex-specific mechanisms of insect attraction by SAP54 could play a role in leafhopper preference to reproduce on plants colonised by a mating partner.

There is evidence for phytoplasma-induced changes in plant volatiles that attract their insect vectors. For example, apple tree infecting ‘Ca. Phytoplasma mali’ alters plant sesquiterpene production, thereby luring the psyllid vector to phytoplasma infected plants (Mayer et al., 2008a,b; Rid et al., 2016). Moreover, changes in plant volatiles may be induced by effector proteins. For example, SAP11 effector homolog from ‘Ca. P. mali’ alters production of volatile organic
compounds that are implicated in pheromone-mediated attraction between insects, albeit this was shown in SAP11 transgenic *N. benthamiana* plants (not apple) and no insect assays were performed to test the attractiveness of SAP11 induced volatile compounds (Tan et al., 2016). Nonetheless, the AY-WB leafhopper vectors appear to use predominantly visual cues to select host plants, and preferentially orientate towards yellow spectrum of visible light (Todd et al., 1990a,b), though olfactory cues may play a role in enhancing insect response to visual cues (Patt and Séta mou, 2007). Volatile attractants may operate at various distances and in combination with visual cues such as colour and shape of the plant.

Plant cuticle thickness and composition play important roles in resistance to both abiotic and biotic stress, including pests and pathogens (Serrano et al., 2014). Similarly, leaf trichomes present an important mechanical barrier to feeding and oviposition of herbivorous insects. For example, increased trichome density on tomato leaves negatively affects whitefly egg-laying (Oriani and Vendramim, 2010). In addition, glandular trichomes may contain secondary chemicals that deter potential herbivores. In contrast to plant species with diverse types of trichomes, such as tomato or *N. benthamiana*, leaves of *Arabidopsis thaliana* (Col-0 ecotype) have only single-cell non-glandular epidermal trichomes typically with 3 branches (Marks, 1997). Thus, any effects of SAP54 on trichome quantity may affect host plant selection by leafhoppers.

Constitutive and induced plant defence responses play an important role in protection against herbivore feeding and egg-laying (oviposition). For example, leafhopper *Dalbulus maidis* is a specialist of maize (*Zea mays*) (Nault, 1990) and cannot survive or reproduce on *A. thaliana*. In contrast, the generalist leafhopper *Macrosteles quadrilineatus* cannot survive and reproduce on maize plants (Weintraub & Beanland, 2006) as efficiently as the specialist *D. maidis*, indicating that the specialist has evolved more effective mechanisms to tolerate or silence constitutive or induced defence responses of maize compared to the generalist. Jasmonate synthesis is induced during leafhopper feeding, and has a negative effect on host selection in the field (Kessler, 2004) and leafhopper reproduction (Sugio et al., 2011). In addition, butterfly *P. brassicae* oviposition elicits plant defence responses via salicylic acid accumulation, which may suppress jasmonate-dependent plant responses to feeding of *S. littoralis* larvae (Bruessow et al., 2010). Thus insect feeding and egg-laying may interfere with each other.
due to defence hormone crosstalk or simply behavioural arrestment of insects on plant where they both feed and lay eggs.

Phloem feeding hemipteran insects also secrete effector proteins into the plant in order to dampen plant defence responses (Hogenhout and Bos, 2011). Therefore, any effects of the phytoplasma effector SAP54 on constitutive or induced plant defence responses to insect colonisation or modulation of insect-derived effector protein functions may play essential roles in plant-insect vector interactions.

The main aim of the current chapter is to explore the possible mechanisms of leafhopper attraction to SAP54 transgenic plants. To this end I analyse various cues acting at a distance (before insect settlement and probing) and contact cues (after insect settlement and probing) that may mediate insect host selection preference and be modulated by SAP54 activity. Furthermore, I explore the attractiveness of plants for male and female insects to identify any sex-specific or sex-dependent effects of SAP54. The main finding is that SAP54 expressing plants attract female insects for egg-laying in male dependent manner by modulating insect induced plant responses.

4.2. Results

4.2.1. Leafhoppers use visual cues in preference to olfactory cues for host location

First I wished to investigate if SAP54-mediated changes in plant visual appearance or volatile emissions could impact leafhopper preference for SAP54 expressing plants. To do this, I developed an experimental setup to measure the effect of volatile compounds in absence or in combination with visual cues (Figure 4.1; detailed setup in Chapter 8). Numerous other experimental designs were tested (see Chapter 8) but were less successful that the assay described herein. The setup described in Figure 4.1 was used in several dual choice assays (Figure 4.2) that directly compared the attractiveness of two different odours or investigated the effect of additional visual stimulus on insect response. Two pots were placed at opposite ends of a choice arena where a group of 20 male and 20 female insects were released. After 8 hours the number of insects stuck to the sticky landing platforms were counted.
Since no positive olfactory stimulus has been reported to attract *M. quadrilineatus*, I used vinegar fly *Drosophila suzukii*, which is attracted to vinegar odour, as a positive control to validate the experimental setup. *D. suzukii* demonstrated a very strong attraction to the vinegar compared to water (Figure 4.2A). This indicates that directional odour gradient has been established, and the assay is suitable for dual-choice experiments. Next, I tested the attraction of *M. quadrilineatus* to a blend of oat and cabbage volatiles from insect non-exposed plants. Oat and cabbage plants are both hosts for *M. quadrilineatus* and are often used to rear leafhopper colonies at the JIC Entomology Facility. Phytoplasma-free leafhoppers were used in all our host choice experiments. Interestingly, leafhoppers demonstrated no response to plant odours neither in daylight or at night (Figure 4.2B,C). However, when a yellow colour trap (known visual attractant of leafhoppers (Todd et al., 1990b) was added to complement either the plant-emitted volatiles or plant volatile-free controls, insects were attracted to the yellow colour trap with or without the presence of plant volatiles (Figure 4.2D,E), suggesting that leafhoppers use primary visual cues in preference to volatiles for orientation in space. Moreover, plant volatile signals neither compete nor complement visual cues in leafhopper attraction. Next, I repeated experiment
B-E but instead of intact plants I used ground plant tissue extract in water (Figure 4.2F-I). Ground plant tissue mimics plant damage and may release different blend of compounds than intact plants. Results were identical to experiments with intact growing plants in that the leafhoppers were attracted by yellow colour irrespective of the presence or absence of ground plant tissue. These data suggested that *M. quadrilineatus* has a strong preference for using visual cues in their orientation to find a host plant and that, compared to the visual cues, any volatiles compounds constitutively produced in intact plants or damage-induced volatiles from the or ground plant tissue have minor impacts on the leafhopper orientation to host plants.

**Figure 4.2. Visual cues have a stronger effect on *Macrosteles quadrilineatus* orientation preference to host plants compared to plant olfactory cues.** Test odours are presented on the left-hand side but controls – on the right-hand side. Experiments were performed either in light or dark conditions as indicated in the diagram. The letters on the diagram correspond to letters in the graph below. In experiments E and I the volatile stimuli are complemented with visual cues. In experiments D and H the volatile stimuli are compared to visual cues. *Drosophila suzukii* has a strong preference for odours from vinegar (t\(4\)=18.86; p≤0.001) (A). *Macrosteles quadrilineatus* demonstrates no attraction to olfactory signals from oat and cabbage plants in light (B) or in dark (C) conditions. *M. quadrilineatus* demonstrates strong attraction to yellow colour visual cue irrespective of absence (t\(4\)=70.82; p≤0.001) (D) or presence (t\(4\)=5.22; p=0.014) (E) of olfactory stimuli from plants. *Macrosteles quadrilineatus* demonstrates no attraction to olfactory signals from grinded oat and cabbage extract in light (F) or in dark (G) conditions. *M. quadrilineatus* demonstrates strong preference for yellow colour visual cue irrespective of absence (t\(4\)=14.07; p=0.001) (D) or presence (t\(4\)=30.21; p≤0.001) (E) of olfactory stimuli from plant extract.
Figure 4.2.
In the dual choice experiments reported above I used equal mix of male and female insects. Given that leafhopper mating occurs on plants, and males emit female-attracting acoustic signals (Heady et al., 1986), I wished to test for any sex-specific leafhopper response to odours and colours. For example, if more males are attracted to SAP54 plants initially, female egg-laying preference may be explained by attractiveness of male odours or singing. I counted both male and female insects in all experiments described in Figure 4.2 and plotted the sex-ratio from all landing platforms that contained (1) only odour but no colour, (2) only colour but no odour and (3) both colour and odour together. I found that volatile and olfactory stimuli alone or in combination trigger equal attraction of male and female insects (Figure 4.3), suggesting that leafhopper may have no sex-specific response to volatile or visual cues acting at a distance prior to physical contact with the plant.

Figure 4.3. The ratio between female and male insects responding to a combination of olfactory and visual cues (A) is similar to insect response to visual cues without additional plant odours (B) and similar to response to plant odours only (C). Male and female insects were trapped after their first landing on platforms with the test or control stimuli in experiments described in Figure 4.2.

I used the setup described in Figure 4.1 to test if volatiles from SAP54 transgenic plants attract leafhoppers, and to investigate if visual stimuli override the effect of olfactory cues from the SAP54 plants. In contrast to the previous setup, I substituted the yellow landing platform with a green one that matches the plant leaf colour. Leafhoppers demonstrated neither preference nor avoidance for volatiles from the SAP54 plants compared to control plant when any
differences in plant odour are complemented with identical visual cues (Figure 4.4A). Furthermore, in absence of visual clues, insects showed no orientation to either SAP54 or the control plant (Figure 4.4B). However, when the visual cue is combined with potential volatile cues from the SAP54 plants or presented on its own without volatile stimuli of the plant, insects strongly preferred to land on the platform with the visual cue irrespective of volatiles released by the SAP54 plants or complete absence of plant volatiles (Figure 4.4C and D). Together, these data suggest that volatiles emitted by SAP54 plants are unlikely to play a major role in the initial choice of plant hosts by leafhoppers. Visual cues dominated over volatile cues in leafhopper attraction, posing the question if the SAP54 plants are perhaps visually more attractive to the leafhoppers.

Figure 4.4. Leafhoppers prefer visual cues over volatile cues from SAP54-expressing plants. (A) *M. quadrilineatus* demonstrates equal landing preference for the odour of SAP54-expressing and control plants when plant volatile signal is complemented with identical green colour landing platforms ($t_{4}=0.29; p=0.788$). (B) Insects show minimal attraction to volatile cues derived from SAP54-expressing and control plants. (C) Leafhoppers show significant preference for SAP54 plant combined with visual stimulus ($t_{4}=4.52; p=0.02$). (D) The visual stimulus alone is an equally potent and significant leafhopper attractant than the combination between plant volatile signature and visual cue ($t_{4}=4.90; p=0.008$).
4.2.2. Phytoplasma effector SAP54 increases yellow spectral reflectance of leaves

Given that the leafhopper *M. quadrilineatus* demonstrated stronger response to visual cues compared to volatile stimuli, I investigated if the leaves of the SAP54 transgenic plants differ in their colour appearance compared to control plants. First, I measured the leaf surface spectral reflectance across the perceived visual spectrum of insect eyes (approximately 400-630nm wavelength). Notably, majority of insect species do not detect wavelength corresponding to the red and far-red spectrum (650-750 nm), whereas wavelength between 500 and 600nm (green-to-yellow) were previously shown to be important in leafhopper orientation (Todd *et al.*, 1990b). Interestingly, leaves of the SAP54 plants showed significantly greater reflectance (approximately 1-2% more reflected light) at wavelengths corresponding to green-orange colours (between 500-650 nm) compared to control plants (Figure 4.5). Independently from surface reflectance measurements, I also measured leaf absorbance using SPAD meter. SPAD values are measures of leaf chlorophyll index derived from absorbance maxima of various leaf pigments and is correlated to the chlorophyll-to-carotenoid ratio in photosynthetic tissue (Ling *et al.*, 2011). Concurrent with spectral reflectance measurements, the SAP54 plants demonstrated significantly lower SPAD readings than control plants (Figure 4.6), suggesting reduction in chlorophyll content and higher proportion of yellow reflecting leaf pigments. To human eye too, SAP54 appeared lighter green and more yellow compared to control plants (Figure 4.6 picture insets), supporting the quantitative measurements of leaf reflectance and absorbance. If the measured differences in leaf colour at the green and yellow spectra can be perceived by insect vision, it may play a role in leafhopper perception and location of host plants.
Figure 4.5. SAP54 transgenic plants demonstrate increased leaf reflectance at green-orange colour spectra compared to control plants. Leaf reflectance was measured across 11 channels corresponding to the approximate spectrum of insect vision. Significant difference in reflectance was measured across 20 independent plants per line (each replicate averaged 3 largest leaves of 8-weeks old plants) using two-tailed t-test at each channel. The resulting p-values were (Bonferroni) corrected for multiple comparisons. Only corrected p-values ≤0.001 are displayed.

Figure 4.6. SAP54 transgenic plants have lower SPAD meter readings compared to control plants. SPAD values are leaf absorbance measurement across different wavelength and correlate to leaf chlorophyll content. SPAD values were averaged from
three largest leaves per plant and measured for 36 independent biological replicate plants per line (8-week old plants). SAP54 significantly reduces SPAD readings compared to the control plants (two-tailed t-test; \( t_{36}=3.58; \ p=0.001 \)). Boxplot demonstrates the 1st and 4th quartiles as vertical line, 2nd and 3rd quartiles as box, median as horizontal line, mean as a cross and outliers as asterisk. Photographs of a representative sample of leaves from the SAP54 and control plants are displayed on the left and right of the boxplots respectively.

4.2.3. Leafhopper preference for SAP54 plants does not involve visual cues

Given the measurable differences in visual appearance between leaves from SAP54 and control plants, I further investigated if these differences might explain the greater attraction of leafhoppers to the SAP54 plants. I caged the whole rosettes of the SAP54 and control plants in completely transparent polycarbonate material to allow the permeation of leaf-reflected light but prevent any release of volatiles. I covered the surface of the polycarbonate cage with transparent glue and arranged plant rosettes in a choice arena to measure leafhopper first landing choice (for details see materials & methods). I previously determined that the polycarbonate material and the glue used in this experiment were completely transparent and did not alter the profile of reflected light from leaves. Surprisingly, I did not find a significant effect in leafhopper landing on SAP54 plants compared to controls (Figure 4.7A), suggesting that the observed difference in leaf reflectance at green-yellow spectra between SAP54 and control plants (Figure 4.5) did not affect leafhopper orientation behaviour towards the plant. Next, I used a yellow replica of A. thaliana rosette and observed significant landing preference for yellow compared to the SAP54 plants (Figure 4.7A). The yellow colour rosette had greater reflectance at green-orange spectra of light compared to the leaf surface from SAP54 and control plants (Figure 4.7B). Taken together, these data indicated that the increased reflectance at green-yellow wavelengths can be more attractive to M. quadrilineatus, however the difference between the spectral reflectance of SAP54-expressing and control plants is relatively minute to have a significant effect on leafhopper host selection by vision.
Figure 4.7. SAP54 has no effect on visual cues that leafhoppers use for host plant selection. (A) *M. quadrilineatus* has similar landing preference for SAP54 and control plants (paired t-test; $t_{12}=0.83; p=0.428$). Yellow is a strong attractant of leafhoppers compared to the green appearance of the plant (paired t-test; $t_4=33.66; p=0.001$). Diagrammatic representation of the visual image of the landing platforms in the choice experiment is given at the sides of the bars. (B) The yellow sticky trap reflects more light at the green-orange visual spectrum compared to both SAP54 and control plants.
4.2.4. Rosette leaf trichome morphology and density do not differ between SAP54 and control plants

The results above suggest that, although leafhoppers respond to distance (mainly visual) stimuli to locate host plants, the difference in visual or olfactory cues between the SAP54 and control plants may not explain preferential attraction of leafhoppers. Instead, leafhoppers may decide to choose to stay on a plant after the first landing and exploring the surface of the leaf. This presents a possibility that leaf mechanical barriers such as epidermal trichomes and cuticle may determine host selection or insect arrestment on the plant. Trichomes are likely to interfere with leafhopper locomotion already before feeding or egg-laying, whereas the cuticle can impose resistance to insect mouthparts or ovipositor. From laboratory observations, leafhoppers are likely to land and first encounter the adaxial (top) side of the leaf but feed and lay eggs on the abaxial (under) side. Furthermore, leafhoppers penetrate leaf surface with their stylets at various distances from the primary or secondary leaf veins, and lay eggs at leaf margins and the intersection between the midvein and leaf blade. For these reasons, measurements of cuticular thickness, structure or composition may be technically challenging and require high spatial resolution over the entire leaf area to accurately correlate with feeding or egg laying preference. However, measurements of leaf trichome morphology and density are technically less challenging and would provide valuable insight in whether the SAP54 plants demonstrate altered trichome phenotype that is correlated with increase in egg-laying. To test this, I first visually compared the distribution pattern of trichomes on 35S:GFP-SAP54 and 35S:GFP plants by taking scanning electron micrographs (SEM) of the adaxial surface of leaves and leaf-like flowers. Interestingly, leaf-like flower tissue and cauline leaves appeared to have greater trichome density compared to rosette leaves (Figure 4.8). Moreover, as demonstrated previously, leafhoppers prefer to reside on rosette parts compared to stems, cauline leaves and floral or leaf-like flower tissues (Chapter 3). This prompted to investigate the density of rosette leave trichomes in greater detail. I counted the trichomes on both adaxial and abaxial leaf sides using optical stereo-microscope. To avoid variance in trichome density due to local patchiness, I quantified the number of trichomes over the entire leaf blade and normalised for leaf area (Figure 4.9). 35S:GFP-SAP54 plants show slight decrease in trichome density on adaxial leaf side but such effects are not significant and may be
caused by natural variation in trichome density among replicate plants. The
difference in trichome density on abaxial side was less pronounced, and the total
number of trichomes on abaxial side (where insects feed and lay eggs) was
significantly less than adaxial side on both 35S:GFP-SAP54 and 35S:GFP plants
(Figure 4.9). Moreover, no trichomes were found on the typical *M. quadrilineatus*
oviposition sites near midvein and leaf edges on adaxial side of the leaves from
SAP54 or control plants. This indicated that leafhoppers may prefer to feed and
lay eggs in trichome-free zones on the leaf. However, the variation in adaxial
trichome density could be important for the initial decision (or acceptance) of the
leaf to stay and commence feeding and egg-laying.

Figure 4.8. Scanning electron microscopy images display increased trichome
densities on leaf-like flowers of 35S:GFP-SAP54 plants and cauline leaves of both
35S:GFP-SAP54 and 35S:GFP plants compared to rosette leaves. Representative
images from rosette leaves, cauline leaves are displayed. Leaf-like flowers are found
only on 35S:GFP-SAP54 plants. Plants were 8-week old. Fully expanded older rosette
leaves were imaged. Bars are 100 µm.
Figure 4.9. Quantification of trichomes on 8-week old 35S:GFP-SAP54 or 35S:GFP plant rosette leaves. Ectopic expression of phytoplasma protein SAP54 does not significantly affect adaxial ($t_{2,16}=1.25; p=0.223$), abaxial ($t_{2,16}=1.1; p=0.281$) and total trichome number ($t_{2,16}=1.6; p=0.122$). Trichomes were counted over the entire leaf blade and normalised per unit of leaf area.

In order to investigate potential role of trichome density in leafhopper reproductive preference for SAP54 plants, I correlated the total number of trichomes per unit of leaf area with leafhopper egg number. I performed a single-leaf choice experiment where I counted both trichome density and egg number on single leaves from 35S:GFP-SAP54 or 35S:GFP plants. Surprisingly, trichome density does not correlate with leafhopper egg-laying preference for SAP54 expressing or control plants (Figure 4.10). Therefore, differences in trichome density between SAP54 and control plants are not likely to cause any significant effect on leafhopper oviposition choice.
Figure 4.10. Egg laying preferences of the aster leafhopper *M. quadrilineatus* on *A. thaliana* leaves are not correlated with leaf trichome numbers. The number of *M. quadrilineatus* eggs is not correlated with the total number of trichomes on the adaxial and the abaxial side of a single rosette leaf from SAP54 (Pearson r=0.24; p=0.337) and GFP (Pearson r=0.423; p=0.073) plants. Trichomes and eggs were counted on the same leaf under stereomicroscope.

In addition to the quantification of trichome density, I also took scanning electron micrographs (SEM) of adaxial trichomes of rosette leaves on 35S:GFP-SAP54 and 35S:GFP plants to detect any changes in trichome morphology. From a representative sample of leaf surface images I did not detect any obvious difference in trichome height and branch length between SAP54 expressing and control plants (Figure 4.11). Absolute majority of trichomes on both 35S:GFP-SAP54 and 35S:GFP plants had the characteristic tripartite branching. Only few trichomes on rosette leaves of SAP54 plants had two or four branches (Figure 4.11). Most trichome branches were straight, although branch tip bending, as depicted in Figure 4.11, could be observed in a small fraction of trichomes on 35S:GFP-SAP54 as well as 35S:GFP plants.

Taken together, I conclude that SAP54 plants do not show significant differences in trichome morphology and density compared to control plants. Furthermore, total leaf trichome density is not a strong predictor of leafhopper egg-laying preference.
Figure 4.11. Scanning electron microscopy images of individual trichomes on epidermis of 8-week old 35S:GFP-SAP54 or 35S:GFP plant rosette leaves. The length of trichome stalk and branches is similar between SAP54 and control plants. While the majority of branches on 35S:GFP-SAP54 or 35S:GFP plants are straight, some branches are uneven and curved on both plants. Images display rare examples of reduced branch number (top right) or increased branch number (bottom) on SAP54 expressing rosette leaves. Scale is identical in all images for ease of comparison.

4.2.5. Female egg-laying preference for SAP54 plants is dependent on the presence of male leafhoppers

Hitherto, the experimental results suggest that leafhopper oviposition preference for SAP54 plants may be mediated neither by constitutive visual or olfactory signals acting at a distance to orientate the insect vector towards host plant nor constitutive local leaf surface barriers, such as trichomes, that influence insect decision to stay on the plant. Therefore, I hypothesise that female leafhopper egg-laying preference for 35S:GFP-SAP54 plants is determined after insect settlement and perhaps during feeding or oviposition. This may involve either constitutive differences in plant quality between SAP54 and control plants or differences in insect-induced defence responses. During probing and feeding leafhoppers may determine plant quality and suitability in order to decide to continue feeding or begin oviposition on the current plant or move to another host. Alternatively, during probing, feeding or oviposition leafhoppers induce plant
responses that may be altered by phytoplasma effector SAP54 to favour insect colonisation. Because male insects feed on the plant but cannot lay eggs like female leafhoppers, the induction of plant responses may be sex-specific.

In order to test whether egg-laying preference for SAP54 plants depends on insect (sex-specific) induced plant responses and whether these induced responses enhance insect egg-laying as well as feeding I devised a series of dual-choice experiments and separated males and females in time and space (Figure 4.12A). All choice experiments were carried out by releasing mixed sex leafhopper population or male-only or female-only populations (with specifications outlined in Figure 4.12A) into a choice arena with one 35S:GFP-SAP54 and one 35S:GFP (control) plant. Leafhoppers produced more progeny on the SAP54 transgenic plants when equal number of male and female leafhoppers are given a choice between the SAP54 and control plants (Figure 4.12B experiment 1). Next, I introduced only female leafhoppers in the choice arena to see if females demonstrate the same preference for SAP54 plants as mixed-sex insect population. Surprisingly, females chose to lay the same number of eggs on the SAP54 and control plants in absence of males (Figure 4.12AB experiment 3). This indicated that female leafhoppers may not rely on constitutive differences in plant quality to select SAP54 plants over the control. Instead, male leafhopper induced plant responses may be required for female oviposition preference for SAP54 plants. To test this further, I exposed the SAP54 and control plants to equal number of male insects before removing them and adding female leafhoppers which were allowed to choose to lay eggs between the two plants (Figure 4.12A experiment 4). Thus, males and females were temporarily and spatially separated on the plant. Surprisingly, female insects still did not show any preference for SAP54 plants that were exposed to males prior to addition of females (Figure 4.12A experiment 4). However, the male-induced plant responses could have faded after removal of the male insects. Therefore, I caged equal number of male insects on two rosette leaves of SAP54 and control plants and simultaneously released female leafhoppers to freely choose between the two plants before counting the progeny (Figure 4.12A experiment 5). Thus males and females were only spatially separated from any contact. Now females demonstrated significant reproductive preference for SAP54 plants (Figure 4.12B experiment 5). These data suggest that the SAP54 plants could modulate male-induced responses to favour female leafhopper oviposition compared to the
control plants. Moreover, female preference for the SAP54 plant was independent from male insect preference for the SAP54 or control plant (Figure 4.12B) because the experiment 5 mimicked equal male choice.

In order to find out whether the male-dependent enhancement of female reproduction on SAP54 plants was associated with preferential feeding of male or female insects, I assessed leafhopper feeding by measuring the amount of insect excreted honeydew after giving leafhoppers the choice between the SAP54 or control plants (Figure 4.12C). Insect choice experiments were performed as described in Figure 4.12A. However, this time leafhoppers were choosing between single leaves of the SAP54 and control plants (see materials and methods). I measured the amount of insect-deposited honeydew around each leaf. Intriguingly, mixed population of male and female insects demonstrated slightly greater honeydew deposition around the leaf of 35S:GFP-SAP54 plant compared to the control plant (Figure 4.12C experiment 1), suggesting greater amount of ingested phloem sap and more intense feeding. Nevertheless, this effect was not statistically significant due to the great variation among experimental replicate cages. Noticeably, the slight feeding preference for SAP54 plants disappeared when either males or females were removed (Figure 4.12C experiments 2 and 3). Thus, neither male nor female feeding in a single-sex population was enhanced on the SAP54 plants. However, similar to the female oviposition preference for SAP54 plants (Figure 4.12B), females showed tendency of increased feeding on SAP54 leaf when other leaves were simultaneously exposed to male insects (Figure 4.12C experiment 5) but not when the males were removed before adding the females for the choice tests (Figure 4.12C experiment 4).
Figure 4.12.
Figure 4.12. Male leafhopper presence on SAP54 plants shows significant attraction of female for egg-laying. Experimental design of experiments 1-5 is depicted in panel A. Female leafhopper reproduction preference is measured as nymph count between 35S:GFP-SAP54 and 35S:GFP plants and displayed in panel B. Leafhopper feeding preference is measured as honeydew deposition, shown in panel C. Experiment 1. When both male and female leafhoppers are allowed to feed on SAP54 or control plants, leafhoppers show significant reproduction \( t_6=6.67; p=0.001 \) and slight feeding \( t_6=1.07; p=0.333 \) preference for SAP54. Experiment 2. In absence of females, male leafhoppers do not show any feeding preference for SAP54 plants \( t_6=0.3; p=0.775 \). Experiment 3. In absence of males, female leafhoppers do not show any reproduction \( t_6=0.33; p=0.753 \) or feeding \( t_6=0.1; p=0.923 \) preference for SAP54 plants. Experiment 4. When females are given a choice between male pre-exposed plants after male removal, female leafhoppers do not show any oviposition \( t_6=0.12; p=0.91 \) or feeding \( t_6=1.43; p=0.211 \) preference for SAP54 plants. Experiment 5. SAP54 and control plants are exposed to equal numbers of male leafhoppers, contained in clip-cages separate from females during female feeding. Female leafhoppers do show significant oviposition \( t_6=3.37; p=0.028 \) and slight feeding \( t_6=1.02; p=0.353 \) preference for SAP54 plants. All pairwise comparisons done with paired t-test based on 6 biological replicates. Bars are one standard error from the mean.

Taken together, male-insect presence on 35S:GFP-SAP54 plants shows significant attraction of females for egg-laying but not feeding. Nevertheless, female feeding and reproduction are positively correlated (Figure 4.13), suggesting that one may influence the other via female arrestment on the plant. To verify how significant is the potential effect of SAP54 on insect feeding, I used an alternative method to ingested honeydew measurements and instead quantified insect probing/feeding sites by staining the stylet punctures and tracks in various regions of the leaf (Figure 4.14). When male and female insects were allowed to feed together, they demonstrated detectable probing and feeding activities compared to background staining of insect non-exposed leaves (Figure 4.14A). I counted the probing/feeding sites of mixed-sex population of leafhoppers near the primary and secondary veins as well as mesophyll tissue. Interestingly, leafhoppers demonstrate slightly more probing on SAP54 expressing leaves compared to controls (Figure 4.14B) similar to the honeydew measurements. However, the difference is not significant due to great variation among the choice cages.
Figure 4.13. Correlation between female *Macrosteles quadrilineatus* feeding (measured as honeydew excretion) and egg-laying. The relative amount of insect feeding preference on either 35S:GFP-SA54 plants or 35S:GFP control plants is strongly correlated (Pearson’s $r=0.973$; $p \leq 0.001$) with the relative host plant preference for egg-laying. Correlated are the female data from Figure 4.12 experiments 1 and 3-5.

Given that male presence is required for female oviposition choice, this presents a tantalising hypothesis that phytoplasma effector SAP54 is modulating the male mating behaviour or signals that operate beyond close physical contact to attract female leafhoppers. To verify the plausibility of such hypothesis, I tested female egg-laying preference for male exposed wild-type Col-0 plants compared to male free plants. Male insects were confined on the plant using clip-cages similar to experiment 5 in Figure 4.12. Interestingly, females showed no significant oviposition preference for plants with or without males (Figure 4.15). This suggests that with the given experimental conditions, females are not likely to be attracted to male acoustic or chemical signals. Conversely, females are not deterred by male induced plant responses.
Figure 4.14. (A) Trypan blue staining of leafhopper probing sites at two magnifications of a representative sample from insect exposed and insect-free *A. thaliana* leaves. (B) Number of probing sites per 1cm$^2$ of leaf tissue near vasculature, in mesophyll or mesophyll and vascular tissue combined (total per leaf). Four different measurements were taken for a single leaf to represent the mean probing sites per each leaf. Six independent plant leaves from 35S:GFP-SAP54 and 35S:GFP control lines are represented in the graph. P-values given for paired-t-test. Bars are one standard error from mean.
Figure 4.15. Leafhopper *Macrosteles quadrilineatus* females has no egg-laying preference for male-exposed plants ($t_6=1.09; p=0.325$). 10 female leafhoppers were allowed to choose to lay eggs between insect-free *A. thaliana* Col-0 plants and Col-0 plants with 10 male insects confined in clip-cages. Eggs laid by females were counted over the entire plant. Data analysed using paired t-test.

4.3. Discussion

Within this chapter I analysed multiple possible mechanisms that could be involved in host plant selection by leafhoppers, and found that SAP54-mediated enhancement of female oviposition requires the presence of male insects (Figure 4.12). This finding supports the hypothesis that SAP54 may suppress male insect-induced plant responses in order to attract and/or arrest female leafhoppers on the plant for feeding and egg laying, thus resulting in greater egg and nymph production on the SAP54 plants. Phytoplasma effector SAP54 may have an effect on plant visual appearance, volatile production or trichome density. However, these alterations do not appear to be important for leafhopper attraction to the plant and could not explain female reproduction preference for the SAP54 plants. Therefore, current data are in favour of a model where female attraction to SAP54 plants depends on modulation of insect sex-specific induced plant responses contrary to manipulation of constitutive distance or direct contact cues that act in host location and settlement (Figure 4.16).
Figure 4.16. Expression of phytoplasma effector SAP54 results in numerous altered plant phenotypes, including generation of leaf-like flowers and enhanced attraction of female insects for egg-laying (red block arrows). Plant signals induced by male direct contact with the plant are required for female reproductive preference for the SAP54 plants (green tick). This is an adaptive trait that enhances plant colonisation by phytoplasma vectors (green block arrow). This chapter investigated the mechanisms that could play a role in female leafhopper oviposition choice: 1) manipulation of constitutive signals such as visual, olfactory cues before insects come in contact with a plant (dash-dot lines); 2) manipulation of constitutive mechanical barriers such as trichomes or cuticle that may determine insect settlement or feeding and egg-laying (dash-dot lines); 3) manipulation of male-induced signals directly required for female egg laying alone (solid lines) or 4) manipulation of male-induced signals required for female egg-laying indirectly mediated via female feeding preference or male-induced volatiles (dashed lines). Red crosses indicate SAP54-induced changes that were shown not to play a significant role in female attraction and oviposition choice.

Plant colour and shape together with emitted volatile organic compounds are important stimuli for host plant location and selection by herbivorous insects and their natural enemies (Reeves, 2011; Mumm & Dicke, 2010). These cues can orientate and attract insects from a distance. In the Cicadellidae family (incl. leafhoppers) vision is one of the main signals used for host location (Todd et al., 1990a,b), and olfactory cues can function to enhance insect responsiveness to visual stimuli (Patt and Sétamou, 2007). Results from this chapter agree with the importance of visual over chemosensory signals in host plant choice by leafhoppers (Figures 4.2). I also investigated the combination of the two types of
distance cues and found no significant interaction between the two stimuli for leafhopper orientation and responsiveness (Figures 4.2 and 4.4). Interestingly, changes in plant appearance may not be limited to spectral reflectance at different wavelength. In addition, polarisation of the reflected light can be affected by leaf surface properties such as formation of cuticular waxes: plant viruses can change the light polarisation from leaves that may be attractive to insects among which polarisation-sensitive visual systems are prevalent (Maxwell et al., 2016).

Although the SAP54 plants may show alterations in their volatile production or visual phenotype (Figure 4.5), such changes are not likely to attract insects from a distance (Figures 4.4 and 4.7). Insect choice experiments involving single leaves of the SAP54 and control plants (chapter 3) also showed that leafhopper attraction may not be caused by changes in rosette architecture or leaf shape that could be perceived by insect vision or interact with volatile cues (Patt and Sétamou, 2007).

If not for their effect of light reflection from the leaf surface, plant trichomes and cuticle are important mechanical barriers to affect insect behaviour after leafhoppers have landed on the plant surface. For example, trichomes are important for insect locomotion (Björkman and Ahrné, 2005) and can be induced upon herbivore attack (Dalin and Björkman, 2003). I directly compared trichome density on SAP54 and control plants before insect exposure and found no differences in trichome number (Figure 4.9). In addition, I investigated the relationship between the number leafhopper eggs and number of trichomes found on leaves after exposure to adult insects and found no correlation on either 35S:GFP-SAP54 or 35S:GFP plants (Figure 4.10). This strongly suggests that any constitutive or insect-induced changes in trichome density may not be responsible for increased egg-laying on SAP54 plants. Moreover, females lay eggs in trichome-free zones on abaxial leaf side (Supplemental Figure 3; Appendix C). There is no difference in the abaxial trichome density between SAP54 and control plants before or after insect exposure (Supplemental Figure 2; Appendix C). Abaxial oviposition sites are typically localised in leaf pedicel, at the intersection between midvein and mesophyll tissue and leaf edges (Supplemental Figure 3; Appendix C). Therefore, any detailed measurements about leaf surface structure, like cuticle, would have to be performed with high spatial resolution in multiple sites on the leaf surface. A. thaliana cuticle is about 100nm thick and consists of cutin matrix with embedded waxes (Shumborski et
Transmission electron microscopy is a common method used to study the ultrastructure and thickness of the cuticle. For these reasons cuticular measurements can be labour intensive and were not performed here.

Leafhopper mating behaviour may also be important for locating host plant with a potential mate. Data presented in this chapter support the conclusion that female attraction and settlement on SAP54 plants is dependent specifically on male insect presence (Figure 4.12B). Male leafhoppers did not show any feeding preference for SAP54 plants (Figure 4.12C). Furthermore, experiments with equal number of males confined to leaves of SAP54 and control plants still demonstrated female oviposition preference for SAP54 plants (Figure 4.12B). Together this suggests that higher male abundance is not likely to explain female reproductive preference for SAP54 plants. However, I cannot exclude the possibility that SAP54 plants may modulate male emitted mating calls or vibration signals that travel through the plant. Male leafhoppers possess specialised tymbal organs or use their entire bodies to emit acoustic courtship calls and attract females (Heady et al., 1986). Acoustic signals may travel not only in the air but also via plant tissue as a medium. In fact, substrate vibrational communication is found in more than 90% of all insect species (Cocroft and Rodríguez, 2005), including leafhoppers (Eriksson et al., 2011). It is tantalising to hypothesise that SAP54 could have an indirect effect on leafhopper mating calls or modulate the vibrational communication by modulation of the plant tissue structure. Thus, despite equal number of male insects on SAP54 and control plants, males on SAP54 plants may communicate differently to females. Nevertheless, the observation that male exposed plants are not significantly more attractive for female egg-laying (Figure 4.15) contradicts this hypothesis and suggests that perhaps in the given experimental setup males are not using such acoustic signals to attract females.

Instead of direct male produced acoustic signals, female egg-laying preference for SAP54 expressing plants could be stimulated by male-induced plant responses. Plants are likely to perceive insect attack using damage or chemical cues that trigger phytochormone signalling to mount defence response (Erb et al., 2012). Mechanical damage from penetration of male insect piercing-sucking mouthparts (stylets) or chemicals released during and male salivation and feeding could induce specific signals in plants that are modulated by SAP54 or complement SAP54 effector activity to render plants more attractive for female
egg-laying. Alternatively, such male-dependent effect may stimulate female feeding on SAP54 plants and thus enhance female arrestment, indirectly resulting in greater oviposition time on SAP54 plants. Due to limited knowledge about leafhopper feeding and oviposition behaviour, it is difficult to establish the cause-effect relationship between feeding and egg-laying. *M. quadrilineatus* feeding and oviposition are strongly correlated (Figure 4.13). In other insect families, however, feeding and oviposition host choice can be different or use distinct cues. For example, *Drosophila melanogaster* (Diptera) alternate between hosts plants with different nutritional quality and prefer to feed on carbohydrate-rich substrates but lay eggs on protein-balanced substrates (Lihoreau *et al.*, 2016). Cereal stemborer *Busseola fusca* (Lepidoptera) uses plant surface chemicals to accept host plant for egg-laying. Furthermore, egg laying or insect feeding can induce plant volatile production. For example, eggs from another species of stemborer, *Chilo partellus* (Lepidoptera), have been shown to induce volatile production in maize (Mutyambai *et al.*, 2016). Likewise, phloem feeding hemipteran insects trigger release of plant volatiles (Machado *et al.*, 2014). However, in most cases herbivore induced volatiles function to directly harm the attacking insect or attract its natural enemies (Pare and Tumlinson, 1999; Machado *et al.*, 2014; Veyrat *et al.*, 2015) rather than attract conspecifics. It still remains to be elucidated whether *M. quadrilineatus* male induced volatiles may play a role in female attraction to or arrestment on SAP54 plants for feeding or oviposition. Females may use such male induced volatiles as direct close-distance oviposition cues or general attractants for feeding and oviposition.

Male requirement for female attraction to SAP54 plants presents a tantalising hypothesis that phytoplasma effector SAP54 manipulates insect induced plant responses in sex-dependent manner. Given that coinciding male presence is required for female oviposition choice, plants are simultaneously exposed to herbivore feeding and oviposition induced signals. There is a crosstalk in defence responses from feeding and egg-laying (Bruessow *et al.*, 2010), therefore male feeding in addition to female feeding may strengthen the feeding induced signals compared to oviposition induced defence signals. In addition, male insects may present a plant response elicitor (a sex-specific chemical or damage by mouthparts) that is absent in females but is key to female preference for SAP54 plants.
The current studies of plant-leafhopper interactions are limited by certain end-point measurements such as egg production or amount of insect excreted honeydew. In order to get more detailed understanding about plant-insect interactions, high-tech real-time observation methods such as 3D insect tracking systems (Thoen et al., 2016) would allow recording movements of male and female insects separately in time and space. This would allow to better understand whether male and female leafhoppers make synchronised selection of a host plant. By using an insect-tracking video system, it would be possible to confirm whether female leafhoppers preferentially fly to (are attracted to) and spend more time (are arrested on) male-colonised SAP54 plants and how often females change host plants. In addition, electro-penetration graph (EPG) studies paired with real-time imaging would allow to understand whether oviposition and feeding occur simultaneously or sequentially. For this purpose, the feeding characteristic electrical signals (waveforms) would have to be characterised first like in the studies of other pathogen vectoring insects from Cicadellidae family (Almeida and Backus, 2004). Similarly, our knowledge is limited about the plant-derived kairomones or conspecific-produced pheromones that play a role in mate attraction or initiation of feeding and oviposition behaviours in leafhoppers. Laser-based methods in detecting leafhopper vibratory communication, such as described by (Eriksson et al., 2011), may aid to address role of intra-specific communication in leafhoppers.
“To produce a really good biological theory one must try to see through the clutter produced by evolution to the basic mechanisms lying beneath them, realizing that they are likely to be overlaid by other, secondary mechanisms. What seems to physicists to be a hopelessly complicated process may have been what nature found simplest, because nature could only build on what was already there.”

Francis Crick (1988), What Mad Pursuit: A Personal View of Scientific Discovery, p 139

Chapter 5
Phytoplasma Effector SAP54 Modulates Insect-Induced Plant Responses

5.1. Introduction

In Chapter 4 I presented the surprising discovery that female leafhoppers prefer to lay eggs on male-colonised plants expressing phytoplasma effector SAP54 compared to male-colonised plants not expressing this gene. Crucially, the female oviposition preference disappears in the absence of male insects, suggesting that SAP54 may modulate insect-induced plant responses rather than constitutive defences. Therefore, the hypothesis is that SAP54 specifically alters male insect-induced plant responses leading to the attraction and/or arrest of female leafhoppers on a host plant for egg laying.

Plants balance the investment into defences against pests and pathogens versus investment into growth and reproduction, and this is key for plant fitness (Figure 5.1). Maintaining constitutive mechanical barriers to herbivores in the form of spines, hairs or trichomes or constitutive high levels of defence chemicals in the plant tissue can be energetically costly strategies to resist herbivore attack and require reallocation of resources from “primary” metabolism (Koricheva, 2001; Schwachtje & Baldwin, 2008; Firn & Jones, 2009). Therefore, plants have adapted to perceive various biotic signals such as herbivore attack to activate defence responses only when needed (Fürstenberg-Hägg et al., 2013). Moreover, plants allocate the defences to selected organs such as young leaves regardless of the location of the original herbivore attack to potentially maximise the fitness benefits of defence response (Eisenring et al., 2017). Interestingly, epigenetic changes of plants that experienced herbivory attack may prime these plants against insect attack in the next generations (Rasmann et al., 2012). And neighbouring plants may transmit insect-induced signals via underground mycorrhizal networks to warn their conspecifics that aphid attack may occur (Babikova et al., 2013). Thus, plants can protect themselves against attack at individual level and also at population and community levels.

In nature plants are attacked by a range of different pathogens and herbivores in addition to being exposed to various environmental stresses such as suboptimal temperatures, light or water availability. Therefore, identifying the stress and fine-tuning the strengths and specificities of signal responses to a range of different biotic and abiotic stimuli are important for allocating and optimally partitioning resources to effectively induce defence while also sustaining growth. Plant resistance to insect herbivore attack relies on multiple layers of induced immunity: 1) recognition of herbivore molecules or herbivore
damage by plant membrane or intracellular receptors; 2) signal transduction and amplification through protein kinases and plant hormone-dependent defence pathways; 3) mounting effective defence responses such as production of anti-herbivore chemical compounds (Howe and Jander, 2008; Fürstenberg-Hägg et al., 2013). An exogenous molecule from a vector-borne microbial plant pathogen that could manipulate any of these aspects of herbivore-induced plant immunity to the benefit of its vector would be a remarkable adaptation of the pathogen to aid its transmission in nature.

Figure 5.1. Plants balance their investment into defence and stress responses versus growth and reproduction. Plant resources are limited. Therefore, trade-offs exist between response to biotic or environmental stress and generation of more biomass or reproduction. Plant primary resources are not only consumed (reduced) by herbivores (Zangerl et al., 2001) but may also function as signals and precursors for synthesis of defence compounds (Schwachtje & Baldwin, 2008). Plant hormones like jasmonate are regulators of metabolite partitioning in plants (Havko et al., 2016). Global changes in “primary” and “secondary” metabolism pathways after biotic and abiotic stresses have been characterised by several –omics studies and plant physiological measurements (Hui et al., 2003, Ralph et al., 2006; Papazian et al., 2016).

Plants detect conserved molecules of herbivores and pathogens that antagonize them. These molecules are known as elicitors. Hitherto, the best studied elicitors are present in the oral secretions (saliva) of chewing insects. Sometimes plants recognize insect-derived amino acids conjugated with plant fatty acids (Halitschke et al., 2001). Not only insect oral secretions or gut-derived molecules but also egg-laying fluids contain elicitors of plant defences (Reymond, 2013). Moreover, in addition to direct detection of herbivore-derived molecules,
plants can perceive molecular changes induced by insects. For example, insects damage plant tissues by chewing, snipping or tearing, resulting in the generation of damage-associated molecular pattern peptides such as Pep1 or systemin from their plant precursors (Schaller and Ryan, 1996; Bartels et al., 2013). Similarly, insects feeding may produce inceptins derived from chloroplast ATP synthase subunits in the ingested plant tissues (Schmelz et al., 2006). It is thought that the different kinds of herbivore- or damage-associated molecular patterns (HAMPs or DAMPs) are perceived via plant pattern recognition receptors (PRRs) to induce the first stage of plant immunity, named PAMP-triggered immunity (PTI). A gene cluster of three rice membrane lectin receptor kinase, has been associated with resistance to rice brown planthopper Nilaparvata lugens (Liu et al., 2015), but what planthopper elicitors these receptors recognize is not yet known. Plant PRRs resemble animal and other eukaryotic Toll and Toll-like receptors that play important roles in immunity (Shiu and Bleecker, 2001), suggesting common origins of membrane receptors among eukaryotes.

Plant leucine-rich repeat receptor-like kinases (LRR-RLKs) often function as PRRs to elicit downstream immune signaling events, involving activation of mitogen-activated protein kinases (MAPKs), induction of Ca\textsuperscript{2+} waves and reactive oxygen species (ROS) bursts (Fürstenberg-Hägg et al., 2013). These signals are key for triggering both general defence responses such as callose depositions or induction of certain defence genes. The cell surface-localized receptor BAK1 has been shown to be important in herbivory-induced defence responses in Arabidopsis thaliana (Prince et al., 2014; Chaudhary et al., 2014) and Nicotiana attenuata (Yang et al., 2011a), and is required for activation of jasmonic acid (JA)-mediated defences (Yang et al., 2011b). Nevertheless, a single PRR, like BAK1, does not function alone in the perception of HAMPs or DAMPs. Instead, various different PRRs may act in concert with BAK1 to integrate various stimuli from wounding, oral secretions or oviposition fluids. Herbivory activates a combination of salicylic acid (SA)-induced protein kinases (SIPKs), wound-induced protein kinases (WIPKs) and mitogen-activated protein kinases (MAPKs) (Wu et al., 2007; Yang et al., 2011b), suggesting that these signaling cascades may integrate signals perceived by a multitude of different receptors. Moreover, activation of several protein kinase cascades may be important to ensure simultaneous elicitation of different defence responses as well as ensuring the robustness of the immune response. Wu et al. (2007) demonstrated that SIPKs
and WIPKs signals converge to upregulate JA and ethylene (ET)-associated signaling in *N. attenuata* attacked by lepidopteran herbivore *Manduca sexta*. Plant hormones SA, JA and ET are important integrators of signals induced by generalist or specialist herbivores and their cross talk may be important for the specificity of plant responses to insect attack (Diezel *et al.*, 2009). JA-activated defences were shown to be key in plant selection of leafhoppers in the field (Kallenbach *et al.*, 2012).

Downstream of plant hormone signaling transcription factors regulate various secondary metabolite pathways for the production of alkaloids, glucosinolates, terpenoids, phenolics and their numerous derivatives. Upon insect attack, plants activate production of various compounds, such as protease inhibitors, lectins, polyphenol oxidases or chitinases, which directly target insect digestive tracts (Howe and Jander, 2008). One of the most important groups of anti-herbivore chemicals in Brassicaceae, including *A. thaliana*, are glucosinolates, which function as insect toxins and feeding deterrents. Quantity and composition of indole and aliphatic forms of glucosinolates were shown to be key in host plant selection by aphids (Kim and Jander, 2007; Zust *et al.*, 2012). Many phenolics are activated upon herbivore attack and aid plant resistance to insect colonization via structural changes in plants (Barakat *et al.*, 2010). For example, fortification of cell walls by deposition of lignins impedes the penetration of hemipteran stylets. Simple phenolic compounds such as phytoalexins and other phenylalanine-derived propanoids such as caffeic, ferulic and coumaric acids are important plant chemicals induced by insect attack and mediate insect selection of host plants (Robert *et al.*, 2012). Terpenoids (monoterpenes and sesquiterpenes) are common plant components that produce blends of volatile compounds with various functions – from directly deterring attacking aphids to the attraction of predatory and parasitoid insects (Fürstenberg-Hägg *et al.*, 2013) – and their synthesis is induced upon insect attack.

Herbivorous insects either adapt to the plant-derived chemical warfare or try to modulate the plant ability to induce these. An emerging paradigm in plant-insect interactions is the presence of specialized effector proteins in insect saliva that suppress defense responses of plants (Hogenhout and Bos, 2011). Moreover, molecules (putative effectors) in insect frass also suppress induced plant responses (Ray *et al.*, 2016). However, plants possess numerous intracellular nucleotide-binding leucine-rich repeat (NBS-LRR) receptor-like kinases
(RLKs) that may recognize such insect effectors and may reinstate defence signaling leading to effector-triggered immunity (ETI). For example, the NBS-LRRs *Mi* confers resistance to potato aphids, *Vat* to cotton/melon aphid in melon, and *Bph14* was identified to confer resistance to planthopper *N. lugens* in rice (Vos *et al.*, 1998; Du *et al.*, 2009; Boissot *et al.*, 2016). However, so far there are no insect effectors/elicitors identified that are recognized by plant NBS-LRRs. Like extracellular PRRs, the intracellular NBS-LRRs share many structural and functional similarities with animal immune receptors (Maekawa *et al.*, 2011).

It is not known whether males and females of sexually dimorphic insect species secrete different defence elicitors or effectors into the plant and thus induce plant responses differentially. Moreover, could vector-borne plant pathogens, such as phytoplasmas, modulate plant responses that are differentially induced by male and female vectors? In this Chapter I focus on investigating how SAP54 may modulate insect-induced plant transcriptional responses. I find that (1) plants respond differentially to male and female leafhoppers, and that (2) insect-induced plant defence responses are altered in the presence of SAP54 in insect sex-specific manner. I discuss the implications of RNA-seq results for novel hypothesis about potential mechanisms how SAP54 may modulate insect induced plant responses to promote female egg-laying.

### 5.2. Results

#### 5.2.1. Experimental design of the RNA-sequencing experiment

In order to elucidate *A. thaliana* transcriptional response to insect feeding and oviposition and how SAP54 may modulate these, I exposed 35S:GFP and 35S:GFP-SAP54 plants separately to male and female adult leafhoppers and compared to plants which were not exposed to insects (Figure 5.2).
Figure 5.2. The design of RNA-seq experiments. Each plant was exposed to five male or five female insects or, as control treatments, insect-free clip cages. Each treatment was repeated four times (i.e. four plants each) to generate four independent samples that were each processed for RNA isolation, library construction and sequencing.

Insects were confined to single fully-expanded leaves using clip cages. To investigate if crowding of insects inside the clip cages may have density-dependent effects on insect performance such as egg laying, I first experimentally quantified female egg-laying as a function of insect density per clip cage over a period of 48h (Figure 5.3). Five females per clip-cage were found to be the most optimal insect density for the RNA-seq experiment based on the highest egg count. Standardizing this among the treatments and repeats is important because oviposition is shown to induce plant defence responses (Reymond, 2013). Insect crowding appears to negatively correlate with female reproductive output. Low insect density, on the other hand, did not ensure egg production in every experimental replicate, and, therefore, may not induce egg-laying specific defence responses. Clip-cages with 5 male or 5 female insects contained similar amount of honeydew excretions (observations under microscope), suggesting similar levels of feeding by males and females. Together, these experiments indicated that 5 insects per clip cage is the most optimal density in the 48h duration. Hence, these conditions were used to obtain the 24 plant samples as shown in Figure 5.2 for subsequent RNA extractions, library constructions and sequencing.
5.2.2. Assessment of technical and biological variation in RNA-seq data

The RNA-seq experiment yielded great read depth and good coverage. Across the 24 RNA-seq libraries generated, the average coverage was 33.29 per (±0.84 SEM) million reads per library with 96.14% (±0.48% SEM) mapping onto the reference genome (*A. thaliana* TAIR10). I further assessed the dataset for any confounding biological variation due to differential expression of transgenes in the experimental lines or technical batch-effects associated with RNA extraction and sequencing. To do this, I first mapped all library reads to the SAP54 and GFP nucleotide sequences. Given that SAP54 and GFP are not part of the *A. thaliana* genome I used these sequences to confirm the genotype of the plant and to assess the variation in SAP54 and GFP expression levels using normalised expression values (Figure 5.4). As was hoped, samples extracted from 35S:GFP contained reads matching GFP only and no reads matching SAP54. Moreover, in the samples derived from the 35S:GFP-SAP54 plants, the number of reads matching SAP54 was perfectly correlated with those matching GFP (Figure 5.4). Only one replicate from the non-insect exposed samples was excluded from further analysis (leaving three replicates) due to the absence of reads matching SAP54, indicating that SAP54 was not expressed in this plant. These data
indicate that there were no significant differences in *SAP54* or *GFP* expression levels between male, female or non-insect exposed 35S:GFP-SAP54 and 35S:GFP plants. Therefore, differences in *SAP54* or *GFP* expression levels are unlikely to contribute to plant responses to males/females.

**Figure 5.4.** Correlation between normalised reads (FPKM counts) corresponding to nucleotide sequences of *SAP54* and *GFP* of samples derived from 35S:GFP (left panel) and 35S:GFP-SAP54 (right) plants. Since *SAP54* is GFP-tagged, reads derived from these plants demonstrate perfect positive correlation to *SAP54* and GFP sequences (right). It was confirmed that no reads corresponding to *SAP54* were present in the 35S:GFP plants (left). Plants exposed to female (f), male (m) or no insects (n) do not differ in number of normalized reads matching either *SAP54* (F$_{2,10}$=2.67; p=0.129; ANOVA) or *GFP* (F$_{2,11}$=0.19; p=0.83; ANOVA) across biological replicates (numbered 0,1,2,3 on the graph).

Whereas there is no statistical difference in *SAP54* and *GFP* expression between male, female and no-insect treated plants, the biological replicates of each treatment represent a range of *SAP54* and *GFP* expression values, and this may be reflected in the variance in normalised read counts for male and female induced plant transcripts, thus lowering the number of significantly induced genes in both treatments. Therefore, I wanted to test if the observed variation in the expression of *GFP* and *SAP54* within each treatment may confound the global expression patterns of all sequenced transcripts. To assess this, I plotted the
median of FPKM normalised expression of all sequenced transcripts against the GFP expression in all samples (Figure 5.5). Since SAP54 is GFP-tagged, GFP alone accounts for the transgene expression in both 35S:GFP-SAP54 and 35S:GFP plants. There were no effects of the biological variation in SAP54 and GFP expression on global gene expression in either male, female or no-insect treated plants (Figure 5.5).

Figure 5.5. Variations in GFP and SAP54 expression levels of 35S:GFP (left) and 35S:GFP-SAP54 (right) plants do not positively correlate with the median expression levels of all sequenced transcripts. Plants exposed to female (f), male (m) or no insects (n) demonstrate similar spread of transgene and global gene expression across all 4 biological replicates (labelled 0-3). Median was chosen as a statistical measure of non-normal distribution of expression values in all sequenced transcripts.

Samples were randomly allocated to four sequencing lanes on the Illumina HiSeq2000 platform to minimize the possibility that all samples from same treatment or day of RNA extraction would enter the same sequencing lane. In order to address the possibility of technical variation among sequencing lanes, GFP expression levels of samples run on different lanes were plotted (Figure 5.6). Since SAP54 is GFP-tagged in 35S:GFP-SAP54, GFP alone accounts for the
transgene expression in both 35S:GFP-SAP54 and 35S:GFP plants. There were no significant differences in GFP expression levels across the different sequencing lanes (Figure 5.6). Thus, technical variation is unlikely to explain plant gene expression differences among the samples.

![Figure 5.6](image.png)

**Figure 5.6. There are no significant differences in GFP expression levels among the four Illumina HiSeq2000 sequencing lanes.** Biological replicates of all insect treatments in each genetic background were randomized among lanes of the sequencing platform and showed no significant difference in the FPKM normalised GFP read count ($F_{3,19}=0.78; p=0.522; \text{ANOVA}$).

Taken together, these results suggest that technical errors in the preparation of the experiment nor expression-dependent effects of SAP54 and GFP in the transgenic lines are unlikely to confound the effects of male and female insect feeding and oviposition on plants.
5.2.3. SAP54 alters plant response to leafhoppers in sex-specific manner

I previously demonstrated that female leafhoppers prefer male-colonised 35S:GFP-SAP54 plants for egg-laying but show no oviposition preference for 35S:GFP-SAP54 plants in absence of males (Chapter 4). This suggests that SAP54 may alter male-induced plant responses. To investigate this further, I compared the overlap between all significantly differentially expressed (DE) transcripts in male- and female-exposed SAP54 and control plants. I found considerably more differentially regulated transcripts on male-exposed 35S:GFP-SAP54 compared to 35S:GFP plants (Figure 5.7). A similar number of transcripts (about 900) were upregulated in male- and female-exposed 35S:GFP plants, indicating considerable overlap between male and female-induced responses. Interestingly, there is a significant proportion (39% upregulated and 82% downregulated) of genes specifically regulated by females but not males in 35S:GFP plants, suggesting that females potentially regulate additional plant processes to males. Surprisingly, this trend is completely reverted on 35S:GFP-SAP54 plants where male leafhoppers upregulate and downregulate a considerably greater proportion of transcripts than female insects. This suggests that SAP54 may primarily alter plant responses to male leafhoppers.

![Significantly upregulated vs downregulated transcripts](image)

Figure 5.7
Figure 5.7. Male insects differentially regulate more genes on 35S:GFP-SAP54 plants compared to 35S:GFP control plants. The overlap between female and male insect upregulated (red) and downregulated (green) genes on 35S:GFP and 35S:GFP-SAP54 plants. Each circle represents the number of DE transcripts combining 4 biological replicates in male or female exposed plants compared to the 4 biological replicates in no-insect (empty clip-cage) treatment (DEseq; DE transcripts called for each-treatment vs control at p<0.05 and q<0.05). Brackets contain the percentage of differentially regulated transcripts from the total in each pairwise comparison.

Next, I wanted to investigate which plant processes are changed in response to male and female insects on SAP54 and control plants. Therefore, I took all significantly up- and down-regulated plant transcripts in response to male and female insects and tested for pathway enrichment using MapMan functional annotation tool (see materials & methods). Biotic stress transcripts are DE in male- and female-exposed control plants. For comparisons, other plant processes such as photosynthesis are enriched in DE transcripts only in female-but not male-exposed plants (Table 5.1). Interestingly, no single pathway is enriched with DE transcripts in female-exposed SAP54 plants, suggesting that plant responses to female leafhoppers may not be induced on these plants or were silenced by SAP54 after female induction. However, consistent with the data presented in Figure 5.7, male insect exposure elicits significant changes in plant biotic stress and hormone responses in the SAP54 plants (Table 5.1).

Since the leafhopper effects on plant biotic stress responses could be one of the key components mediating plant-insect interactions, I decided to visualise up- and down-regulated transcripts in different components of biotic stress responses in the SAP54 and control plants. I used the MapMan functional annotation tool to plot fold change (log scale) of insect sex-specific induced plant transcripts. More biotic stress-related transcripts were DE in female- compared to male-exposed control plants (Figure 5.8). Intriguingly, many of the female and male induced transcripts are not DE in the SAP54 plants. Furthermore, pathways involved in stress signalling, protein degradation and abiotic (heat) stress are significantly downregulated only in male-treated SAP54 plants (Figure 5.8).

In summary, SAP54 appears to modulate plant stress responses to leafhopper-treated plants. Moreover, biotic stress transcripts are significantly suppressed in male-exposed SAP54 plants.
Table 5.1. Enrichment of biological functions in female and male exposed 35S:GFP and 35S:GFP-SAP54 plants compared to insect free plants. Enrichment is calculated taking together fold change in all up-regulated and down-regulated transcripts. Biological functions are ranked using Wilcoxon rank test with Benjamini-Hochberg correction for multiple comparisons. The number of elements in each pathway corresponds to the total number of genes within each functional group, based on in-built TAIR9 functional annotation in MapMan.

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Figure 5.8. Female and male leafhopper induced plant (biotic) stress responses differ between 35S:GFP and 35S:GFP-SAP54 plants. Female insects (A) differentially regulate more transcripts than males (B) on 35S:GFP plants compared to no-insect treated plants. Female leafhoppers (C) elicit fewer changes than males (D) in plant stress responses on 35S:GFP-SAP54 plants compared to no-insect treated plants.

The analyses hitherto did not reveal whether the same DE genes in insect-exposed control plants were also DE or were regulated in opposite direction in insect-exposed SAP54 plants. To test this, I compared the identity of all DE transcripts (biotic stress together with other processes) in insect exposed SAP54 and control plants (Figure 5.9). Surprisingly, almost none of the DE transcripts in insect-treated control plants show DE in the opposite direction in the insect-exposed SAP54 plants (Figure 5.9), suggesting that male and female insects may regulate a different set of transcripts on SAP54 compared control plants. However, a certain proportion of upregulated and downregulated transcripts in insect-exposed control plants are not DE in SAP54, suggesting that SAP54 may be modulating the expression of certain insect-regulated plant transcripts. A large
The proportion of DE transcripts in the leafhopper-treated control plants are DE in the same direction in the SAP54 plants.

Together, these finding suggest that SAP54 may largely modulates the type of plant response to the insects independently from silencing insect-induced plant responses. In other words, SAP54 modulates plant response in a given pathway, such as biotic stress response, by altering expression of leafhopper-induced genes as well as differentially regulating another set of genes in insect-dependent manner.

Figure 5.9. Male and female insects regulate a different set of transcripts on 35S:GFP-SAP54 plants compared to 35S:GFP control plants. Male and female insect upregulated (red) and significantly downregulated (green) genes on 35S:GFP are compared the equivalent treatments on 35S:GFP-SAP54 plant. Each circle represents the number of significantly regulated transcripts (DEseq; p<0.05 and q<0.05) in male or female exposed plants compared to the no-insect (empty clip-cage) treatment. Brackets contain the percentage of differentially regulated transcripts from the total in each four-way comparison.

There is a possibility that DE transcripts in the female-exposed control plants may be oppositely regulated in the male-exposed SAP54 plants. This may be important for female attraction to male-colonised plants. I investigate the overlap between plant responses to males and females in Figure 5.10. Interestingly, most genes that were up- or down-regulated in the male-treated
SAP54 plants are not DE in the female-exposed control plants, suggesting that males in SAP54 plants may not oppositely regulate the transcripts which females would normally induce in the control plants.

Figure 5.10. Male insect regulated transcripts on 35S:GFP-SAP54 plants are different from female regulated transcripts on 35S:GFP plants. Female insect significantly upregulated (red) and significantly downregulated (green) genes on 35S:GFP are compared the differentially regulated gene set in male exposed 35S:GFP-SAP54 plants. Each circle represents the number of significantly regulated transcripts (DEseq; p<0.05 and q<0.05) in male or female exposed plants compared to the no-insect (empty clip-cage) treatment. Brackets contain the percentage of differentially regulated transcripts from the total in each four-way comparison.

Taken together, data in Figures 5.7-5.9 demonstrate that male-elicited responses in the SAP54 plants are largely different from male as well as female insect induced responses in the control plants because different sets of genes are DE in insect-exposed SAP54 and control plants. Nevertheless, genes that are not DE in insects exposed 35S:GFP-SAP54 plants compared to 35S:GFP could be silenced by SAP54 or not induced by insects in presence of SAP54.

It could be that SAP54 alters plant transcriptional responses independently from insect exposure, thus explaining why there is a large proportion of DE transcripts in insect-exposed SAP54 plants that are not DE in the control plants. To test if the effects of the effector were insect-dependent, I compared the DE
transcripts between 35S:GFP-SAP54 and 35S:GFP plants exposed to only males, only females or no insects (Figure 5.11). I found that most SAP54-induced changes in plants are dependent on insect exposure. Furthermore, the greatest effect of SAP54 is significant upregulation of 341 transcript in male-dependent manner.

Figure 5.11. SAP54 differentially regulates plant genes in insect-dependent manner. The number of significantly upregulated (red) and significantly downregulated (green) genes in 35S:GFP-SAP54 plants compared to 35S-GFP plants depends on the insect presence and are specific to male and female leafhoppers. Each circle represents the number of significantly regulated transcripts (DEseq; p<0.05 and q<0.05) in insect exposed or non-exposed 35S:GFP-SAP54 compared to insect exposed or non-exposed 35S:GFP plants. Brackets contain the percentage of differentially regulated transcripts from the total in each three-way comparison.

In summary, female insects can elicit different plant responses to males in 35S:GFP plants. However, both male and female induced plant responses are altered in 35S:GFP-SAP54 plants in leafhopper sex-specific manner. In addition to altering expression of transcripts initially induced by males or females in 35S:GFP plants, another set of transcripts are DE in the SAP54 plants in insect-dependent manner.
5.2.4. *One half of A.thaliana genes are expressed in vegetative state and define insect sex-specific plant responses*

Analysing only the significantly differentially regulated transcripts may not reflect the cumulative effect of small, yet un-significant, changes in insect-induced plant pathways. For example, differential expression of a transcription factor may cascade to co-ordinated and additive changes in genes representing a certain defence pathway. To characterise such effects and identify which pathways may be paramount for male-dependent preference of female egg laying on SAP54 plants, I decided to analyse the transcriptional changes of all expressed plant genes.

To do this, I first identified the set of all expressed transcripts distinct from transcripts that show very low expression in any of the experimental conditions. Plotting the normalised expression level of all genes in *A.thaliana* transcriptome against the fold change in the gene expression between 35S:GFP-SAP54 and 35S:GFP plants in male, female-or no-insect treatments showed that transcripts with the greatest fold change have very low relative expression (Figure 5.12).

![Figure 5.12. Relationship between differential gene regulation (fold change between SAP54 and GFP) and gene expression values in female, male and no insect exposed plants. Genes with very low expression demonstrate greatest change.](image-url)
This means that a great proportion of genes with minute expression in both the experimental treatment and the control can demonstrate massive differences in fold change. In a hypothetical example, transcript A changing from $1 \times 10^{-20}$ FPKM (control) to $1 \times 10^{-18}$ FPKM (after male insect exposure) shows 100 fold increase in expression but transcript B changing from 50 FPKM to 200 FPKM only 4 fold. Given the average read depth of $33 \times 10^6$ cDNA fragments and assuming the length of transcripts A and B to be approx. 1000 bp, the approximate number of reads for transcript A in male exposed plants would be negligible, only $33 \times 10^{-12}$, whereas transcript B would be detected with $66 \times 10^8$ reads. To avoid such low expression bias and increase the resolution of reliably detectable differences among the treatments, I decided to define an expression threshold. Defining the threshold would help to eliminate the negligibly expressed transcripts that would give enormous fold-changes. I plotted the frequency of transcripts as the function of gene expression. About 25% of the total transcriptome across all biological replicates in each treatment has lower expression than one FPKM (Figure 5.12). Therefore, for further analysis I considered only those transcripts that demonstrate $\geq 1$ FPKM expression (i.e., approximately 1 read per million reads of 1kb transcript) in more than a half of all biological replicates in at least one of the six experimental treatments (Figure 5.2) sequenced. This would capture any meaningful increase or decrease in gene expression regardless of the treatment of interest. However, in order to not exclude significant changes that result from very small but consistent expression changes across biological replicates in any treatment, I kept all significantly changed transcripts in the final set of 17153 transcripts for further analysis (Figure 5.13).

Together, this approach defines a working list of genes that would characterise the global transcriptome changes within the transcribed genome including but not restricted to small number of significant fold changes (which are defined by statistical assumptions in calculating DE transcripts in Cufflinks (see section 8.12)). This would give greater power to subsequent enrichment analysis of functional pathways that display the greatest transcriptional changes and minimise the “noise” of transcripts that are not expressed in all biological replicates.
Figure 5.13. Combination of all expressed transcripts and transcripts that demonstrate significant fold-change in expression levels between any pairwise comparisons between treatments. All cDNA libraries show similar distribution of normalised gene expression with overrepresentation of low-expressed transcripts. All transcripts that demonstrate ≥1 FPKM expression in any of the treatments (top panel) were selected together with all transcripts that show significant fold-change irrespective of their expression (red; bottom panel) to obtain the final list of 17'153 genes (out of total ~33'000 mapped transcripts).
I next performed multi-dimensional distance-similarity (MDS) analysis to visualise the differences between 35:GFP-SAP54 and 35:GFP plant responses to male and female insects based on the expression of the 17’153 genes (Figure 5.14). Female treated samples group separately from male and no-insect exposed plants. The grouping of biological replicates by male, female and no-insect treatment is stronger than distinction between all GFP or SAP54 plants. Interestingly, more distinct subgrouping of SAP54 and GFP plants is observed within the group of male-exposed plants compared to females or no-insect treatment. This suggests, that the list of expressed 17’153 transcripts captures the difference between male and female insects and the potential male-dependent effects of SAP54.

![Multi-dimensional plot displays similarity and differences among all 24 cDNA libraries](image)

**Figure 5.14. Multi-dimensional plot displays similarity and differences among all 24 cDNA libraries.** Numbers 0 to 3 label biological replicates in male, female and no-insect control (UTCTRL) on 35S:GFP and 35S:GFP-SAP54 plants. Male (green) and female insects (red) group separately from no-insect exposed plants (blue). These groupings were highlighted manually after MDS analysis. Within the male group, SAP54 and GFP plants form more distinct sub-groups than within the groups of female and no-insect exposed plants. The only exception were biological replicate SAP54_MALE_2 and GFP_MALE_2 (confirmed transgene expression in Figure 5.4) grouping to male treated GFP and SAP54 plants respectively.
5.2.5. Female leathoppers show differential effects on plant primary and secondary metabolism compared to males

In the previous sections I revealed that SAP54 changes plant responses to insects in sex-specific way. Therefore, such effect should rely on the difference between male and female induced plant responses. Understanding the differential recognition of insect sexes by plants and characterisation of plant responses is a paramount for developing a testable hypothesis about the mechanism of SAP54- and male-dependent female oviposition preference.

I visualised the response of all 17'153 expressed *A. thaliana* transcripts to female and male *Macrosteles quadrilineatus* on 35S:GFP plants. Interestingly, both males and females affect similar metabolic pathways in the plant, although the extent to which certain pathways are induced appears to be sex-specific (Figure 5.15). Both male and female insects induce changes in photosynthesis, amino acid metabolism, protein transport as well as alteration in gene regulation related to chromatin remodelling and transcription factor activation (Table 5.2). Interestingly, many defence-related pathways are enriched in transcripts regulated by both male and female insects. These include receptor-like kinases, JA and ET pathways as well as secondary metabolites.

I used Iterative Group Analysis (IGV) approach to further discriminate which functional pathways are enriched in upregulated and downregulated transcripts by male and female insects (Table 5.3). Both males and females show downregulation of gibberellin pathway and upregulation of jasmonic acid and biosynthesis of glucosides and monoterpenes.

Functional group enrichment analysis also suggest that certain groups of secondary metabolites (glucosides and monoterpenes) show correlated change with regulation of phytohormone signalling and, therefore, might be situated downstream insect perception to mount resistance against herbivore attack (Table 5.3). Moreover, some responses in secondary metabolisms appear to be sex-specific: cellulose synthesis is significantly enriched with upregulated transcripts in male exposed plants but lignin biosynthesis – in female treated plants (Table 5.3). Although all components of cell wall synthesis (including cellulose and lignin synthesis) are affected in plants exposed to both sex insects (Figure 5.15), the degree to which each of these pathways are altered is insect sex-dependent. This suggests that modulation of cell wall composition may be a general response to leathoppers, and there could be sex-specific differences in
regulation of cell wall components. Similarly, photosynthesis pathway is significantly enriched with downregulated transcripts in both male and female exposed plants (Table 5.2). However, female insects demonstrate much stronger suppression of photosynthetic pathways compared to males (Figure 5.15 and relative p-value difference in Table 5.2).

Table 5.2. List of pathways in MapMan annotation that show significant enrichment of differentially regulated transcripts in both male and female exposed plants. For convenience, redundant functional groups (bins) already represented by higher order groupings are omitted from this list. Enrichment analysis is based on Benjamini-Hochberg correction for multiple pathway comparisons using Wilcoxon rank test.

<table>
<thead>
<tr>
<th>MapMan Bin</th>
<th>Pathway description</th>
<th>Nr elements in pathway</th>
<th>p-value (male insects)</th>
<th>p-value (female insects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.1</td>
<td>light reactions photosystem II</td>
<td>59</td>
<td>0.009582</td>
<td>1.26E-16</td>
</tr>
<tr>
<td>1.3.13</td>
<td>calvin cycle rubisco interacting proteins</td>
<td>6</td>
<td>0.007271</td>
<td>0.0212272</td>
</tr>
<tr>
<td>13.1.3</td>
<td>amino acid metabolism: synthesis of aspartate</td>
<td>48</td>
<td>7.66E-04</td>
<td>0.0119167</td>
</tr>
<tr>
<td>13.1.7</td>
<td>amino acid metabolism: synthesis of histidine</td>
<td>10</td>
<td>0.040206</td>
<td>0.0048714</td>
</tr>
<tr>
<td>16.1.4</td>
<td>secondary metabolism: isoprenoids and carotenoids</td>
<td>13</td>
<td>0.004834</td>
<td>0.0020084</td>
</tr>
<tr>
<td>17.5.2</td>
<td>hormone metabolism: ethylene signal transduction</td>
<td>35</td>
<td>1.26E-08</td>
<td>1.31E-04</td>
</tr>
<tr>
<td>17.7.3</td>
<td>hormone metabolism: jasmonate signal transduction</td>
<td>15</td>
<td>0.004335</td>
<td>0.0212272</td>
</tr>
<tr>
<td>20.1.7</td>
<td>biotic stress: PR-proteins</td>
<td>502</td>
<td>0.001538</td>
<td>1.55E-10</td>
</tr>
<tr>
<td>27.3.3</td>
<td>transcriptional changes: APETALA2/Ethylene-responsive element binding protein family</td>
<td>117</td>
<td>5.17E-06</td>
<td>4.42E-04</td>
</tr>
<tr>
<td>27.3.32</td>
<td>transcriptional changes: WRKY domain transcription factor family</td>
<td>72</td>
<td>2.53E-06</td>
<td>6.56E-12</td>
</tr>
<tr>
<td>27.3.44</td>
<td>transcriptional changes: Chromatin Remodeling Factors</td>
<td>36</td>
<td>1.49E-07</td>
<td>0.0280811</td>
</tr>
<tr>
<td>29.1.30</td>
<td>protein activation: pseudouridylate synthase</td>
<td>15</td>
<td>0.042528</td>
<td>0.0054951</td>
</tr>
<tr>
<td>29.2.1</td>
<td>protein synthesis ribosomal proteins</td>
<td>439</td>
<td>0.011931</td>
<td>1.23E-77</td>
</tr>
<tr>
<td>29.3.4</td>
<td>protein secretory pathway</td>
<td>128</td>
<td>0.029239</td>
<td>3.57E-04</td>
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<tr>
<td>30.2.17</td>
<td>signalling receptor-like kinases: DUF 26 family</td>
<td>50</td>
<td>0.003154</td>
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<tr>
<td>30.2.20</td>
<td>signalling receptor-like kinases: LRK10 family</td>
<td>12</td>
<td>0.048369</td>
<td>0.013881</td>
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<tr>
<td>35.1.5</td>
<td>not assigned no ontologypentatricopeptide (PPR) repeat-containing protein</td>
<td>455</td>
<td>8.15E-63</td>
<td>2.72E-15</td>
</tr>
</tbody>
</table>
Figure 5.15. Both female (A) and male (B) insects induce global transcriptional changes in *A. thaliana*. Insects have marked effect on numerous metabolic pathways, including carbohydrate, lipid and amino-acid metabolisms. Pathways involved in photosynthesis light and dark reactions demonstrate sex-specific degree of regulation. All transcripts are coloured based on log$_2$(fold change) when compared insect exposed plants to no insect exposed controls.
Table 5.3. Iterative group analysis reveals functional groups that are significantly overrepresented within the list of upregulated or down-regulated transcripts. The method estimates enrichment of certain pathways based on fold-change ranking of differentially expressed transcripts compared to random un-ranked list of the same gene-set. P-value threshold is adjusted for false-discovery rate.

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Total nr of genes</th>
<th>Nr of genes changed</th>
<th>P-Value</th>
<th>% genes changed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calvin cycle</td>
<td>39</td>
<td>11</td>
<td>1.92E-05</td>
<td>28.205</td>
</tr>
<tr>
<td>gibberellin biosynthesis II (early C-3 hydroxylation)</td>
<td>2</td>
<td>2</td>
<td>3.82E-05</td>
<td>100</td>
</tr>
<tr>
<td>cyanate degradation</td>
<td>8</td>
<td>3</td>
<td>0.0001</td>
<td>37.5</td>
</tr>
<tr>
<td>gibberellin biosynthesis I (non C-3, non C-13 hydroxylation)</td>
<td>3</td>
<td>2</td>
<td>0.00011</td>
<td>66.667</td>
</tr>
<tr>
<td>gibberellin biosynthesis III (early C-13 hydroxylation)</td>
<td>3</td>
<td>2</td>
<td>0.00011</td>
<td>66.667</td>
</tr>
<tr>
<td>flavonoid biosynthesis</td>
<td>21</td>
<td>3</td>
<td>0.0008</td>
<td>14.286</td>
</tr>
<tr>
<td>gibberellin biosynthesis II (early C-3 hydroxylation)</td>
<td>2</td>
<td>2</td>
<td>9.67E-05</td>
<td>100</td>
</tr>
<tr>
<td>gibberellin biosynthesis I (non C-3, non C-13 hydroxylation)</td>
<td>3</td>
<td>2</td>
<td>0.00029</td>
<td>66.667</td>
</tr>
<tr>
<td>gibberellin biosynthesis III (early C-13 hydroxylation)</td>
<td>3</td>
<td>2</td>
<td>0.00029</td>
<td>66.667</td>
</tr>
<tr>
<td>jasmonic acid biosynthesis</td>
<td>15</td>
<td>8</td>
<td>5.76E-08</td>
<td>53.333</td>
</tr>
<tr>
<td>monoterpene biosynthesis</td>
<td>2</td>
<td>2</td>
<td>4.78E-05</td>
<td>100</td>
</tr>
<tr>
<td>lipoxygenase pathway</td>
<td>11</td>
<td>5</td>
<td>5.11E-05</td>
<td>45.455</td>
</tr>
<tr>
<td>glucoside biosynthesis</td>
<td>49</td>
<td>8</td>
<td>5.74E-05</td>
<td>16.327</td>
</tr>
<tr>
<td>lignin biosynthesis</td>
<td>30</td>
<td>8</td>
<td>0.00086</td>
<td>26.667</td>
</tr>
<tr>
<td>jasmonic acid biosynthesis</td>
<td>15</td>
<td>10</td>
<td>1.73E-08</td>
<td>66.667</td>
</tr>
<tr>
<td>lipoxygenase pathway</td>
<td>11</td>
<td>8</td>
<td>5.62E-08</td>
<td>72.727</td>
</tr>
<tr>
<td>monoterpene biosynthesis</td>
<td>2</td>
<td>2</td>
<td>5.84E-05</td>
<td>100</td>
</tr>
<tr>
<td>glucoside biosynthesis</td>
<td>49</td>
<td>7</td>
<td>0.00012</td>
<td>14.286</td>
</tr>
<tr>
<td>anthocyanin biosynthesis</td>
<td>5</td>
<td>3</td>
<td>0.00052</td>
<td>60</td>
</tr>
<tr>
<td>cellulose biosynthesis</td>
<td>31</td>
<td>5</td>
<td>0.00085</td>
<td>16.129</td>
</tr>
</tbody>
</table>

In order to analyse the relative differences between males and females, I tested for enrichment of transcripts that demonstrate the greatest difference in fold change between male and female exposed plants (Table 5.4). The strength of regulation of anabolic primary metabolism pathways (light reactions and Kalvin cycle) as well as catabolic reactions (glycolysis and TCA cycle) is different in female compared to male exposed plants. Similarly, female exposed plants display stronger induction of secondary metabolism pathways such as synthesis of glucosinolates, phenylpropanoids and flavonoids (Table 5.4).
Taken together, I detected differences in plant transcriptional response to male and female insects, suggesting that different insect genders may elicit distinct responses in plants. RNA-seq data support a hypothesis that female insect attack may cause greater suppression of photosynthesis genes and stronger upregulation of secondary metabolism pathways compared to male leafhoppers. There might also be qualitative differences in regulation of cell wall composition in male and female exposed plants. Further metabolomic analysis could verify such hypotheses about plant responses to different insect genders.

Table 5.4. Enrichment of pathways that show the greatest differences between female and male exposed plants. Pathways are derived from MapMan functional annotation and ranked based on Benjamini-Hochberg correction for multiple pathway comparisons using Wilcoxon rank test. The number of elements in each pathway correspond to the total number of expressed genes within each functional bin.

<table>
<thead>
<tr>
<th>BinCode</th>
<th>Pathway description</th>
<th>elements in pathway</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Photosystem light reaction</td>
<td>133</td>
<td>8.742E-36</td>
</tr>
<tr>
<td>9</td>
<td>mitochondrial electron transport / ATP synthesis</td>
<td>109</td>
<td>4.841E-06</td>
</tr>
<tr>
<td>16.5</td>
<td>secondary metabolism of glucosinolates</td>
<td>54</td>
<td>2.104E-05</td>
</tr>
<tr>
<td>10.7</td>
<td>cell wall modification</td>
<td>41</td>
<td>4.200E-04</td>
</tr>
<tr>
<td>16.2</td>
<td>secondary metabolism of phenylpropanoids</td>
<td>60</td>
<td>2.730E-03</td>
</tr>
<tr>
<td>4</td>
<td>glycolysis</td>
<td>56</td>
<td>5.946E-03</td>
</tr>
<tr>
<td>10.8</td>
<td>cell wall pectin esterases</td>
<td>31</td>
<td>9.194E-03</td>
</tr>
<tr>
<td>2.2.2</td>
<td>starch degradation</td>
<td>26</td>
<td>1.252E-02</td>
</tr>
<tr>
<td>19</td>
<td>tetrapyrrole synthesis</td>
<td>41</td>
<td>1.690E-02</td>
</tr>
<tr>
<td>1.3</td>
<td>Kalvin cyle</td>
<td>34</td>
<td>2.167E-02</td>
</tr>
<tr>
<td>16.8</td>
<td>secondary metabolism of flavonoids</td>
<td>56</td>
<td>2.207E-02</td>
</tr>
<tr>
<td>5</td>
<td>fermentation</td>
<td>11</td>
<td>2.567E-02</td>
</tr>
<tr>
<td>10.2</td>
<td>cell wall cellulose synthesis</td>
<td>32</td>
<td>3.156E-02</td>
</tr>
<tr>
<td>8.1</td>
<td>TCA cycle</td>
<td>37</td>
<td>3.321E-02</td>
</tr>
<tr>
<td>16.10</td>
<td>secondary metabolism of phenols</td>
<td>5</td>
<td>4.185E-02</td>
</tr>
</tbody>
</table>
5.2.6. Plants differentially perceive male and female insects

The evidence for sex-specific insect effects on plant primary and secondary metabolism suggest that plants may perceive and distinguish between male and female insect attack. I aimed to determine whether *M. quadrilineatus* differentially regulates plant pattern recognition receptors and triggers signalling cascades characteristic to microbial pathogen recognition via PTI and ETI.

To investigate this, I compiled a list of all published genes involved in pathogen/herbivore perception and signal transduction, and visualised insect induced changes within various functional categories (Figure 5.16A). I found that leafhoppers upregulate several classes of membrane PRR as well as cytoplasmic receptors, including NBL receptors. I also highlighted the families of receptors that are significantly enriched in differentially regulated transcripts by either males or females (Figure 5.16B). Interestingly, SD, LRKL and L-lectin receptor families are significantly differentially expressed only in plants exposed to female insects (Figure 5.16A). In contrast, cytoplasmic receptors are significantly enriched by both sex insects (Figure 5.16A-B).

I also quantified the difference in fold change between male and female insects to determine which families of receptors show the greatest sex-specific responses. Interestingly, induction of cytoplasmic NBL and membrane SD and LRR receptors is significantly different between female and male treated plants (Table 5.5). There is a significantly large number of NBL transcripts that are induced in female-specific manner (Figure 5.16A). Among them are many members of TIR-domain containing TNL clade and CC_RPWS-domain containing CNL clade of NBL receptors which demonstrate 1.5 to 2 fold greater induction in females compared to males. Similarly, many LLR are regulated in female-specific manner (Table 5.5). In particular, type I LRR are upregulated approximately 4 to 16 fold stronger by female compared to male exposed plants. In contrast, type III LRR are induced 0.6 to 1 fold stronger by males compared to females. SD and L-lectin receptors are significantly upregulated by females but not males (Figure 5.15C), constituting a significant difference between female and male exposed plants (Table 5.5).

Taken this evidence together, the differences between males and females could be perceived by plant membrane and cytoplasmic receptors to trigger PTI and ETI responses.
Figure 5.16.
Figure 5.16. Males and females induce JA, SA and ET defence pathway through activation of PTI and ETI. Female (A) and male (B) induced defence responses are represented by functional categories of all upregulated (red) and downregulated (green) transcripts. Panel C summarises the statistical significance of all male and female altered defence pathways represented in panels A and B. DE gene enrichment analysis is based on Benjamini-Hochberg correction for multiple pathway comparisons using Wilcoxon rank test. P-Values <0.05 are shaded in C and highlighted in panels A and B.

Table 5.5. Enrichment of gene families that show the greatest differences in defence gene expression between female and male exposed plants. Groups are ranked based on Benjamini-Hochberg correction for multiple comparisons using Wilcoxon rank test. The number of elements in each pathway correspond to the total number of expressed genes within each functional group.

<table>
<thead>
<tr>
<th>Pathway description</th>
<th>elements in pathway</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLR</td>
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<td>1.00E-20</td>
</tr>
<tr>
<td>LRR</td>
<td>139</td>
<td>2.61E-11</td>
</tr>
<tr>
<td>SD</td>
<td>26</td>
<td>1.92E-07</td>
</tr>
<tr>
<td>DUF26</td>
<td>33</td>
<td>2.25E-06</td>
</tr>
<tr>
<td>WAKL</td>
<td>11</td>
<td>6.94E-06</td>
</tr>
<tr>
<td>CDPK</td>
<td>21</td>
<td>3.39E-05</td>
</tr>
<tr>
<td>L-lectin</td>
<td>21</td>
<td>3.62E-05</td>
</tr>
<tr>
<td>JA SIGNALLING</td>
<td>32</td>
<td>2.34E-04</td>
</tr>
<tr>
<td>MEKK</td>
<td>15</td>
<td>4.82E-04</td>
</tr>
<tr>
<td>RAF</td>
<td>37</td>
<td>5.35E-04</td>
</tr>
<tr>
<td>SA signalling</td>
<td>24</td>
<td>5.51E-04</td>
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<tr>
<td>LRKL</td>
<td>10</td>
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<tr>
<td>CrRLK</td>
<td>12</td>
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</tr>
<tr>
<td>JA BIOSINTHESIS</td>
<td>18</td>
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<tr>
<td>MAP4K</td>
<td>9</td>
<td>0.0181</td>
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<td>SnRK</td>
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<tr>
<td>RLCK</td>
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</table>
5.2.7. Leafhoppers induce plant hormone signalling through calcium dependent and mitogen activated kinases

Leafhoppers upregulate calcium-dependent protein kinases (CDPK) and MAP kinase cascade (Figure 5.16). CDPK and MEKK are significantly upregulated by females (Figure 5.16A) and demonstrate the greatest difference between females and males compared to other gene families involved in defence signal transduction (Table 5.5). Some of the male and female induced signals may converge via MAP2K which appear to be significantly upregulated in both males and females (Figure 5.16C) with little difference between insect sexes.

Given that there could be male and female specific regulation of membrane and cytoplasmic receptors and potentially sex-specific signal propagation via calcium-dependent or mitogen-activated protein kinases, I wanted to further investigate any sex-specific differences in plant hormone production and signalling. Both male and female leafhoppers showed enrichment of differentially regulated transcripts in ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) related pathways relative to other defence signalling components (Figure 5.16). Furthermore, there appear to be significant differences in insect induced SA, JA and ET signalling between male and female exposed plants (Table 5.5). To characterise which transcripts may contribute to such differences I arranged all plant hormone production and signalling associated transcripts based on their differential induction or suppression by leafhoppers (Table 5.6). Interestingly, most transcripts in SA, JA or ET-related signalling pathways are regulated in the same direction by both males and females. However, females show much stronger relative up- or down-regulation of majority of transcripts compared to males. Only female regulated transcripts are significantly induced or suppressed relative to no-insect exposed plants, suggesting more consistent and less variable response than male insects. The significant differences between plant defence hormone response to females and males (Table 5.5) can thus be largely explained by the relative strength of JA and SA pathway induction (Table 5.6). In contrast, ET signalling genes EIN2/4/5 and EIL3 appear to be up-regulated by females but suppressed by males, suggesting potential sex-specific regulation. EINs and EILs are known inducers of defence genes PDF1.2, PR3 and PR4 (HEL). Moreover, EIN2 has been implicated in mediating the cross-talk between SA-JA pathways by potentially upregulating SA and NRP1 dependent defence responses (Leon-Reyes et al., 2009).
Interestingly, lipoxygenase LOX1, which is involved in jasmonate synthesis, is down-regulated in males but upregulated by females, while other lipoxygenases LOX2/3/4 also show stronger induction by females compared to weaker upregulation by males. Together, this suggests that males and females induce SA and JA pathways at different strength but ET pathway may be induced specifically by females.

Table 5.6. Transcriptional regulation of production and signalling of SA, JA and ET pathways by male and female leafhoppers in 35S:GFP plants compared to no-insect treated 35S:GFP plants. Table displays log2 fold change of gene expression in insect exposed compared to insect non-exposed plants. Genes are ranked based on the difference between female and male exposed plants. Significantly up- or down-regulated transcripts are bold (DEseq of 4 biological replicates; p<0.05; q<0.05).

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**ETHYLENE PRODUCTION**

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To validate the findings from RNA-seq, expression of several marker genes for JA, SA and ET signalling pathways was measured by rt-qPCR (Figure 5.17). These genes were selected independently from the results revealed by RNA-seq experiment. The transcriptional changes in defence gene signalling are likely to be an integrated measure of the upstream response to insect-specific stimuli (e.g., receptors in Figure 5.16A) and downstream hormonal cross-talk (Figure 5.17A) (Pieterse et al., 2012). For example, the ET response factor ERF1 is activating PDF1.2 and PR4 (HEL) while simultaneously suppressing VSP2 and LOX2. In contrast, JA response factors MYC2 and ORA37 positively regulate VSP2 and LOX2 but suppress PDF1.2 and HEL. Therefore, focus on individual marker genes may not be a measure of independent variables but rather reflect an inter-correlated network of transcriptional regulation and cross-talk. qPCR data indicate that both male and female insects induce SA, JA and ET marker genes, although there is large variation among biological replicates (Figure 5.17B-F). PR1 was upregulated by both sex insects (Figure 5.17B). JA-induced LOX2 expression in female exposed plants was significantly increased compared to no-insect treatment (Figure 5.17F). VSP2 was significantly upregulated by male leafhoppers (Figure 5.17E). PR4 showed significant induction by female leafhoppers and was also strongly upregulated by males (Figure 5.17D), while PDF1.2 was not induced by females and showed slight induction by males (Figure 5.17C).

In agreement with RNA-seq data, rt-qPCR data suggest that ET, JA, SA pathways are upregulated by male and female insects alike. However, RNA-seq data indicate that many defence genes are upregulated stronger in female treated plants compared to slightly lower induction by males. For example, JA-response genes MYC2 and ORA37 are more strongly upregulated by females than males (Table 5.6). Similarly, JA responsive genes ERF1 and ORA59 (positive regulators of PDF1.2 and HEL), are also more strongly upregulated by females compared to males (Table 5.6). Therefore, the interpretation of insect-sex specific plant signalling responses may depend on the functional delimitation of each signalling pathway and the effects of hormonal cross-talk.
Figure 5.17. Plant exposure to male and female *M. quadrilineatus* upregulate marker genes of SA response (*PR1*), and JA (*VSP2, LOX2*) and ET response (*HEL, PDF1.2*). (A) Herbivory induced JA pathway consist of two branches – MYC activated VSP2 branch and ERF1/ORA59 activated PDF1.2 branch. ET and SA signals engage in a complex cross-talk with each other and the JA signalling (Pieterse *et al.*, 2012). Pointed arrows indicate positive regulation but block arrows – negative. Solid lines indicate direct regulation; dashed lines - indirect downstream response. (B-F) Gene expression (rt-qPCR) measurements for selected JA, SA and ET marker genes in no-insect (X), male
(M) and female (F) exposed 3S:GFP-SAP54 and 3S:GFP plants. Different letters above the bars indicate treatments that are significantly different (Tukey test; p<0.05) in any pairwise comparison within each panel. Bars are one standard deviation from the mean. Expression measured in 3 independent experiments 48h after exposure to insects in identical setup as the RNA-seq experiment. This work is a contribution from Gatsby summer student Hannah Smith (University of Manchester), supervised by Z. Orlovskis.

5.2.8. *M. quadrilineatus* alters regulation of plant secondary metabolism in sex-specific manner

Given the involvement of JA, SA and ET signals in insect-induced defence responses, I decided to characterise the transcriptional changes relating to secondary metabolite biosynthesis pathways that may be the downstream the hormonal defence signals. I visualised the differentially regulated secondary metabolism genes in male and female exposed 3S:GFP plants and found induction of several pathways (Figure 5.18). The most enriched pathways with insect regulated transcripts relate to phenylpropanoid, carotenoid and anthocyanin synthesis (Table 5.7).

### Table 5.7. Enrichment of secondary metabolism pathways that show the greatest differences in female and male exposed 3S:GFP plants compared to insect non-exposed 3S:GFP plants.

Groups are ranked based on Benjamini-Hochberg correction for multiple comparisons using Wilcoxon rank test. The number of elements in each pathway correspond to the total number of expressed genes within each functional group. Significant enrichment is highlighted in bold.

<table>
<thead>
<tr>
<th>bin</th>
<th>pathway name</th>
<th>elements</th>
<th>p-value (male)</th>
<th>p-value (female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.1.4</td>
<td>isoprenoids; carotenoids</td>
<td>14</td>
<td>9.80E-03</td>
<td>6.27E-03</td>
</tr>
<tr>
<td>16.8.1</td>
<td>flavonoids; anthocyanins</td>
<td>16</td>
<td>1.79E-02</td>
<td>9.44E-03</td>
</tr>
<tr>
<td>16.2</td>
<td>phenylpropanoids</td>
<td>60</td>
<td>7.26E-02</td>
<td>1.74E-03</td>
</tr>
<tr>
<td>16.8.5</td>
<td>flavonoids; isoflavonols</td>
<td>7</td>
<td>3.20E-01</td>
<td>1.12E-01</td>
</tr>
<tr>
<td>16.1.5</td>
<td>isoprenoids; terpenoids</td>
<td>9</td>
<td>4.17E-01</td>
<td>1.12E-01</td>
</tr>
<tr>
<td>16.5.1</td>
<td>sulfur-containing glucosinolates</td>
<td>50</td>
<td>4.31E-01</td>
<td>3.35E-02</td>
</tr>
<tr>
<td>16.10</td>
<td>simple phenols</td>
<td>5</td>
<td>4.39E-01</td>
<td>1.05E-02</td>
</tr>
</tbody>
</table>
Figure 5.18. Enrichment of the secondary metabolism pathways with female (A) and male (B) regulated transcripts. Genes are coloured based on log₂(fold change) when compared insect exposed plants to no insect exposed controls. Red indicates upregulated transcripts and green – downregulated transcripts.

The parts of the phenylpropanoid pathway affected by insects are involved in lignin biosynthesis and may reflect remodelling of cell wall composition as a direct mechanical defence to insect feeding and egg-laying. Moreover, since amino acids tyrosine and phenylalanine are involved in the first steps of phenylpropanoid-derived lignin synthesis, these results confirm the observed changes in upregulation of several modules for amino-acid metabolism (Figure
Another important branch of phenylalanine and phenylpropanoid metabolism is the synthesis of salicylic acid (SA), which I found to be transcriptionally upregulated by both male and female insects (Figures 5.16 and 5.17). MapMan analysis reveal that several methyl-transferases are upregulated by males and females equally, suggesting upregulation of transcripts involved in volatile methyl-salicylate production (Figure 5.19).

Interestingly, the carotenoid pathway, especially synthesis of lycopene and its volatile derivatives, is downregulated by both male and female insects (Figure 5.19). Carotenoids are conjugated tetraterpenoids (isoprenoids) that protect plant photosynthetic machinery from oxidative stress like ROS as well as function in light absorption. Decrease in carotenoid levels could, therefore, be associated with general downregulation of photosynthetic processes by insects. In addition, carotenoids are precursors of ABA synthesis. ABA biosynthetic pathway is not significantly altered by male (p=0.48) or female (p=0.28) insects.

Figure 5.19. Enrichment of the phenylpropanoid (green) and carotenoid (orange) pathways with female (A) and male (B) regulated transcripts. Genes are coloured based on log₂(fold change) when compared insect exposed plants to no insect exposed controls. Red indicates upregulated transcripts and green – downregulated transcripts. Synthesis of cummarate and its derivatives are the first steps in synthesis of diverse range of phenylpropanoids. Methyl-SA is derived from SA and functions as herbivore-induced volatile signal. Carotenoid pathway depicts synthesis of lycopene and its volatile derivatives.
In contrast to suppression of carotenoid pathway, most oxidoreductases and transferases involved in anthocyanin anabolism are upregulated in both females and males (Table 5.7). Anthocyanins are flavonoid compounds that accumulate in response to various biotic and abiotic stresses (Nicholson and Hammerschmidt, 1992) and can be upregulated by JA (Li et al., 2014). Anthocyanins are implicated to function as deterring signal to foliar herbivores (Gould, 2004), although detailed mechanistic biochemical analyses on their defence role in insect feeding are absent. It could be that anthocyanin accumulation is a secondary side-effect of JA signalling without a direct role in protection from herbivore attack. Interestingly, phytoplasma infection also change leaf pigmentation reminiscent of anthocyanin accumulation (Orlovskis et al., 2017), suggesting that this may be a general response to biotic stress.

When analysing only the transcripts that show significant differential expression in male and female treated plants compared to non-exposed controls, the terpenoid pathway (includes carotenoid synthesis) (p=0.0268) appears to be significantly upregulated in females, whereas no secondary metabolism pathways are enriched by significantly differentially regulated transcripts in male treated plants. This suggests that insect colonisation may induce a plethora of subtle changes in secondary metabolism pathways that are better described by the cumulative effect of all transcripts rather than only the significantly regulated transcripts. Nevertheless, relative to other elements of secondary metabolism, terpenoid pathway shows the greatest change (p=0.12) in significantly differentially regulated genes in male treated plants as well. In addition, monoterpenoid pathway displays significant enrichment with upregulated transcripts in both male and female exposed plants by IGA analysis (Table 5.3). Terpenoids are diverse class of secondary metabolites. They include many herbivore induced volatiles (HIV) such as isoprene (C\textsubscript{5}), monoterpenes (C\textsubscript{10}) and sesquiterpenes (C\textsubscript{15}) which function in attraction of herbivore natural enemies like parasitoids (Mumm et al., 2008).

Having looked at male and female elicited responses separately, I calculated the relative difference in fold-change between males and females in order to characterise secondary metabolite pathways that are most different between male and female treated plants. Interestingly, the greatest difference between male and female exposed plants can be observed in glucosinolate synthesis and degradation (p=5.8E-6) as well as phenylpropanoid (p=0.0027)
and terpenoid ($p=0.0069$) pathways. The individual transcripts that could contribute to the male-female difference in phenylpropanoid and terpenoid pathways is discussed in figure 5.19. I further analyse transcripts in glucosinolate pathway which are most different between male and female exposed 35S:GFP plants. Interestingly, transcription factor *MYB34* (AT5G60890), known inducer of indole glucosinolate synthesis, is significantly upregulated by both male and female insects but only female exposed plants demonstrate significant induction of monooxygenases and sulfotransferases required for synthesis of indole glucosinolates (Table 5.8). In contrast, transcription factor *MYB28* (AT5G61420), an inducer of the aliphatic glucosinolate pathway, is significantly suppressed only by males. This correlates with downregulation of CYP79 family P450 and several other transcripts required for aliphatic glucosinolate synthesis in male but not female exposed plants (Table 5.8).

Taken together, *M. quadrilineatus* males and females appear to transcriptionally induce phenylpropanoid (incl. lignins), flavonoid (anthocyanin) and terpenoid (incl. carotenoids) biosynthesis. However, these findings would require further verification by metabolic analysis. Based on comparison of plant transcriptional response to male and female insects, I hypothesise that the greatest difference between male and female insects could be stronger induction of indole glucosinolate pathway and stronger lignification of cell walls (as part of the phenylpropanoid pathway) in female compared to male exposed 35S:GFP plants.
Table 5.8. Transcriptional regulation of glucosinolate synthesis and degradation by male and female leafhoppers compared to no-insect treated 35S:GFP plants. Changes in gene expression are expressed as log2(fold change compared to insect non-treated 35S:GFP plants).

<table>
<thead>
<tr>
<th>Id</th>
<th>Description</th>
<th>Known and Putative Function</th>
<th>Log2(fold change) Male vs No Insect (35S:GFP)</th>
<th>Log2(fold change) Female vs No Insect (35S:GFP)</th>
<th>Log2(fold change) Male vs Female (35S:GFP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G12140</td>
<td>CYP79F1; (CYTOCHROME P450 FAMILY 79 SUBFAMILY F MEMBERS); oxidoreductase</td>
<td>synthesis</td>
<td>1.7279567</td>
<td>0.53767973</td>
<td>1.2012322</td>
</tr>
<tr>
<td>AT1G16400</td>
<td>CYP79F2; oxidoreductase</td>
<td>synthesis</td>
<td>0.5202076</td>
<td>0.82240066</td>
<td>1.2023122</td>
</tr>
<tr>
<td>AT1G64400</td>
<td>CYP79F2; (CYTOCHROME P450 FAMILY 79 SUBFAMILY F MEMBERS); oxidoreductase</td>
<td>synthesis</td>
<td>0.1083005</td>
<td>0.14608237</td>
<td>0.9542342</td>
</tr>
<tr>
<td>AT2G25270</td>
<td>FMO GS-OX1 (FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 1);</td>
<td>synthesis</td>
<td>0.61784907</td>
<td>0.55947804</td>
<td>0.7570217</td>
</tr>
<tr>
<td>AT2G25680</td>
<td>CYP79F3; oxidoreductase</td>
<td>synthesis</td>
<td>0.5660541</td>
<td>0.2409004</td>
<td>0.9454232</td>
</tr>
<tr>
<td>AT3G59600</td>
<td>CYP79F4; (CYTOCHROME P450 FAMILY 79 SUBFAMILY F MEMBERS); oxidoreductase</td>
<td>synthesis</td>
<td>0.5432867</td>
<td>0.6570217</td>
<td>1.2012322</td>
</tr>
<tr>
<td>AT4G22310</td>
<td>CYP79F5; oxidoreductase</td>
<td>synthesis</td>
<td>0.5432867</td>
<td>0.6570217</td>
<td>1.2012322</td>
</tr>
<tr>
<td>AT5G44300</td>
<td>CYP79F6; oxidoreductase</td>
<td>synthesis</td>
<td>0.1083005</td>
<td>0.14608237</td>
<td>0.9542342</td>
</tr>
<tr>
<td>AT2G25270</td>
<td>FMO GS-OX1 (FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 1);</td>
<td>synthesis</td>
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<td>0.55947804</td>
<td>0.7570217</td>
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<td>synthesis</td>
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<td>0.2409004</td>
<td>0.9454232</td>
</tr>
<tr>
<td>AT3G59600</td>
<td>CYP79F4; (CYTOCHROME P450 FAMILY 79 SUBFAMILY F MEMBERS); oxidoreductase</td>
<td>synthesis</td>
<td>0.5432867</td>
<td>0.6570217</td>
<td>1.2012322</td>
</tr>
<tr>
<td>AT4G22310</td>
<td>CYP79F5; oxidoreductase</td>
<td>synthesis</td>
<td>0.5432867</td>
<td>0.6570217</td>
<td>1.2012322</td>
</tr>
<tr>
<td>AT5G44300</td>
<td>CYP79F6; oxidoreductase</td>
<td>synthesis</td>
<td>0.1083005</td>
<td>0.14608237</td>
<td>0.9542342</td>
</tr>
</tbody>
</table>
5.2.9. Insect induced plant stress responses are central in multi-functional gene network

RNA-seq results in section 5.2.5 suggested that biotic stress related pathways are among the strongest plant responses to insects in addition to insect effect on primary metabolic processes like photosynthesis. Why so many different plant processes are altered at the same time by insect exposure? Regulation of plant defence, growth and nutrition can be linked in a regulatory gene or protein interaction network. To assess the extent to which defence related genes are wired up to other plant processes, I took all expressed genes from the RNA-seq dataset (Figure 5.13) and analysed their interaction network using all publically available protein-protein and transcription factor-target gene interactions from STRING and JASPAR databases respectively (for details see materials and methods section) to generate network of all expressed genes in my RNA-seq dataset (red circle Figure 5.20).

Within this network, I highlighted the total interactome (green circle Figure 5.20) of all defence genes annotated in Figure 5.16 to look at the functional enrichment of all genes that are directly interacting with genes encoding receptors, signalling cascades and phytohormone signals (Figure 5.21). I found the 693 annotated and expressed defence genes are well connected with nodes that have diverse biological functions in primary and secondary metabolism. Protein modifications, immune response and developmental regulation are examples of biological functions that are significantly enriched with defence gene interactors (Figure 5.21).

I found that the sub-network including all defence genes and their first-level interactors (green circle Figure 5.20) has an average of 10 connections to other expressed genes, half of which are interactions between defence genes with each other (network analysis, Cytoscape). This connectivity is similar to the mean number of edges (interactions) per each node within the network of all expressed transcripts (red circle Figure 5.20), suggesting that any similar size random list of genes could produce a network with similar connectivity. Therefore, it could have been difficult to isolate a hypothetical network of defence gene interactors based on network topology alone. I found that many biological functions that are enriched with interactors from defence-related nodes (Figure 5.21) are also part of plant transcriptional response to insect attack (Table 5.1). This suggests that, given the co-expression of the neighbouring (interacting)
genes in the network, perception of insect attack and downstream signalling cascade may affect a variety of other biological processes in addition to activating plant defence. Hence, the properties (topology) of gene regulatory and protein-protein interaction network may underlie the trade-offs between growth and defence (Figure 5.1) and confound the multitude of changes that are observed in plant response to insects (Figure 5.15).

Figure 5.20. Schematic diagram describing the interaction network of *A.thaliana* defence genes. I annotated 1082 genes (blue circle) relating to the defence signalling from membrane and cytoplasmic receptors to kinases and SA, JA, ET signalling (displayed in Figure 5.16). 693 of these genes are expressed in the RNA-seq dataset and have direct interaction with each other or with any other expressed gene, constituting a network with 16 763 interactions or edges (green circle). 15 430 or 89% out of the total of 17 153 expressed genes have identifiable interactions, comprising a network of 183 835 interactions (red circle). The defence genes interact with each other (green + blue interaction) and with other genes of various functions (red + green intersection). The defence genes (4.4% of total expressed network nodes) constitute approximately 9.1% of all interactions within the total interaction network of expressed genes (red circle), indicating considerable inter-connectivity or centrality.
**Figure 5.21.** Visualisation of the biological function enrichment within the interaction network of all defence-related genes and their direct (first level) interactors. Genes are assigned biological function based on GO terms and clustered according on their function and network topology using BINGO enrichment tool within Cytoscape network visualisation package. Enrichment analysis is done by hypergeometric test and Bejamini-Hochberg (BH) False Discovery rate correction. Two pathways relating to defence signalling and developmental regulations are highlighted to demonstrate the broad biological context of the interactors of the 693 gene set of selected receptors, kinases and signalling pathways. For clarity, enrichment of only few biological functions is displayed in the table.

**5.2.10. Plant perception of male and female insects is altered on SAP54 plants**

Upon exposure to male or female leafhoppers, plants differentially regulate membrane and cytoplasmic receptors (Table 5.5), which may be directly involved in insect sex-dependent recognition. These results suggest that plants could potentially recognise the difference between female or male insect presence and activity. I, therefore, wanted to investigate whether SAP54 may change the male and female differentially induced plant responses and identify functional groups.
of genes that do not any longer show the observed difference between male and female insect induced responses in 35S:GFP-SAP54 plants.

To do this, I took the fold change for each transcript in response to male (and, separately, female) insects (relative to no-insect treatment) on 35S:GFP and 35S:GFP-SAP54 plants. Then I calculated the difference between male (and, separately, female) effect in 35S:GFP and 35S:GFP-SAP54 plants. Finally, I calculated the difference between male and female differentially induced responses in 35S:GFP and 35S:GFP-SAP54 plants. I investigated which plant responses are enriched in this parameter. Surprisingly, I found that SAP54 has the greatest effect on the difference in how male and female insects affect expression of plant signalling genes, especially receptor kinases (Table 5.9; Analysis 1). This suggests that SAP54 may alter how plants perceive male and female leafhoppers. Moreover, there are significant differences between male and female effects on transcriptional regulation, protein translation and basal metabolism on 35S:GFP-SAP54 plants compared to 35S:GFP plants (Table 5.9; Analysis 1). Because many members of plant receptor families are wired up in a dense network of genes with diverse range of functions from defence to development (Figure 5.21), 35S:GFP-SAP54 plants may demonstrate different responses in many biological functions to male and female insects compared to 35S:GFP plants.

To characterise which receptors are most affected, I performed similar enrichment analysis specifically for all defence signalling genes manually annotated in Figure 5.16 and found that leucine-rich repeat receptors (LLRs, especially subfamily III and XI), nucleotide binding LLR receptors (NLRs) as well as MAP3K (RAF sub-family) demonstrate the greatest sex-specific effects of SAP54 (Table 5.9; Analysis 2). In addition, male and female induced changes in phytohormone (SA, JA, ET) biosynthesis are significantly different on 35S:GFP-SAP54 plants compared to 35S:GFP plants (Table 5.9; Analysis 2).

This provides strong case for further in-depth comparative analysis of male and female effects on defence signalling and potential downstream responses of secondary metabolites in 35S:GFP-SAP54 and 35S:GFP plants.
Table 5.9. Functional enrichment analysis of transcripts that demonstrate SAP54 dependent changes in the difference between female and male induced plant responses. Analysis 1 describes the enrichment of all *A.thaliana* pathways (17153 expressed genes). Analysis 2 was performed independent of analysis 1 and specifically describes the SAP54 effects on female-male difference in manually curated defence signalling pathway (693 expressed genes). Both analysis are based on Benjamini-Hochberg correction for multiple pathway comparisons using Wilcoxon rank test and are ranked according to significance.

<table>
<thead>
<tr>
<th>Bin Code</th>
<th>Bin Name</th>
<th>Number of genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis 1: Enrichment of all functional pathways</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>signalling</td>
<td>872</td>
<td>&lt;1E-100</td>
</tr>
<tr>
<td>30.2</td>
<td>signalling via receptor kinases</td>
<td>294</td>
<td>&lt;1E-100</td>
</tr>
<tr>
<td>29.2.1</td>
<td>protein.synthesis.ribosomal protein</td>
<td>361</td>
<td>1.73E-71</td>
</tr>
<tr>
<td>29.2</td>
<td>protein.synthesis</td>
<td>511</td>
<td>1.12E-49</td>
</tr>
<tr>
<td>27.3</td>
<td>RNA.regulation of transcription</td>
<td>1451</td>
<td>1.62E-09</td>
</tr>
<tr>
<td>29</td>
<td>mitochondrial electron transport / ATP synthesis</td>
<td>109</td>
<td>2.65E-09</td>
</tr>
<tr>
<td>28.1</td>
<td>DNA.synthesis/chromatin structure</td>
<td>262</td>
<td>1.96E-07</td>
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<tr>
<td>31.1</td>
<td>cell organisation</td>
<td>257</td>
<td>3.42E-07</td>
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<td>27.3.44</td>
<td>RNA.regulation of transcription.Chromatin Remodeling Factors</td>
<td>24</td>
<td>3.70E-07</td>
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<td>20.2.1</td>
<td>stress abiotic heat</td>
<td>141</td>
<td>1.30E-05</td>
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<tr>
<td>30.2.3</td>
<td>signalling via receptor kinases (leucine rich repeat III)</td>
<td>27</td>
<td>1.88E-05</td>
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<tr>
<td>30.11</td>
<td>signalling (light reactions)</td>
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<td>2.07E-05</td>
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<tr>
<td>31.2</td>
<td>cell division</td>
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<td>4.28E-05</td>
</tr>
<tr>
<td>34.16</td>
<td>ABC transporters and multidrug resistance systems</td>
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<tr>
<td>29.5.1</td>
<td>protein degradation (subtilases)</td>
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<td>1.70E-04</td>
</tr>
<tr>
<td>9.7</td>
<td>mitochondrial electron transport / ATP synthesis.cytochrome c oxide</td>
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<td>2.25E-04</td>
</tr>
<tr>
<td>30.2.11</td>
<td>signalling via receptor kinases (leucine rich repeat XI)</td>
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<td>2.46E-04</td>
</tr>
<tr>
<td>29.4</td>
<td>protein posttranslational modification</td>
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<td>2.48E-04</td>
</tr>
<tr>
<td>27.1.2</td>
<td>RNA.processing (helicases)</td>
<td>32</td>
<td>2.73E-04</td>
</tr>
<tr>
<td>2</td>
<td>major Carbohydrate metabolism</td>
<td>77</td>
<td>3.44E-04</td>
</tr>
<tr>
<td>29.3.4.99</td>
<td>protein targeting (secretion)</td>
<td>48</td>
<td>5.69E-04</td>
</tr>
</tbody>
</table>

| Analysis 2: Enrichment of defence signaling transcripts |
| manual curation | LRR | 139 | <1E-20 |
| manual curation | NLR | 85 | <1E-20 |
| manual curation | RAF | 37 | <1E-20 |
| manual curation | DUF26 | 33 | 0.0006 |
| manual curation | WAKL | 11 | 0.0083 |
| manual curation | ET BIOSYNTHESIS | 5 | 0.0129 |
| manual curation | SD | 26 | 0.0149 |
| manual curation | CRLK | 12 | 0.0149 |
| manual curation | MEKK | 15 | 0.0163 |
| manual curation | extensin | 5 | 0.0203 |
| manual curation | MAP4K | 9 | 0.0210 |
| manual curation | SA synthesis | 12 | 0.0272 |
| manual curation | JA BIOSYNTHESIS | 18 | 0.0318 |
| manual curation | CDPK | 21 | 0.0510 |
| manual curation | ET SIGNALLING | 15 | 0.0566 |
| manual curation | PERKL | 5 | 0.0995 |
| manual curation | ZIK | 9 | 0.1072 |
| manual curation | NB | 14 | 0.1628 |
| manual curation | CRAL | 4 | 0.1630 |
| manual curation | OTHER RLK | 14 | 0.1879 |
| manual curation | RLCK | 72 | 0.2131 |
| manual curation | JA SIGNALLING | 32 | 0.4997 |
| manual curation | CRK | 7 | 0.6555 |
| manual curation | LRKL | 10 | 0.7036 |
| manual curation | MAPK | 18 | 0.7496 |
| manual curation | SHRK | 30 | 0.7731 |
| manual curation | L.lectin | 21 | 0.7787 |
| manual curation | SA signalling | 24 | 0.9872 |
| manual curation | MAP2K | 6 | 0.9882 |
5.2.11. Phytoplasma effector SAP54 alters male leafhopper induced plant defence signals

Since female leafhoppers demonstrate preference for male-colonised 35S:GFP-SAP54 plants (Chapter 4), and the specificity of male and female induced defence responses is altered on 35S:GFP-SAP54 compared to control plants, I hypothesise that insect induced plant defence signals may be changed by SAP54 in insect sex-specific manner.

In order to evaluate this hypothesis and tease apart which elements of insect-induced plant responses are modulated by SAP54, I analysed the transcriptional changes in defence signalling of male and female exposed 35S:GFP-SAP54 plants and compared to 35S:GFP plants. Intriguingly, SAP54 appears to suppress male-induced defence signalling (Figure 5.22B) but not female-induced defence responses (Figure 5.22A). Most of the female-induced transcripts in 35S:GFP plants remain upregulated in female exposed 35S:GFP-SAP54 plants. In contrast, male-exposed plants show considerable downregulation of cytoplasmic NBLs proteins, membrane PRRs as well as MAP and Ca-dependent kinase cascades in SAP54 plants. Surprisingly, even though the effect of SAP54 on the expression of different functional groups of defence signalling genes in female treated plants is barely noticeable, the families that demonstrate the greatest change relative to other categories of defence genes between SAP54-expressing and non-expressing plants within male or female treatments are similar (Figure 5.22C). These include NLR, LRR, SD and L-lectin receptor families which are more upregulated in female-, compared to male-, exposed 35S:GFP plants. These receptors are largely downregulated in male-exposed 35S:GFP-SAP54 plants but are changing only slightly in female-exposed 35S:GFP-SAP54. This suggests that the degree of suppression of plant pattern recognition receptors may be highly insect sex dependent. However, this would require further validation with RNA-seq independent methods.

Dramatic downregulation of receptors as well as defence signalling through kinases can be observed only in male-exposed plants. Kinases in MEKK and CDPK families were significantly induced by female but not male insects in 35S:GFP plants (Figure 5.16C). Interestingly, particular CDPK transcripts, for example, CDPK29, which are primary induced by both male and female insects in 35S:GFP plants, are downregulated only in male-exposed 35S:GF-SAP54 plants. In addition, many MAP3K, that were not induced by insects in 35S:GFP
plants, are downregulated in male colonised SAP54 plants as well (Figure 5.22B). This suggests that SAP54 may modulate both insect induced and non-induced plant defence signals predominantly in male-dependent manner.

Figure 5.22. (continued next page)
### Functional groups significantly altered by SAP54

<table>
<thead>
<tr>
<th>Functional group</th>
<th>Nr of expressed genes</th>
<th>p-value (female)</th>
<th>p-value (male)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLR</td>
<td>85</td>
<td>1.27E-13</td>
<td>8.22E-21</td>
</tr>
<tr>
<td>SD</td>
<td>26</td>
<td>7.78E-06</td>
<td>4.32E-06</td>
</tr>
<tr>
<td>LRR</td>
<td>139</td>
<td>2.81E-05</td>
<td>3.61E-16</td>
</tr>
<tr>
<td>RAF</td>
<td>37</td>
<td>3.56E-05</td>
<td>1.40E-06</td>
</tr>
<tr>
<td>CDPK</td>
<td>21</td>
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Figure 5.22. The effect of SAP54 on female (A) and male (B) leafhopper induced plant defence signalling responses. For each panel insect treated plants are compared to no-insect exposed control to characterise differentially expressed transcripts. Insect induced responses are compared between SAP54 non-expressing plants (left) and SAP54 expressing plants (right). Colour scale indicates log2(fold change). Panel C summarises the overrepresentation of SAP54-altered expression of different functional groups in the defence pathway by taking the difference in fold change between female induced responses in SAP54 expressing plants (right) and female induced responses in SAP54 non-expressing plants (left). The enrichment analysis is based on Benjamini-Hochberg correction for multiple pathway comparisons using Wilcoxon rank test. P-Values <0.05 are shaded in C and highlighted on the right-hand side in panels A and B.

Interestingly, SA or JA synthesis and signalling are not significantly altered in female exposed SAP54 compared to the control plants. Nevertheless, transcripts in ET signalling show significantly different induction by females in 35S:GFP-SAP54 compared to 35S:GFP plants (Figure 5.22A,C). While SA pathway is not significantly altered by males, the transcripts involved in both JA synthesis and ET signal transduction are significantly changed in male exposed SAP54 plants compared to GFP controls (Figure 5.22B,C). The alteration of both female and male induced ET signalling related transcripts in SAP54 plants may explain the significant effects of SAP54 on the difference between male and female induced ET synthesis and signalling (Table 5.9; Analysis 2).

This prompted me to further investigate which particular transcripts may best demonstrate sex-specific induced responses to leafhoppers in 35S:GFP-SAP54 plants. In Table 5.10 I compare regulation of all SA, JA and ET synthesis and response genes in male and female exposed plants. ET signalling pathway demonstrates most dramatic male-specific regulation in 35S:GFP-SAP54 plants relative to JA or SA pathways. Ethylene response factors EIN2/5 and EIL3 are significantly downregulated only in male-exposed SAP54 plants. Moreover, several other transcripts (e.g., EIM3/5, CTR1, ETR1, EIL1) show greater downregulation in male-exposed 35S:GFP-SAP54 plants compared to male-treated 35S:GFP plants or any of the female treatments (Table 5.10).

Majority of male and female induced SA synthesis and signalling genes in 35S:GFP plants remain induced in 35S:GFP-SAP54 plants. Only SA synthesis
gene EDS1 and SA response factor TGA6 are downregulated (yet not significantly) in male-exposed SAP54 plants. The expression of PR1 was independently confirmed in insect exposed SAP54 plants by rt-qPCR. Similar to RNA-seq data, PR1 transcripts did not display significant change in male or female treated SAP54 plants compared to insect exposed GFP controls (Figure 5.17B).

JA synthesis genes LOX1, MFP1 are significantly suppressed in both female and male treated 35S:GFP-SAP54 plants compared to 35S:GFP controls. While several other JA synthesis genes (e.g., LOX2/3, OPR2/3, ACX1, FAD7) and jasmonate-amido synthetase JAR1 are significantly upregulated in female exposed SAP54 and control plants, these transcripts are not significantly regulated in male exposed SAP54 or control plants (Table 5.10). This further suggests that strength of induction of JA synthesis transcripts may be sex-specific but SAP54 may not alter these responses. Furthermore, female and male regulated expression of JA signalling transcripts is very similar in both 35S:GFP and 35S:GFP-SAP54 plants. JA signalling genes largely demonstrate the same fold change in response to female and male insects compared to no-insect treatments in 35S:GFP and 35S:GFP-SAP54 plants. Nevertheless, male-induced transcripts in 35S:GFP-SAP54 are significantly upregulated compared to non-significant but similar fold induction in 35S:GFP, suggesting less variable 35S:GFP-SAP54 plant response to males across the 4 biological replicates compared to male response in GFP plants. Independent qPCR measurements confirm RNA-seq data and display no significant effect of SAP54 on male or female induced expression of VSP1 (Figure 5.17E). However, the marker gene for other JA signalling branch – PDF1.2 - was significantly upregulated only in male exposed SAP54 plants compared to GFP controls (Figure 5.17C). In contrast, the expression of PDF1.2 in female exposed SAP54 was not significantly changed compared to GFP plants (Figure 5.17C).

Regulation of PDF1.2 is partially dependent on the ET pathway. Hence, sex-specific transcriptional changes in PDF1.2 in SAP54 plants are consistent with the marked downregulation of several ethylene response factors in male exposed SAP54 plants compared to any other treatment (Table 5.10). In addition, such effect may also be mediated by ET biosynthesis genes like CEV1 (CONSTITUTIVE EXPRESSION OF VSP1), which is downregulated in male exposed 35S:GFP-SAP54 plants. CEV1, also known as CESA3, is a cellulose
synthase gene and a negative regulator of JA and ET synthesis (Ellis et al., 2002). For example, PDF1.2 expression is increased in cev1 mutant (Ellis et al., 2002). Similarly, another example of only male downregulated transcript in 35S:GFP-SAP54 plants, is a chloroplast protein MFP1 (AT3G16000) which may influence the distribution of plastid nucleoid-associated transcription factors such as JASMONATE- AND ETHYLENE-RESPONSIVE FACTOR3 (JERF3) (Melonek et al., 2012).

Taken together, male leafhoppers significantly downregulate many families of plant receptors as well as kinases, involved in defence signal transduction, and ethylene signalling in 35S:GFP-SAP54 plants compared to female exposed plants. This supports a hypothesis that phytoplasma effector SAP54 may modulate plant (defence) response to insects in sex-specific manner.

### Table 5.10. Transcriptional regulation of production and signalling of salicylic acid, jasmonic acid and ethylene pathways by male and female leafhoppers in 35S:GFP and 35S:GFP-SAP54 plants compared to no-insect treated controls.

Changes in gene expression are expressed as log2(fold change compared to insect non-exposed plants) and colour coded based on magnitude of fold change. All significantly differentially regulated transcripts (DEseq; p<0.05; q<0.05) are bold. Genes within each functional category are ranked based on the difference between female induced responses in 35S:GFP and male exposed 35S:GFP-SAP54 plants.

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SA production
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5.2.12. SAP54 suppresses glucosinolate pathway in both female and male exposed plants

Manipulation of insect induced defence responses in SAP54-expressing plants prompted to further investigate if there are any sex-specific leafhopper effects on plant secondary metabolite production in 35S:GFP-SAP54 compared to 35S:GFP plants. Interestingly, female triggered plant responses are largely very similar between SAP54 and control plants (Figure 5.23A), whereas male insects downregulate several transcripts in isoprenoid (non-mevalonic acid)

| AT1G19180 | JAZ1 | 3.3158224 | 1.992522 | 2.0527022 | 1.0722562 | 2.2435662 |
| AT1G30135 | JAZ8 | 5.6011035 | 4.181439 | 4.965972 | 3.633433 | 1.9676975 |
| AT1G17380 | JAZ5 | 4.1672425 | 3.3774276 | 3.1219172 | 2.282001 | 1.8852415 |
| AT5G24770 | VSP2 | 3.4108935 | 3.592643 | 3.4761329 | 2.6144319 | 1.6964616 |
| AT5G24780 | VSP1 | 6.312277 | 5.941676 | 5.2881503 | 4.6462083 | 1.6657072 |
| AT1G32640 | MYC2 | 1.9626862 | 1.3016942 | 1.3749169 | 0.6179589 | 1.3447273 |
| AT5G22570 | WRKY38 | 3.595954 | 2.8239894 | 3.6389165 | 2.7153137 | 1.2446403 |
| AT5G13220 | JAZ10 | 6.470595 | 6.2967223 | 5.740018 | 5.2448177 | 1.225337 |
| AT1G7250 | Thz2.1 | -3.8823571 | -4.167722 | 2.6024313 | 2.7487748 | 1.1335823 |
| AT5G01900 | WRKY62 | 6.274398 | 6.820461 | 5.190764 | 5.1679066 | 1.1066311 |
| AT3G56400 | WRKY70 | 1.767238 | 1.5156872 | 1.3786796 | 0.7960569 | 0.9711816 |
| AT1G74950 | JAZZ | 1.9626862 | 1.3016942 | 1.3749169 | 0.6179589 | 1.3447273 |
| AT1G28480 | GGRX480 | 6.288935 | 6.0329523 | 5.7966275 | 5.5204635 | 0.7684715 |

**Table 5.10**
pathway as well as in phenylpropanoids (incl. lignin biosynthesis) pathways in 35S:GFP-SAP54 compared to 35S:GFP plants (Figure 5.23B). Nevertheless, no single pathway is significantly enriched in transcripts that are differentially regulated by males on SAP54 compared to control plants (Figure 5.23C). This suggests that there are small transcriptional changes relatively evenly distributed in most secondary metabolite pathways in male exposed SAP54 plants. Surprisingly, glucosinolate pathway (p=0.0067) is significantly enriched in female regulated transcripts that show the greatest difference between 35S:GFP-SAP54 and 35S:GFP plants. Glucosinolates also demonstrate the greatest difference between male exposed 35S:GFP-SAP54 and 35S:GFP plants relative to other male-regulated pathways (Figure 5.23C).

I further investigated which transcripts involved in regulation and production of glucosinolates are most changed in insect exposed 35S:GFP-SAP54 compared to 35S:GFP plants. I found that numerous transcripts involved in indole and aliphatic glucosinolate synthesis as well as mirasonase production are significantly downregulated in male-treated SAP54 plants (Table 5.11). The male-suppressed mirasonases TGG1 and 2 are hydrolysing insect ingested plant glucosinolates into toxic thiocyanates within herbivore guts (Bennett and Wallsagrove, 1994).
Figure 5.23. The effect of SAP54 on female (A) and male (B) leafhopper induced secondary metabolism. For each panel insect treated plants are compared to no-insect exposed control to characterise differentially expressed transcripts. Insect induced responses are compared between SAP54 non-expressing plants (left) and SAP54 expressing plants (right). Colour scale indicates log2(fold change). Panel C summarises the overrepresentation of SAP54–altered expression of different functional groups in the secondary metabolite pathway by taking the difference in fold change between insect induced responses in SAP54 expressing plants (right) and insect induced responses in SAP54 non-expressing plants (left). The enrichment analysis is based on Benjamini-Hochberg correction for multiple pathway comparisons using Wilcoxon rank test. P-Values <0.05 are shaded in C and highlighted on the right-hand side in panels A and B. Only a selection of pathways with top-ranked p-values are selected.
Table 5.11. Transcriptional regulation of indole and aliphatic glucosinolates in male and female leafhoppers exposed 35S:GFP and 35S:GFP-SAP54 plants compared to no-insect treated controls. Changes in gene expression are expressed as log2(fold change compared to insect non-exposed plants) and colour coded based on magnitude of fold change. All significantly differentially regulated transcripts (DEseq; p<0.05; q<0.05) are bold. Genes within each functional category are ranked based on the difference between female induced responses in 35S:GFP and male exposed 35S:GFP-SAP54 plants.

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</tbody>
</table>
Next, I aimed to investigate in which other secondary metabolism groups the difference between male and female induced responses is most significantly altered by SAP54. Surprisingly, SAP54 does not have significant effects on the difference between male and female induced responses (Table 5.12). These results suggest that SAP54 may similarly affect the same processes in male and female exposed plants but have greater effect only in male-exposed plants. Thus, expression of SAP54 may simply amplify the already existing differences between male and female differentially induced secondary metabolite transcripts. Nevertheless, the carotenoid and lignin biosynthetic pathways demonstrate the greatest effect of SAP54 on differences between male-to-female induced responses relative to other pathways (Table 5.12).

**Table 5.12. Functional enrichment analysis of transcripts that demonstrate SAP54 dependent changes in the difference between female and male induced plant responses in secondary metabolite biosynthesis.** Pathways are ranked according to significance, applying Benjamini-Hochberg correction for multiple pathway comparisons after Wilcoxon rank test. Some pathways demonstrate nested redundancy for greater discrimination of overrepresented changes due to SAP54 activity.

<table>
<thead>
<tr>
<th>Bin Code</th>
<th>Bin Name</th>
<th>Number of genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.1.4</td>
<td>isoprenoids; carotenoids</td>
<td>14</td>
<td>0.1264</td>
</tr>
<tr>
<td>16.2.1</td>
<td>phenylpropanoids; lignin biosynthesis</td>
<td>31</td>
<td>0.2268</td>
</tr>
<tr>
<td>16.4.2</td>
<td>betaine</td>
<td>3</td>
<td>0.2425</td>
</tr>
<tr>
<td>16.8.3</td>
<td>flavonoids; dihydroflavanols</td>
<td>16</td>
<td>0.4590</td>
</tr>
<tr>
<td>16.10</td>
<td>simple phenols</td>
<td>5</td>
<td>0.4744</td>
</tr>
<tr>
<td>16.5.1</td>
<td>glucosinolates</td>
<td>50</td>
<td>0.5215</td>
</tr>
<tr>
<td>16.1.2</td>
<td>isoprenoids; mevalonate pathway</td>
<td>15</td>
<td>0.5920</td>
</tr>
<tr>
<td>16.1.5</td>
<td>isoprenoids; terpenoids</td>
<td>9</td>
<td>0.6646</td>
</tr>
<tr>
<td>16.8.2</td>
<td>flavonoids; chalcones</td>
<td>9</td>
<td>0.6651</td>
</tr>
<tr>
<td>16.8.4</td>
<td>flavonoids; flavonols</td>
<td>9</td>
<td>0.6918</td>
</tr>
<tr>
<td>16.1.3</td>
<td>isoprenoids; tocopherol biosynthesis</td>
<td>8</td>
<td>0.7324</td>
</tr>
<tr>
<td>16.2</td>
<td>phenylpropanoids</td>
<td>60</td>
<td>0.7417</td>
</tr>
<tr>
<td>16.1.1</td>
<td>isoprenoids; non-mevalonate pathway</td>
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</tr>
<tr>
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<td>flavonoids; anthocyanins</td>
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</tr>
<tr>
<td>16.8.5</td>
<td>flavonoids; isoflavonols</td>
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</tr>
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<td>16.7</td>
<td>wax</td>
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<td>13.1.6.4</td>
<td>synthesis of tyrosine</td>
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<td>0.9650</td>
</tr>
<tr>
<td>13.1.6.3</td>
<td>synthesis of phenylalanine</td>
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</tr>
<tr>
<td>16.4.1</td>
<td>misc. alkaloid-like</td>
<td>18</td>
<td>0.9857</td>
</tr>
</tbody>
</table>
Detailed analysis of male and female regulation of the carotenoid pathway reveal that transcripts involved in lycopene biosynthesis and conversion are more strongly downregulated in male than female exposed 35S:GFP-SAP54 plants compared to 35S:GFP controls (Figure 5.24). A greater difference between male and female induced responses in SAP54 plants can be observed in phenylpropanoid (lignin) pathway (Figure 5.24). While some PAL transcripts that catalyse conversion of phenylalanine to cinnamic acid are upregulated in both male and female exposed 35S:GFP plants, all PAL transcripts are downregulated in SAP54 plants in male-specific manner (Figure 5.24). In concordance with such changes in PALs, similar effects can be observed for several 4CL transcripts which have previously been identified to show similar expression profile with PAL (Lois and Hahlbrock, 1992) and may be regulated by the same set transcription factors. In contrast to PAL and 4CL, males significant upregulate CCoAOMT genes (p=0.089) that methylate caffeate, ferrulate and sinapate in the lignin biosynthesis pathway in 35S:GFP-SAP54 plants compared to 35S:GFP. It is possible that upregulation of these genes is a result of lower abundance of their substrates due to male-specific suppression of 4CL in SAP54 plants (Figure 5.24). Caffeic, ferrulic and sinapic acids form various conjugates with different solubility. The composition and bioavailability of these different phenolic acid derivatives is implicated in the induced systemic resistance to herbivory (Erb et al., 2015). Limiting the transformation of phenylalanine into these phenolic acids may suppress plant resistance to leafhoppers and enhance host acceptance for oviposition.

In summary, glucosinolate production is significantly downregulated in male-exposed SAP54 plants but much less reduced in female-exposed SAP54 plants. Moreover, male insects specifically downregulate transcripts involved production of anti-herbivore phenolic acids in 35S:GFP-SAP54 plants while such effects are not characteristic in female-exposed plants.
Figure 5.24. The effects of SAP54 on phenylpropanoid and carotenoid pathways. Insect exposed control plants are on the left column and SAP54 expressing plants are on the right. All plants are exposed to either female (top) or male (bottom) insects. Scale bars represent log2(fold change) for phenylpropanoid and carotenoid pathways. Pathway images adopted from MapMan visualisation interface. PAL (phenylalanine ammonia-liase) genes are involved in phenylalanine conversion to cinnamate. Cinnamate or cinnamic acid are further converted to cummarate and caffeic acid (represented by multiple sequential arrows), then ferrulic and sinapic acids (single arrows). These organic acids are conjugated to Co-A by Co-A ligase (4CL). CCoAOMT
(caffeoyl–coenzyme A (CoA) O-methyltransferase) genes (marked as B) are involved in methylation pathway of lignin biosynthesis using Co-A conjugated caffeate, ferrulate, hydroxyferrulate and sinapate as substrates (marked by single arrows). SAP54 downregulates PAL and 4CL catalysed steps and upregulates CCoAOMT in male exposed plants. The carotenoid pathway depicts conversion of two geranylgeranylpyrophosphates (GGPP) into phytoene, zeta-carotene and lycopene. Lycopene is later converted into various carotenoids and their volatile derivatives. This pathway is downregulated by both male and female insects. SAP54 induces even stronger suppression in male-exposed plants.

5.2.13. Downregulation of defence signalling in male-exposed plants is linked to transcriptional suppression of plant secondary metabolism via interacting protein network

A key step to understand the potential mechanism of SAP54 effect on plant-insect interactions is to analyse the regulatory links between insect perception, signal transduction and defence responses such as changes in plant chemistry. These components could be linked via dense network of protein-protein interactions (PPI) and transcription factor interactions (TFI) with their targets. I wanted to investigate whether the male-specific downregulation of defence signalling genes could be linked with secondary metabolism transcripts and demonstrate co-ordinated regulation in 35S:GFP-SAP54 plants.

I used the available knowledge about experimentally validated as well as strongly predicted PPI and TFI to construct an interaction network consisting of all expressed and annotated defence signalling genes (Figure 5.16), their interactions with each other and with the secondary metabolism pathways. This way I will test whether transcriptional responses of various plant defence genes that demonstrated sex-specific response to insect exposure demonstrate correlated changes in expression with their interacting partners in the network. I chose to visualise the network interactions between defence signalling genes with each other and the glucosinolate, phenylpropanoid (lignin biosynthesis) and carotenoid pathways. The glucosinolates demonstrated greatest difference between male and female leafhopper exposed 35S:GFP plants (section 5.2.7) and showed the greatest difference between 35S:GFP and 35S:GFP-SAP54 plants in response to male and female insects (5.23C). Lignin biosynthesis and carotenoid pathways demonstrated the strongest effect of SAP54 on the
difference between plant responses to female and male insects (Table 5.12). Hitherto, I demonstrated male-specific suppression of defence responses (Figure 5.16) and secondary metabolite pathways (Figure 5.20) in 35S:GFP-SAP54 plants. For this reason, I plotted the change in gene expression in male-exposed SAP54 plants within the interaction network (Figure 5.25).

Interestingly, plant receptors, protein kinases and phytohormones (JA, SA, ET synthesis and signalling) grouped into a network of well-connected and functionally distinct modules that showed coordinated changes in gene regulation (Figure 5.25). Moreover, glucosinolate pathway was more connected to defence signalling modules via PPI compared to carotenoid or phenylpropanoid pathway.

There is experimental evidence for plant hormone-induced transcription factors that regulate receptor-like kinases. For example, WRKY60 is induced by SA-dependent signalling and regulates PR gene expression, and together with WRKY40 and 18 form an integrated hub in plant biotic and abiotic stress responses (Chen et al., 2010). Moreover, WRKY60 represses cysteine-rich receptor-like kinase CRK5 (Lu et al., 2016). Interestingly, the network in Figure 5.25 (largest cluster within SA signalling group) displays WRKY18/40/60 transcription factors to be linked with several down-regulated receptor-like kinases. There may be positive feedback loops between phytohormone-dependent defence signals and membrane receptors. Therefore, it is plausible to hypothesise that the defence signalling network may be multi-directional and not strictly hierarchical.

Most of the interactions between genes in the defence network in Figure 5.25 are PPI. LRR kinases demonstrate ample interactions with CDPK and MAPK which are appear to be the main interacting modules with JA, SA and ET signalling genes. Interestingly, many NLR receptors are likely to interact with LRR, SD and RCLK membrane receptors as well as Ca-dependent and mitogen-activated kinases. This suggests that membrane and cytoplasmic receptor complexes may function together in defence signalling via kinase cascades in agreement with recent findings (Peng et al., 2016). Furthermore, many membrane receptors demonstrate interactions with each other, for example, WAKL family kinases have multiple confirmed and predicted interactions with LRR, SD, DUF26, CrRKL receptors suggesting similar functions. In addition, LRR, WAKL, SD and DUF26 families demonstrate the greatest difference between male and female insect perception (Table 5.5), supporting the finding
about their interconnectivity and potential common regulation. Male leafhoppers
downregulate most of the receptor kinases in 35S:GFP-SAP54 plants, but
predominantly LRR (Table 5.9; analysis 2). The observed connectivity of LRR
with other defence modules may further explain the coordinated downregulation
of MAPK, SA, JA and ET pathways (Figure 5.25). Intriguingly, ET, JA and SA
biosynthesis and signalling modules interacts via PPI suggesting that in
additional to the known transcriptional crosstalk there might be extensive
crosstalk at post-translational level as well.

Interestingly, lignin synthesis and carotenoid pathways demonstrate very
few PPI, suggesting that most of the regulation of these secondary metabolites
could be transcriptional. Lack of the connectivity between these secondary
metabolism modules with JA, SA or ET modules at protein-protein interaction
level does exclude potential upstream regulation by hormone-dependent signal.
In contrast, glucosinolate pathway has more PPI with SA, JA and ET signalling
modules as well as few Ca-dependent protein kinases.

Taken together, the analysis of PPI network of defence responses
suggests that many insect-sex specific transcriptional responses in SAP54 plants
may be correlated due to the inter-connectivity of particular functional modules in
the network. Together with analysis from previous sections, the current results
induce a hypothesis that downregulation of chemical defences against insects
may be mediated by SAP54-dependent modulation of insect recognition in plants.
I further hypothesise that altered insect perception may be mediated through the
kinase and phytohormone dependent signalling cascades to potentially induce
different defence chemicals.

**Figure 5.25.** The PPI and TFI network linking Membrane receptor like kinase gene
families (LRKL, L-lectin, LRR, CrRLK, DUF26, WAKL, SD), cytoplasmic receptors
(NLR, NB, RLCK), Ca-dependent kinase cascade (CDPK, CRK, SnRK), mitogen-
activated kinases (MAPK, MAPK2, MAPK3, MAPK4), JA, SA and ET biosynthesis
and signalling, lignin, glucosinolate and carotenoid synthesis. The network depicts
all experimentally confirmed PPI as black solid lines (edges), predicted PPI as grey
dashed lines and all confirmed TFI as blue lines (only one was identified). Each box
(node) represents a single transcript. Colours represent log2(fold change) of the
difference between SAP54 expressing and non-expressing male exposed plants.
Figure 5.25
5.3. Discussion

In this chapter I investigated the differences between male and female leafhopper induced transcriptional responses in 35S:GFP and 35S:GFP-SAP54 plants. The aim of this research was to better understand if phytoplasma effector SAP54 alters insect triggered plant responses. Hitherto, I have discovered that male insect induced responses in 35S:GFP-SAP54 plants are significantly different from female regulated transcripts in 35S:GFP-SAP54 plants and different from both male and female induced responses in 35S:GFP plants. Moreover, male and female leafhoppers differ in their induced responses in 35S:GFP plants. Together this strongly suggests that SAP54 remolds plant responses to leafhoppers in male-dependent manner by interacting with herbivore sex-specific induced plant reactions. Plant defence responses to biotic stress are among the most strongly downregulated plant functions in male exposed SAP54 plants compared to GFP controls. Surprisingly, downregulation of biotic stress responses was not observed in female exposed plants. Detailed analysis of plant transcriptional responses relating to insect perception, signal integration via plant hormone network and mounting potential defences has suggested the following hypothesis (Figure 5.26).
Figure 5.26. Hypothetical model of insect-sex specific remodelling of herbivore induced plant defence responses by phytoplasma effector SAP54. (A) Female leafhoppers demonstrate stronger induction of plant Nucleotide Binding Site Leucine-Rich Repeat (NBL) receptor family together with stronger upregulation of Receptor-like kinases (RLKs) belonging to Leucine Rich Repeat (LRR) family, Domain of Unknown Function 26 (DUF26) RLKs, and S-domain (SD) RLKs compared to male insects. Receptor activity induces cascading effects in the interconnected plant regulatory network to activate jasmonic acid (JA) and salicylic acid (SA) production and defence signalling in both males and females. Ethylene production and signalling is much weaker induced in males compared to females. The cumulative effects of insect induced defence signalling upregulate plant secondary metabolism pathways. The induction of glucosinolate, phenylpropanoid (incl. phenolic acid and lignin), and flavonoid (anthocyanin) pathways is stronger in female compared to male exposed plants. Together, anti-herbivore chemicals could act as toxicants or deterrents and contribute to plant defences against herbivores. (B) Phytoplasma effector SAP54 remodels male insect induced plant responses resulting in downregulation of plant RLKs, NBL and signal transduction via protein kinases. This may result in suppression of ET signalling and certain defence metabolite, including glucosinolate, pathways. Such changes may weaken plant defences against insects and be required for female attraction to male colonised SAP54 plants.

Plant responses to insects show significant enrichment with biotic stress and signalling related transcripts (Table 5.2). Further analysis of manually annotated plant defence pathway (Figure 5.15) revealed that insects upregulate numerous families of RLKs as well as cytoplasmic receptors, predominantly NBS-LRR proteins, which often function as plant resistance (R) genes. This suggests that insects are recognised by plants and, similar to microbial pathogens, could trigger plant responses characteristic to pattern triggered immunity (PTI) as well as effector-triggered immunity (ETI). Moreover, male and female insects differ in the specificity and average fold change of induced receptor families. The greatest differences between male and female induced receptors were within NBS-LRR proteins, LRR, S-domain and DUF26 RLKs (Table 5.5). This was mainly due to greater fold change and larger number of female induced NBS-LRRs and RLKs compared to males. Many RLK transcripts were specifically induced by female but not male insects. Insect induced RLKs have multiple functions in defence and development. For example, S-domain serine/threonine RLKs are one of the
largest and fastest evolving plant groups of receptor-like/Pelle kinases and function in reproductive self-compatibility mechanisms (Xing et al., 2013). The Domain of unknown function-26 receptors, also known as Cysteine-rich Receptor-like Kinases (CRKs), play important roles in pathogen defence and programmed cell death, and can be regulated by SA-dependent signals (Wrzaczek et al., 2010). This also suggests that insect induced phytohormone signalling may show positive feedback to regulate plant receptors. SD and L-lectin RLKs were significantly induced by female leafhoppers (Figure 5.16A). Interestingly, lectin RLKs were also identified in the rice resistance locus to brown planthopper *N. lugens* (Du et al., 2009). SERK family RLKs, such as BAK1, is a ubiquitous co-receptor of many RLKs in recognition of pathogens (Couto and Zipfel, 2016). Therefore, many HAMP or DAMP activated RLKs may not function alone but associate with co-receptors. Furthermore, tomato homolog of SERK1 may also bind coiled-coil domain NBS-LRR proteins to recognise aphid effector and confer resistance to the herbivore (Peng et al., 2016). Although female leafhoppers upregulate a greater number of NBS-LRR proteins, both sex insects mainly regulate TIR-domain containing NBS-LRR proteins (MapMan analysis for Figure 5.16). The TIR- and LRR domains are likely to regulate the activity of these receptors via binding to co-receptors or downstream targets (Belkhadir et al., 2004). While no leafhopper effectors have yet been characterised, the potential differences in male and female secreted effectors could contribute to sex-specific induced plant responses. Such differences would mean that effector-containing salivary secretions during male or female feeding could potentially trigger different ETI responses.

Nevertheless, the most obvious difference between male and female insects is that males can feed from the plant but females can also lay eggs in addition to feeding. During egg-laying female leafhoppers penetrate plant surface with a specialised egg-depositing organ, called ovipositor, and produce oviposition fluids that facilitate deposition and attachment of eggs into the leaf. Leafhoppers lay eggs that are partially or fully embedded in leaf tissue (Supplemental Figure 3; Appendix C). Therefore, egg surface may come into direct contact with sub-epidermal layers of plant tissue. Thus, during egg-laying female leafhoppers present plants with additional set of oviposition-related HAMPs and DAMPs compared to feeding-related signal elicitors. The potential elicitors of oviposition-triggered defence responses are bruchins in female
oviposition fluids or benzyl cyanide and indole compounds on the egg-surface (Reymond, 2013). Interestingly, oviposition is known to induce similar plant responses to PTI: deposition of callose, activation of ROS as well as upregulation of CDPK and MAPK induced genes in the SA pathway (Little et al., 2006; Gouhier-Darimont et al., 2013). Furthermore, egg laying induced responses can modulate plant-insect interactions. For example, *Pieris brassicae* butterfly eggs induce responses in *A. thaliana* that result in deterrence of females for egg-laying (Groux et al., 2014). In addition, *P. brassicae* egg-laying can enhance local arrestment of egg-parasitoid *Trichogramma* wasp during host location (Fatouros et al., 2005). Plant responses to egg-laying could be not only local but also systemic. *P. brassicae* egg triggered upregulation of SA-dependent defences negatively regulate JA signals and reduces plant resistance to generalist chewing herbivore (Bruessow et al., 2010). This may also suggest that eggs or female oviposition fluids may contain effectors that manipulate certain aspects of plant defence responses.

I hypothesise that egg-laying could contribute to female-specific induction of PTI by differential regulation of certain RLKs and activation of ETI via upregulation of female-specific NLR proteins. Furthermore, CDPK and MAP3K (MEKK subfamily) are also specifically induced by females (Figure 5.16) and could potentially transduce the female-specific signals. *M. quadrilineatus* females induce both JA- and SA-related defences which is characteristic to oviposition and wounding responses (Reymond, 2013). Because male leafhoppers also induce JA- and SA-responses that are hardly distinguishable from female exposed plants (Table 5.6), there could be overlap between some of the feeding and oviposition induced defence signalling downstream insect recognition by plant receptor kinases or NBS-LRR proteins. In contrast, ethylene receptors ETR1 and EIN4 as well as their downstream relays EIN2/5 and EIL3 are downregulated by male but upregulated by female leafhoppers. Moreover, ET marker genes ORA59 and ERF1 are significantly induced in females but not males (Table 5.6). Differential regulation of ET signalling pathway is one of the most evident differences between male and female induced plant responses and could originally arise from differential ETI and PTI signals. Furthermore, this suggests that ET signalling may be an important element of oviposition-related plant responses. Hitherto, ET signalling has been little studied in context of insect oviposition. Egg secretions of sawfly *Diprion pini* reduces ethylene production in
pine *Pinus sylvestris* (Schröder *et al.*, 2007). It is known that exogenous application of ethylene in olive fields can reduce olive moth *Prays oleae* egg production (Ramos *et al.*, 2008). However, mechanistic links between egg-laying and induction of ET-dependent defence genes has not yet been reported (Reymond, 2013). Similar to JA, ET production is induced during plant wounding and therefore could play a role in responses to egg deposition within leaf tissue. ETHYLENE RECEPTOR1 (ETR1) and ET signalling component EIN2 are key in ETI-dependent hypersensitive response and cell death (van Loon *et al.*, 2006). These transcripts are upregulated by female leafhoppers compared to males. Together, the transcriptome data support the hypothesis that female oviposition may induce sex-specific plant responses that are similar to PTI and ETI and result in upregulation of ethylene signalling pathway via female-specific Ca\(^{2+}\)-dependent and mitogen activated kinases.

It is most intriguing that SAP54 modulates ET signalling in plants by significantly downregulating several ET signalling transcripts in male-specific manner. This suggests that SAP54 either 1) requires activation of certain male-specific components not present in female exposed plants or 2) interacts with a component that is induced by both male and female insects but may be suppressed by female-specific activities such as egg-laying. The key component for SAP54 activity may be related to or function upstream ET signalling. This is also consistent with the finding that the effect of SAP54 on ET signalling is more consistent than effect on SA or JA signalling pathways in male exposed plants.

It is, however, possible that modulation of ET signalling by SAP54 is only means towards a different aim. Instead of being a principal regulator *per se*, ET signals are often viewed as modulators of SA or JA signals due to the complex crosstalk between these phytohormones (Broekgaarden *et al.*, 2015). ET response factors EIN3 and EIN2 are key regulators that connect ET signalling to other plant hormones (Bisson *et al.*, 2009; Yoo *et al.*, 2009; Yan *et al.*, 2012; Chang *et al.*, 2013). Similarly, SA or JA can alter ET responses. For example, perception of JA signals activate ETHYLENE RESPONSE FACTOR1 (ERF1) and AP2/ERF transcription factor ORA59 (Lorenzo *et al.*, 2003; Pre *et al.*, 2008). Moreover, ERF1 suppresses SA-dependent PATHOGENESIS RELATED (PR) gene expression and increases plant susceptibility to biotrophic pathogens (Pantelides *et al.*, 2013). For this reason pathogenic bacteria like *Pseudomonas syringae* may modulate ET responses to suppress plant defences (Guan *et al.*, 2013).
Interestingly, the male-specific suppression of ET responses in 35S:GFP-SAP54 plants does not correlate with any obvious changes in JA and SA pathways (Table 5.6) which remain similarly regulated in male and female exposed 35S:GFP and 35S:GFP-SAP54 plants. The effect of ET on JA and SA responses is largely mediated via MPK3 and MPK6 (Guan et al., 2015). These kinases do not appear to be differentially regulated by male leafhoppers in SAP54 plants. Together, this indicates that SAP54 may be directly targeting ET responses in male-dependent manner rather than using ET pathway to target signalling by other plant hormones.

ET responses are important for the Induced Systemic Resistance (ISR) in plants (van Loon et al., 2006; Broekgaarden et al., 2015). ISR is often induced by above- and below-ground non-pathogenic beneficial microbes and involves priming of plant defence responses. In contrast, SA responses are required for Systemic Acquired Resistance (SAR) and often effective against biotrophic pathogens. By downregulating ET-related plant responses to insects, SAP54 could be modulating plant ISR and priming of defence responses that function in oviposition. Furthermore, by altering ET responses, phytoplasma effector SAP54 could modulate plant secondary metabolism that is important in plant-insect interactions. Phenolic acids are involved in induced systemic resistance and host plant selection by foliar herbivores (Erb et al., 2015). And, interestingly, the conversion of phenylalanine into phenolic acids (cinnamate, caffeate, ferrulate and sinepate) appear to be downregulated in male exposed 35S:GFP-SAP54 plants (Figure 5.24). ET signals are implicated in production of volatile terpenoids (monoterpenes, sesquiterpenes) that play diverse roles in plant-herbivore and plant-herbivore natural enemy interactions (Fürstenberg-Hägg et al., 2013; Broekgaarden et al., 2015). EIN2-dependent ET responses are also required for broad-spectrum anti-microbial and anti-herbivore phytoalexin synthesis in N. benthamiana (Matsukawa et al., 2013). Many subfamilies of cytochrome P450 genes (CYP79/82/83/84) are involved in synthesis of indole derivatives like glucosinolates, camalexin (phytoalexin) or auxin from tryptophan (Mizutani and Ohta, 2010). Many of the CYP genes involved in glucosinolate synthesis are significantly downregulated in male exposed SAP54 plants (Table 5.8). Interestingly, it has been demonstrated that cyp79b2 cycp79b3 double mutant plants are compromised in indole glucosinolate as well as camalexin accumulation and more susceptible to aphids (Glawischnig et al., 2004; Kim et
CYP79B2 and CYP79B3 are significantly induced by females in 35S:GFP and 35S:GFP-SAP54 plants but downregulated in male exposed 35S:GFP-SAP54 compared to female exposed plants or male exposed 35S:GFP plants (Table 5.11). In addition to CYP79B2/3, many more transcripts with functions in indole and aliphatic glucosinolate synthesis are suppressed by male leafhoppers in SAP54 plants. Several studies highlight the importance of MPK3/6 and their downstream WRKY transcription factors in indole glucosinolate and phytoalexin synthesis (Ren et al., 2008; Ishihama et al., 2011; Lassowskat et al., 2014). Nevertheless, there may be numerous other kinase-dependent regulatory loops of the indole-derived defence chemicals. For example, ethylene receptor CTR1 is a RAF family MAP3K which regulate the ET response factor EIN3 in an opposite manner than MAP3/6-MKK9 kinase branch, thus constituting bifurcate control of ethylene signalling (Yoo et al., 2008). Many mitogen-activated kinases show protein-protein interactions with ET response factors and transcription factor or enzymes involved in glucosinolate synthesis (Figure 5.25). Moreover, these transcripts show correlated suppression specifically in male exposed SAP54 plants. Neither MPK3/6 nor camalexin synthesis enzyme PAD3 and 4 (PHYTOALEXIN DEFICIENT3 and 4) are differentially regulated by SAP54 in male and female exposed plants, suggesting that other branches of indole compounds than phytoalexins could be the targeted by SAP54 via alternative MAP-kinase dependent regulation. Interestingly, Matsukawa et al. (2013) found that a N. benthamiana calreticulin gene confers resistance to Phytophthora infestans by positively regulating ET-dependent indole compound production. This occurs independent from MAPK induced cell death but is mediated via ethylene-induced Cytochrome P450s. Calreticulins are chaperone proteins on plant and animal endoplasmic reticulum where they aid processing of glycosylated membrane receptors (Thelin et al., 2011). A. thaliana has 3 calreticulin genes - CLT1A, CLT1B, and CLT3. All three are significantly upregulated by female leafhoppers in 35S:GFP plants. Surprisingly, CLT1A, CLT1B are significantly downregulated while CLT3 upregulated slightly but not significantly in male exposed 35S:GFP-SAP54 plants.

Taking together, SAP54 may target plant receptors or receptor modifying proteins such as calreticulins to modulate male insect induced PTI and ETI responses that lead to suppression of ET-signalling transcripts involved in plant secondary metabolite such as glucosinolate synthesis in sex-specific manner.
Given that SAP54 enhances female preference for oviposition in male-dependent manner (Chapter 4), an intriguing question arises – how does SAP54 modulate plant responses to simultaneous male and female attack? I investigated plant transcriptional response to males and females separately. The insect choice experiments reported in the previous chapters involved simultaneous release of male and female leafhoppers. Males may differ in their feeding behaviour from females and feed immediately after release whereas females may be more passive and demonstrate delayed choice of the host plant compared to males. This awaits empirical testing. However, this would offer a behavioural context for the hypothesis that SAP54 suppresses male-dependent priming of plant defences to make the host plant appear less-defended and thus positively affect female landing choice.

I would like to close the discussion of this chapter by addressing the questions why plant responses to insects involve so many other plant functions in addition to biotic stress (Figure 5.15) and why SAP54 may differentially regulate many additional transcripts to the insect induced ones (Table 5.9)? Although I cannot exclude the possibility that SAP54 is modulating something other than plant defence responses to enhance female oviposition, it is plausible to hypothesise that alteration of insect induced defence responses are key. I highlighted that plant defence genes involved in the recognition of biotic stress and signalling do not exist in isolation but are positioned in a dense network of interacting proteins with diverse functions, including plant nutrition, anabolic primary metabolism (photosynthesis), growth and development (Figure 5.21). It is often conceived that co-expressed genes may be co-regulated and co-functional (Allocco et al., 2004; Michalak, 2008). Indeed, many plant defence genes show correlated suppression in insect exposed plants which may be explained by their linkage in protein-protein interaction network (Figure 5.25) or common transcriptional regulators. Similarly, many defence related genes are upregulated under insect attack but growth-related transcripts are downregulated (Table 5.3). Such effects may result from the trade-offs between growth and defence (Figure 5.1) and are enabled by the connectivity of diverse functions via common regulators (Figure 5.20). Furthermore, synthesis of various secondary metabolites like glucosinolates or phenylpropanoids requires amino acids methionine, tryptophan or phenylalanine (Fürstenberg-Hägg et al., 2013). This may explain the correlated changes in amino-acid metabolism and transport to
balance the demands for nutrition, growth and defence (Figure 5.15; Table 5.2). Similarly, downregulation of photosynthesis and remodelling of tilakoid membranes may affect many chloroplast localised defence regulators such as MFP1 (Melonek et al., 2012).

Experimental work and modelling approaches have identified ample transcriptional and post-translational regulatory links that ensure coordinated and robust response of plant defence network (Tsuda et al., 2009; Sato et al., 2010). The annotated defence network in Figure 5.25 contained very few transcription factor – target gene interactions that have experimentally verified transcription factor binding motifs (See materials and methods for de novo search for transcription factor-target interactions for this study). Instead, majority of the visualised links were protein-protein interactions. By targeting certain regulators upstream plant hormone responses, SAP54 may modulate various components of plant defences due to cross-talk between plant hormone signalling (incl., JA, SA, ET, ABA, brassinosteroids). For example, by targeting plant receptors, SAP54 may induce a plethora of secondary side-effects which may not be adaptive for the effector function in enhancing insect colonisation but instead may result from the inter- and intra-connectivity of the defence signalling network. Further analysis of predicted transcription factor binding sites within the defence network can be instrumental to better understand the genetic mechanism of SAP54 activity.
"In the strict formulation of the law of causality - if we know the present, we can calculate the future - it is not the conclusion that is wrong but the premise."

Werner Heisenberg, in C.D. Cassedy (2009), Beyond Uncertainty: Heisenberg, Quantum Physics, and the Bomb, p 228

Chapter 6
The Role of MADS-box Transcription Factors in Plant-Phytoplasma-Insect Interactions

6.1. Introduction

Data presented in the previous chapters suggest that SAP54 enhances female insect egg-laying preference for male colonised plants by modulating plant responses to male leafhoppers. Furthermore, SAP54 interacts and destabilises plant MADS-box transcription factors (MTFs) via recruitment of 26S proteasome cargo protein RAD23. SAP54-mediated female leafhopper oviposition preference requires RAD23 (MacLean et al., 2014) but is independent from developmental changes in flowers (Orlovskis and Hogenhout, 2016). Since plant MTF may regulate additional plant processes to their well-established role in reproductive development, I hypothesise that modulation of MTF-regulated plant processes other than flowering could be key to transcriptional reprogramming plants by SAP54 to attract insects for egg-laying.

There is growing body of evidence that members of the same plant transcription factor family do not have discrete functions but instead regulate a myriad of different plant processes. For example, TCPs are recognised for their role in both vegetative and reproductive development as well as phytohormone biosynthesis and crosstalk (Uberti Manassero et al., 2013). TCPs are highly connected proteins within plant regulatory network and have roles in plant immunity (Kim et al., 2014; Lopez et al., 2015). Similarly, in addition to their evolutionary conserved roles in regulating floral meristem and organ identity, MTFs may regulate plant responses to biotic stress. Chip-seq experiments suggest that LEAFY (LFY) targets pattern recognition receptors such as FLS2 (Winter et al., 2011), SHORT VEGETATIVE PHASE (SVP1) regulates JAZ proteins, which are involved in perception of jasmonic acid (Gregis et al., 2013), and SUPPRESSOR OF CONSTANS1 (SOC1) targets TCPs and miRNA319 that are implicated in plant immunity as well (Immink et al., 2012). Interestingly, pathogen effector proteins were shown to target multifunctional plant transcriptional regulators like TCPs to suppress jasmonate production (Sugio et al., 2011) or circadian clock regulating Glycine-Rich Proteins GRP7 to regulate PAMP receptors like FLS2 (Nicaise et al., 2013).

Intriguingly, many plant developmental processes and interaction with pathogens may be mediated by the same pattern recognition receptors (Govers and Angenent, 2010). Plant MLO family receptor-like kinases recognise both pollen tube and powdery mildew hyphae (Kessler et al., 2010). In Chapter 5 I found insect sex specific induction of plant receptors and male-specific
transcriptional regulation by phytoplasma effector SAP54. However, transcriptional regulation of RLK in mammals and plants is yet poorly understood. There is some evidence for the effects of abiotic stress and plant hormone pathways on transcriptional regulation of RLKs (Wrzaczek et al., 2010; Wu et al., 2015).

Along with transcription factors, plant receptor-like kinases are recognised as an important component of coordination of cell division, control of organ shape and tissue specification (De Smet et al., 2009). Plant RLKs are controlled by transcription factors and respond to PAMP-elicited transcriptional reprogramming (Wrzaczek et al., 2010; Wu et al., 2015). The pleiotropic roles of transcriptional regulators like MTFs could be explained by specificity to diverse targets as well as multiple functions of the targets such as RLKs themselves. For example, brassinosteroid receptor BRI1 functions in regulation of growth and pathogen resistance in dicots and monocots (Nam and Li, 2002; Ali et al., 2014).

In this chapter I aim to understand the transcriptional regulation of plant MTFs in response to insects and SAP54. Furthermore, I will test the direct effect of MTFS in plant resistance to insect egg-laying. I will explore the potential targets of SAP54 other than MTFs and investigate the transcriptional circuits that could link SAP54 targets with plant defence genes, including pattern recognition receptors.

6.2. Results

6.2.1. Expression of plant MADS-box transcription factors is altered by SAP54 in insect sex-dependent manner

The effect of SAP54 on leafhopper egg-laying is independent from flowering and floral transition, and leafhoppers prefer to lay eggs on plant vegetative organs expressing SAP54 (Orlovskis and Hogenhout, 2016). Therefore, I wished to find out which of the potential SAP54-targeted MTFs are expressed in the vegetative plant tissue. I clustered all annotated MTFs in A. thaliana genome based on their normalised expression values to see which MTFs are expressed and may functions in leaves. A significant proportion of MTFs demonstrates negligible expression in leaves while other MTFs are expressed during vegetative growth (Figure 6.1). Moreover, numerous MTFs show different
expression in response to insects and SAP54. Interestingly, SAP54 mainly interacts with MTFs expressed in leaves (Figure 6.1). Yeast-two-hybrid studies show that SAP54 interacts with 15 out of the total 107 MTFs. These interactors belong to the type II MICK clade of MTFs and are traditionally considered to function in floral transition and reproductive development. RNA-seq experiment indicates that many of these MTFs, including FUL and SEP4, are actually expressed in the vegetative organs prior to floral transition, and could perform functions other than regulation of reproductive development.

Out of 107 MTFs, I selected only those genes that demonstrated consistent expression above the pre-determined expression threshold (see materials methods) in majority of technical replicates in any single treatment. I discovered 20 genes that met these criteria and were expressed in the vegetative tissue of *A. thaliana*. Out of 20 vegetative stage expressed MTFs four interact with SAP54 directly (Figure 6.2). While most of the 20 expressed MTFs are not direct targets of SAP54, some leaf-expressed MTFs interact with SAP54 interactors and therefore may be targeted indirectly as part of MTF multimer complexes (de Folter *et al.*, 2005).

I calculated the fold change in MTF expression in response to male and female insects compared to no-insect treatment of 35S:GFP and 35S:GFP-SAP54 plants. Interestingly, MTFs exhibit insect sex-dependent regulation (Figure 6.2). For example, AGL30, MAF2, MAF3 and MAF5 are upregulated in female but suppressed in male leafhopper exposed plants. Furthermore, MAF5 is downregulated in male exposed 35S:GFP-SAP54 but upregulated in 35S:GFP plants. Other genes, such as SVP1 and AGL24, are downregulated by both male and female insects in 35S:GFP but upregulated in SAP54 plants.

In summary, I identified a set of type II MTFs that are expressed in vegetative plant tissue prior to floral transition and interact with SAP54 directly or associate with SAP54 interactors. Interestingly, certain MTFs demonstrate sex-specific response to leafhoppers. Furthermore, RNA-seq experiment suggests that the expression of several insect-induced MTFs (MAF5, SVP1, and AGL24) is altered in insect exposed SAP54 plants compared to controls.
Figure 6.1. SAP54 interacts with MADS-box transcription factors which are expressed in leaves. MTF interaction with SAP54 in yeast-two-hybrid is highlighted in yellow colour (MacLean et al. (2014)). Red colour intensity correlates to the absolute
gene expression values (normalised FPKM). Columns from left to right represent all sequenced experimental treatments as follows: (Lane 1) MTF expression in female-exposed SAP54 plants, (Lane 2) MTF expression in female-exposed GFP plants, (Lane 3) MTF expression in male-exposed SAP54 plants, (Lane 4) MTF expression in male-exposed GFP plants, (Lane 5) MTF expression in no insect-exposed SAP54 plants, (Lane 6) MTF expression in no insect-exposed GFP plants. Clustering performed using Euclidean distance via centroid linkage of untransformed FPKM values (Cluster 3.0), viewed in TreeView (contrast 0.05 to highlight the low-expression transcripts).

Figure 6.2. Clustering of MADS-box transcription factors with similar expression in response to male and female insects in SAP54 and control plants. Log\(_2\)(fold change) is displayed as measure of differential regulation of MTF genes, red indicates upregulation and green – downregulation. Lane 1: female vs no insect exposed GFP plants. Lane 2: female vs no insect exposed SAP54 plants. Lane 3: male vs no insect exposed GFP plants. Lane 4: male vs no insect exposed SAP54 plants. Direct SAP54 interactions with MTFs are indicated in yellow (MacLean et al. (2014)). N.A. applies to few MTFs that have not yet been tested for interactions with SAP54. All other interactions
between any pair of expressed MTFs are highlighted in yellow, based on Y2H screen by de Folter et al (2005). Clustering based on Euclidean distance via centroid linkage.

6.2.2. Host plant selection for insect egg-laying is dependent on MADS-box transcription factors

Given that a number of leaf-expressed MTFs interact with SAP54 or demonstrate sex-specific regulation in 35S:GFP-SAP54 plants, I wanted to directly test the role of these MTFs in host plant selection by leafhoppers. I acquired seeds for several homozygous MTF mutant (T-DNA insertion) lines and performed leafhopper oviposition choice tests to test which MTFs play a role in plant resistance to insect colonisation. Interestingly, two MTF mutants – svp1 and maf5 – demonstrate significantly greater leafhopper egg-laying preference compared to the wild-type control plants (Figure 6.3A). These results suggest that destabilisation or downregulation of SVP1 and MAF5 may mimic the leafhopper oviposition preference for SAP54 plants.

In the experiment described above I measured insect reproductive preference for MTF mutant or wild-type plants that are not infected with phytoplasma and have no SAP54. Next, I wanted to determine which MTFs may be required for SAP54-dependent leafhopper attraction. Due to time constraints for generating 35S:GFP-SAP54 plants in MTF mutant background, I infected MTF mutant plants with AY-WB phytoplasma to introduce SAP54 into the plant via phytoplasma. Surprisingly, svp1 and maf5 no longer showed leafhopper oviposition preference (Figure 6.3B), suggesting that SVP1 and MAF5 may act downstream SAP54. Interestingly, two other MTF mutants – agl24 and ful – demonstrated significantly less attraction of leafhoppers compared to wild-type controls (Figure 6.3B), suggesting that AGL24 and FUL may be required for leafhopper egg-laying preference in infected plants similar to RAD23 protein (Maclean et al., 2014). MAF5, SVP1, AGL24 and FUL are not direct targets of SAP54 and may be differentially regulated in SAP54 plants as a result of destabilisation of other MTFs or MTF-complexes.

In conclusion, I demonstrated that MTFs may play an important role in host plant selection by insects in both healthy and phytoplasma infected plants. SVP1, MAF5 may be indirectly targeted by SAP54 to enhance plant colonisation by insects, and such mechanism would require AGL24 and FUL.
Figure 6.3. Measurement of leafhopper *Macrosteles quadrilineatus* egg-laying preference for non-infected (A) or AY-WB phytoplasma infected (B) MTF mutant or wild-type (Col-0) plants. There is significant leafhopper preference for reproduction on \( svp1 \) (t\(_{6}\)=3.31; p=0.021) and \( maf5 \) (t\(_{6}\)=4.78; p=0.005) mutants. \( agl24 \) (t\(_{6}\)=2.02; p=0.100), \( sep4 \) (t\(_{6}\)=2.05; p=0.096), \( maf4 \) (t\(_{6}\)=0.09; p=0.932), \( maf1 \) (t\(_{6}\)=0.54; p=0.615), \( ful-1 \) (t\(_{6}\)=0.27; p=0.796) and \( soc1 \) (t\(_{6}\)=0.18; p=0.865) plants do not show significant leafhopper preference compared to wild-type controls. When both MTF mutant and control plants are infected with AY-WB phytoplasma leafhoppers show significant preference to wild-type plants compared to \( agl24 \) (t\(_{6}\)=3.02; p=0.029) and \( ful \) (t\(_{6}\)=10.22; p≤0.001). Insects showed no preference for wild-type or \( maf5 \) (t\(_{6}\)=1.52; p=0.188), \( svp1 \) (t\(_{6}\)=0.46; p=0.668), \( sep4 \) (t\(_{6}\)=0.68; p=0.531), \( maf4 \) (t\(_{6}\)=0.28; p=0.789) or \( maf1 \) (t\(_{6}\)=0.72; p=0.501) mutants. For each experiment 10 female and 10 male adult leafhoppers were released in a choice
cage with control plant and MTF mutant plant. Adult insects were removed after 5 days and nymphs counted on each plant within the choice cage separately. The graph displays the relative distribution of nymphs on control and mutant plants. Data analysed using paired t-test and p-values <0.05 indicated with an asterisk.

6.2.3. MADS-box transcriptional regulators demonstrate co-expression with defence genes in response to insect attack and SAP54

Due to the insect sex-specific effects of on the expression of MTFs in 35S:GFP-SAP54 plants, genes like MAF5 are the prime suspects to orchestrate the transcriptional changes upstream the defence signalling and secondary chemical pathways which showed male-dependent suppression by SAP54 as described in the chapter 5. To test such potential roles of MTFs, I performed a co-expression clustering analysis with all 20 leaf-expressed MTFs and around 1060 manually annotated genes (see materials methods) with role in plant defence signalling. I aimed to investigate which defence signalling components cluster most closely with MTFs. I identified 6 MTF clusters containing defence-related genes that show correlated expression and similar magnitude of fold change in response to male and female insects on 35S:GFP-SAP54 and 35S:GFP plants (Figure 6.3). Intriguingly, MTFs cluster closely with a range of different families of the membrane and cytoplasmic receptor kinases. In addition, Ca-dependent and mitogen-activated kinases as well as SA or JA signalling genes show similar expression patterns to certain MTFs. SVP1, MAF5, AGL24 and FUL cluster most closely with LRR, SD-domain and L-lectin RLKs (Figure 6.3). Together, this suggests that pattern recognition receptors show similar response to insects as MTFs that are potentially targeted by SAP54 and show altered plant resistance to insect egg-laying.

Furthermore, I wanted to determine whether defence genes in any of the identified 6 MTF clusters in Figure 6.3 are enriched with predicted binding sites for any known families of plant transcription factors, including CArG-box of MTFs. I performed a promoter motif search for predicted transcription factor sites of MTF, WRKY, Ethylene-AP2 and other factors in the -5000 bp non-overlapping upstream promoter regions of all defence genes included in the clustering analysis. I calculated the overrepresentation of binding sites for any particular transcriptional regulator in each defence gene cluster from Figure 6.3. I found
that defence genes that cluster with MTFs are not only enriched with potential MTF binding sites but also other transcription factors, including bZIP, WRKY, DOF and AP2 (Table 6.1).

Next, I highlighted clusters with MTFs that interact with SAP54 directly or MTFs that interact with other SAP54-targetted MTFs from Figure 6.2. These include 10 MTFs found in clusters 1, 3 and 5 in Figure 6.3. From the potential SAP54-targetted proteins only MAF5 cluster contains overrepresentation of defence-related transcripts with MTF and WRKY binding sites (Table 6.1). Within this cluster CArG-box was found in DUF26 and CR4L families of RLKs whereas W-box sites were present in almost all transcripts of cluster 5. Ethylene-AP2 binding sites were significantly overrepresented in the cluster containing AGL24 and SVP1. Interestingly, SOC1 and FUL containing cluster 1 shows overrepresentation of bZIP sites. MYB-factor or DOF binding sites were not overrepresented in any of the clusters with potential SAP54 interactors (Table 6.1).

Taken together, these data suggest that the SAP54 effect on defence gene expression may act not exclusively via direct MTF-defence gene interactions but via a combination of putative MTF effects on other transcription factors that may control the defence network. Further analysis on SAP54 effects on insect induced transcriptional regulation is required to elucidate which transcriptional regulators are likely to mediate the downstream effects of SAP54-MTF interactions on defence signalling and secondary metabolism described in the previous chapter.

**Figure 6.4. MTFs and defence genes demonstrate co-expression in response to insect attack and phytoplasma effector SAP54.** 20 MTFs are clustered together with 1062 defence genes annotated from figure 5.11. Each gene identifier was linked to the belonging of a functional group such as JA signalling or particular PRR family, as displayed in the figure. MTFs are identified with arrows. Clusters 1 and 3 also contain AGL18 and AGL26 respectively (not displayed as they are further from the rest of MTFs in the respective clusters). Column 1 indicates transcript upregulation (red) or downregulation (green) in female exposed plants without SAP54; column 2- female exposed SAP54 expressing plants; column 3 - male exposed plants without SAP54; column 4 - male exposed SAP54 expressing plants. Clustering based on Euclidean distance via complete linkage (Cluster 3.0), viewed in TreeView (colours depict log2(fold change)).
Table 6.1. Summary of the presence of transcription factor binding sites in the upstream -5000 bp non-overlapping promoter regions of defence genes that demonstrate correlated expression patterns with MTFs (cluster numbers based on figure 6.3). The total number of genes in the clusters and number of potential cis-regulatory elements for each transcription factor (TF) family are indicated within each cluster. The probability of random TF binding site discovery is based on cumulative hypergeometric distribution in each cluster relative to the occurrence of TF binding sites in the total population of all expressed defence genes. Any probability below 1/2 tells that the observed cis-element enrichment is against the odds to be discovered at random and hence indicates overrepresentation of the transcription factor binding sites in the cluster. Clusters that contain potential SAP54 interactors of (based on figure 6.2) are highlighted bold.

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<th>Probability of random discovery</th>
<th>Cluster with SAP54 Interactors</th>
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49 CArG box motifs in 707 expressed defence genes
557 W-box motifs in 707 expressed defence genes
6 AP2 motifs in 707 expressed defence genes
81 MYB box motifs in 707 expressed defence genes
324 C2C2 DOF box motifs in 707 defence genes
423 bZIP box motifs in 707 expressed defence genes
6.2.4. Male leafhoppers suppress plant transcriptional regulators in SAP54-dependent manner

To investigate if SAP54 may have insect sex-dependent effect on plant transcriptional regulators, I compared male and female leafhopper induced changes in numerous transcription factor families in 35S:GFP-SAP54 and 35S:GFP plants (Figure 6.5). Interestingly, female insects significantly upregulate plant WRKY (p=1.78E-14), MYB (p=5.22E-4) as well as AP2/Ethylene responsive (AP2/EREBP) (p=0.0546) transcription factors in 35S:GFP plants (Figure 6.5A). In addition, females significantly downregulate histone and histone-binding proteins compared to no insect treatment (p=7.92E-8). Like females, male insects significantly upregulate WRKY (p=8.49E-9) and AP2/EREBP (p=2.53E-5) transcription factors and downregulate histone as well as chromatin remodelling factor genes compared to no insect exposed 35S:GFP plants (p=8.04E-5) (Figure 6.5B).

Surprisingly consistent with data in chapter 5, male insect induced changes in plant transcription factors are dramatically changed in 35S:GFP-SAP54 compared to 35S:GFP plants (Figure 6.5B). Interestingly, such differences are not observed in female exposed 35S:GFP-SAP54 plants (Figure 6.5A). Next, I investigated which functional groups of transcriptional regulators are changing most in male and female 35S:GFP-SAP54 plants compared to the controls. Notably, histone genes, histone acetyltransferases and other chromatin remodelling factors such as SET-domain, PWWP-domain, JUMONJI-domain or GTE transcription factors are significantly altered in both male and female exposed SAP54 plants (Figure 6.5C). However, the magnitude to which these gene groups are downregulated is considerably greater in male compared to female exposed plants (comparing Figure 6.5A to B).

Along with other chromatin remodelling factors, SET-family proteins were most downregulated by SAP54 in female and, especially, male exposed plants compared to GFP control (Figure 6.5C). Among most suppressed SET factors are several SUVH (SU(VAR)3-9 HOMOLOG) genes that encode histone methyltransferases. PWWP and GTF family factors are amongst other most downregulated transcripts in insect exposed 35S:GFP-SAP54 compared to 35S:GFP (Figure 6.5). PWWP-domains are found in nucleosome-binding DNA methyl-transferases and resemble other eukaryotic DNA-binding domains such as SAND and Tudor (Qiu et al., 2002). The General Transcription Factor Group
E6 (\textit{GTE}) are bromodomain-containing nuclear localised proteins involved in leaf development by histone acetylation (Yii \textit{et al.}, 2005). In addition, SAP54 downregulates many JUMONJI or jmjN/C-domain transcription factors, including \textit{EARLY FLOWERING 6 (ELF6)}. ELF6 has a H3K27me3 demethylase activity and is important for the activity of \textit{FLC} in embryos (Crevillen \textit{et al.}, 2014). Furthermore, brassinosteroid response factor \textit{BES1} recruits \textit{ELF6} to regulate target gene expression and coordinate developmental processes and disease responses (Yu \textit{et al.}, 2008). In contrast to the significant effects of SAP54 on chromatin remodelling factors, the phytoplasma effector does not significantly affect the insect-induced \textit{WRKY} or \textit{MYB} factors which largely remain upregulated after leafhopper feeding and oviposition (Figure 6.5A,B). There is significant enrichment of \textit{AP2/EREBP} factors in female exposed plants (p=0.0485) among transcription factor families that change their expression most in response to SAP54 (Figure 6.5C). However, this is explained by a mixture of \textit{AP2/EREBP} transcripts being upregulated and downregulated by SAP54 in female exposed plants (Figure 6.5A). Interestingly, the enrichment of MTFs among SAP54 most changed families of transcription factors in male and female exposed plants is not so high as, for example, alterations in chromatin remodelling factor expression (Figure 6.5C). Chromatin remodelling factors, including PWWP, SET, JUMONJI and others, display stronger downregulation in male exposed SAP54 plants compared to female exposed plants (Figure 6.5A,B).

Having analysed the main effect of SAP54 in male and female exposed plants separately, I wanted to characterise the main difference between female and male induced responses in 35S:GFP plants and investigate how SAP54 affects this male-female difference. To do this, I calculated the difference between male and female effect in 35S:GFP and, separately, 35S:GFP-SAP54 plants. Then I calculated the difference between male-female difference in 35S:GFP and 35S:GFP-SAP54 plants and investigated which transcriptional regulator categories are enriched using MapMan. The regulation of chromatin remodelling factors, including PWWP, GTE and JUMONJI families is most different between the two insect sexes in 35S:GFP plants (Table 6.2). In addition, females demonstrate higher relative induction of \textit{WRKY} and \textit{MYB} factors compared to males. In contrast, males show slight upregulation of Zn-finger GATA transcription factors compared to downregulation of these by females. Interestingly, that SAP54 has the strongest effect on the insect sex-specific
effects in chromatin remodelling factors such as CHROMATIN REMODELING 12 (CHR12), which arrests plant growth in response to environmental stress, as well as GTE and JUMONJI proteins (Table 6.2).

Taken together, these data suggest that SAP54 alters plant responses to insects through chromatin remodelling. Male and female leafhoppers have sex-specific effects on transcriptional regulation of plant chromatin structure and function. Chromatin remodelling factors are downregulated by the phytoplasma effector SAP54 largely in male-dependent manner.
Figure 6.5 (continued next page)
Figure 6.5 (continued next page)
Figure 6.5. The effect of SAP54 on female leafhopper (A) and male leafhopper (B) induced changes in plant transcriptional regulators. Red indicates upregulation and green – downregulation. Scale bar is log2(fold change). The relative enrichment of SAP54 altered transcripts by females (A) or males (B) are summarised in the table (C). P-values are based on Benjamini-Hochberg correction for multiple group comparison using Wilcoxon rank test. All significant SAP54 effects on male and female responses are displayed.
Table 6.2. Main differences in transcription regulators between female and male insect exposed plants are illustrated in the top panel of the table. The effect of phytoplasma effector SAP54 on the difference in transcription regulator expression between female and male leafhopper exposed plants are demonstrated on the bottom panel of the table. Enrichment analysis performed using Wilcoxon rank test and Benjamini-Hochberg correction for multiple comparisons. Only significant differences (p<0.05) are displayed.

<table>
<thead>
<tr>
<th>MapMan bin</th>
<th>Functional group</th>
<th>Nr of expressed genes</th>
<th>p-value</th>
</tr>
</thead>
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<td>28.1.3</td>
<td>chromatin structure (histone modification)</td>
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<td>1.6E-08</td>
</tr>
<tr>
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<td>WRKY domain transcription factor family</td>
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<td>3.0E-06</td>
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<td>Chromatin Remodeling Factors</td>
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<td>9.3E-06</td>
</tr>
<tr>
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<td>MYB domain transcription factor family</td>
<td>75</td>
<td>2.8E-04</td>
</tr>
<tr>
<td>27.3.9</td>
<td>C2C2(Zn) GATA transcription factor family</td>
<td>22</td>
<td>6.2E-04</td>
</tr>
<tr>
<td>27.3.68</td>
<td>PWPP domain protein</td>
<td>8</td>
<td>2.7E-03</td>
</tr>
<tr>
<td>27.3.52</td>
<td>Global transcription factor group</td>
<td>13</td>
<td>5.1E-03</td>
</tr>
<tr>
<td>27.3.67</td>
<td>other putative transcription regulators</td>
<td>123</td>
<td>1.0E-02</td>
</tr>
<tr>
<td>27.3.57</td>
<td>JUMONJI family</td>
<td>12</td>
<td>1.6E-02</td>
</tr>
<tr>
<td>27.3.40</td>
<td>Aux/IAA family</td>
<td>19</td>
<td>1.7E-02</td>
</tr>
<tr>
<td>27.3.60</td>
<td>NIN-like bZIP-related family</td>
<td>5</td>
<td>2.2E-02</td>
</tr>
<tr>
<td>27.3.39</td>
<td>AtSR Transcription Factor family</td>
<td>6</td>
<td>4.8E-02</td>
</tr>
</tbody>
</table>

Main effect of SAP54 on the difference between female and male induced responses

<table>
<thead>
<tr>
<th>MapMan bin</th>
<th>Functional group</th>
<th>Nr of expressed genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Chromatin Remodeling Factors</td>
<td>24</td>
<td>3.7E-07</td>
</tr>
<tr>
<td>27.3.67</td>
<td>other putative transcription regulators</td>
<td>123</td>
<td>6.8E-05</td>
</tr>
<tr>
<td>27.3.57</td>
<td>JUMONJI family</td>
<td>12</td>
<td>1.2E-02</td>
</tr>
<tr>
<td>27.3.52</td>
<td>Global transcription factor group</td>
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<td>1.2E-02</td>
</tr>
<tr>
<td>27.3.4</td>
<td>ARF, Auxin Response Factor family</td>
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<td>2.2E-02</td>
</tr>
<tr>
<td>27.3.68</td>
<td>PWPP domain protein</td>
<td>8</td>
<td>3.1E-02</td>
</tr>
</tbody>
</table>

6.2.5. Plant defence gene promoters contain binding sites for MTFs and other families of insect induced transcriptional regulators

In the previous section I identified potentially significant effect of SAP54 on various transcription factor families and chromatin remodelling factors. However, what are the targets downstream these regulators remains to be elucidated. To better understand such potential regulatory links, I selected plant defence modules from Figure 5.15 and analysed overrepresentation of binding sites for any particular family of transcriptional regulators within promoters of these defence genes. To calculate the enrichment of the known motifs of
regulatory cis-elements (obtained from AtcisDB) for MTF, WRKY, MYB, AP2/EREBP, DOF, bZIP, bHLH and GATA proteins I compared their frequency in promoters of defence-related genes to their overall occurrence in all of the expressed transcripts from A. thaliana genome (Table 6.3). Interestingly, I found that the DUF26 and extensin receptors, NLR genes and RAF family of MAP3K have a considerable overrepresentation of putative MTF binding sites. Similarly, SA biosynthetic genes, glucosinolates and carotenoids are enriched in CArG-box sites in their promoters compared to rest of the genome. WRKY, bHLH and GATA factors are generally overrepresented in all defence genes, including membrane and cytoplasmic receptors, suggesting that defence could be one of the main regulatory functions of these proteins. WRKY sites show the greatest overrepresentation in L-lectin NLR receptors and CDPKs, whereas bHLH and GATA binding sites are most often found in LRR genes. Interestingly, bHLH and GATA binding sites are also considerably overrepres ented in lignin and carotenoid biosynthetic pathways. Glucosinolates showed overrepresentation of several transcriptional regulators, including MYB-like factors, DOF, bZIP, bHLH and GATA regulators, suggesting a very diverse regulation of glucosinolate synthesis. In addition to overrepresentation within glucosinolate biosynthesis gene promoters, MYB-binding sites are enriched in several membrane receptor kinases as well as NLR proteins while DOF and bZIP binding sites are also frequently found in ET and JA signalling and various membrane receptors (Table 6.3).

In contrast to the ubiquitous WRKY, bHLH and GATA binding sites in defence genes, AP2/EREBP sites are not overrepresented in most of the defence modules, indicating that these regulators may function predominantly in other processes than biotic stress. Nevertheless, LRR and DUF26 receptors as well as ET signalling genes are enriched in AP2/EREBP binding sites (Table 6.3). Importantly, these receptors and ET signalling are likely to be important in mediating insect-sex specific responses in plants and were significantly affected by SAP54 (chapter 5). Thus, AP2/EREBP may function in a positive feedback loop with ET-related defence signals.
Table 6.3 (next page). Table of enrichment analysis for various transcription factor binding sites in the promoters of defence gene modules that are affected by SAP54. Table contains information about male and female specific significant (p<0.05) effects of SAP54, indicated by YES/NO. Number of expressed transcripts and transcripts containing the specified cis-regulatory element is provided. Overrepresentation of these sites is calculated using cumulative probability hypergeometric test when compared of the absolute frequency of the transcription factor binding sites in the whole set of 17153 expressed *A.thaliana* transcripts. The total number of binding sites is provided below the table. The p-value indicates the probability to find the indicated number or more cis-elements in any given module at random. Any probability below ½ is indicated in bold as overrepresentation.
236

Total nr
expressed
genes
CArG
W-box
AP2/EREBP
C2C2 DOF

NLR
SD
LRR
RAF
CDPK
L-lectin
RLCK
ET signalling
MAP4K
WAKL
CrRLK
LRKL
ZIK
MEKK
JA synthesis
PERKL
DUF26
ET synthesis
extensin
SA synthesis
glucosinolates
carotenoid
lignin synthesis

Defence and
secondary
metabolism
modules

Table 6.3

1301
11671
128
7870

17153

YES
YES
YES
YES
YES
YES
YES
YES
YES
YES
YES
NO
YES
YES
YES
YES
YES
NO
NO
NO
NO
NO
NO

YES
YES
YES
YES
YES
YES
YES
YES
YES
YES
YES
YES
YES
YES
NO
NO
NO
NO
NO
NO
YES
NO
NO

MYB
bZIP
bHLH
GATA

YES
YES
YES
YES
NO
NO
NO
NO
YES
YES
YES
YES
YES
YES
YES
NO
YES
YES
YES
YES
NO
NO*
NO*

2138
10682
7588
13818

85
26
135
37
21
21
66
15
9
11
12
10
9
15
18
5
33
5
4
13
65
24
41

9
1
9
5
0
1
2
0
0
1
1
0
0
0
0
0
6
0
1
2
6
3
0

SAP54
SAP54
SAP54
Nr of
Nr of
effect in effect in effect on
CArG
expressed
male
female femalebox
genes in
exposed exposed
male
binding
modules
plants
plants difference
sites
1.94E-01
8.72E-01
7.04E-01
1.46E-01
1.00E+00
8.09E-01
9.65E-01
1.00E+00
1.00E+00
5.80E-01
6.12E-01
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.00E+00
3.56E-02
1.00E+00
2.71E-01
2.59E-01
3.71E-01
2.73E-01
1.00E+00
71
23
100
27
21
21
54
11
6
9
8
10
8
10
12
4
29
4
3
11
42
16
27

9.49E-04
1.58E-02
7.65E-02
3.26E-01
3.06E-04
3.06E-04
9.09E-03
4.49E-01
6.84E-01
2.65E-01
6.70E-01
2.12E-02
1.63E-01
6.62E-01
6.56E-01
4.88E-01
8.10E-03
4.88E-01
6.17E-01
1.63E-01
7.68E-01
6.50E-01
6.86E-01

Nr of WCArG
box
W-box
p-value binding p-value
sites
0
0
3
0
0
0
0
1
0
0
0
0
0
0
0
0
1
0
0
0
0
0
0

1.00E+00
1.00E+00
8.02E-02
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.06E-01
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.00E+00
2.19E-01
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.00E+00
1.00E+00

11
3
12
1
3
0
10
3
2
2
1
1
1
2
2
0
1
0
1
1
11
2
4

Nr of
Nr of
AP2/
AP2/
MYB
EREBP EREBP
binding
binding p-value
sites
sites
4.96E-01
6.46E-01
9.24E-01
9.93E-01
4.96E-01
1.00E+00
3.05E-01
2.85E-01
3.12E-01
4.07E-01
7.98E-01
7.36E-01
6.98E-01
5.74E-01
6.76E-01
1.00E+00
9.88E-01
1.00E+00
4.13E-01
8.23E-01
1.81E-01
8.19E-01
7.69E-01

MYB
p-value

30
15
68
16
7
6
36
10
4
9
5
7
3
5
11
3
4
3
1
4
40
7
15

Nr of
DOF
binding
sites
9.82E-01
1.56E-01
1.67E-01
6.85E-01
9.17E-01
9.67E-01
9.85E-02
8.74E-02
6.59E-01
1.71E-02
7.17E-01
1.13E-01
8.63E-01
8.93E-01
1.44E-01
4.23E-01
1.00E+00
4.23E-01
9.14E-01
9.17E-01
7.94E-03
9.70E-01
9.13E-01

DOF
p-value

49
9
77
25
15
13
36
8
7
4
8
7
5
8
12
4
15
3
1
10
51
10
21

Nr of
bZIP
binding
sites
8.40E-01
9.99E-01
9.11E-01
3.14E-01
2.65E-01
6.09E-01
9.22E-01
8.37E-01
2.77E-01
9.80E-01
5.04E-01
4.41E-01
7.79E-01
8.37E-01
4.52E-01
3.77E-01
9.84E-01
7.21E-01
9.80E-01
2.14E-01
3.96E-03
9.88E-01
9.46E-01

26
15
81
20
11
11
37
11
7
5
7
7
5
7
13
2
15
3
2
7
38
12
23

9.96E-01
1.18E-01
1.58E-04
1.50E-01
2.96E-01
2.96E-01
3.53E-02
2.20E-02
4.51E-02
5.83E-01
2.43E-01
9.33E-02
3.61E-01
5.24E-01
1.55E-02
7.32E-01
5.11E-01
3.93E-01
5.97E-01
3.35E-01
1.46E-02
3.56E-01
8.52E-02

Nr of
bZIP
bHLH
bHLH
p-value binding p-value
sites
60
23
125
33
21
21
62
15
9
10
12
10
9
12
17
4
26
5
3
13
60
24
39

9.91E-01
2.27E-01
7.72E-05
1.28E-01
1.06E-02
1.06E-02
2.02E-03
3.90E-02
1.43E-01
3.39E-01
7.46E-02
1.15E-01
1.43E-01
6.69E-01
1.09E-01
7.49E-01
6.95E-01
3.39E-01
8.28E-01
6.01E-02
7.65E-03
5.56E-03
8.24E-03

Nr of
GATA GATA
binding p-value
sites


I further investigated how frequent are the MTF binding sites within the promoters of members of other transcription factor families. Moreover, are transcription factors that have MTF binding sites in their promoters downregulated by male insects in SAP54 plants? To do this I filtered out all transcripts that lack CArG motif in their promoter from Figure 6.5 and displayed separately in Figure 6.6.

Figure 6.6. The effect of SAP54 on male-induced expression of A. thaliana transcriptional regulators (A). Only genes with putative MADS-box binding sites are indicated. The graph plots the log2(fold change) of the difference between male induced
transcripts in presence of SAP54 minus male induced transcripts in absence of SAP54. Green indicates transcripts which are suppressed but red – activated by SAP54 relative to GFP control. (B) There is no enrichment of responsive transcriptional regulators that contain CArG-box in their promoter and show greatest changes between male exposed 35S:GFP-SAP54 and 35S:GFP plants. Analysis performed using Wilcoxon rank test and Benjamini-Hochberg correction for multiple comparisons.

I found that putative MTFs binding sites occur in several other transcription factor families, apart from C2C2 DOF factors and GATA regulators, which do not have predicted CArG-box in their promoters (Figure 6.6A). In many other transcription factor families the probability of CArG-box occurrence is higher than in a random list of genes (Table 6.3). Interestingly, from all transcriptional regulators that have putative MTFs binding sites and are differentially regulated between male exposed SAP54 and control plants, there is no significant enrichment for any transcription factor family (Figure 6.6B). From the genes that have predicted CArG-box, male insects differentially regulate several transcripts in MTF, MYB as well as chromatin modifying protein families in 35S:GFP-SAP54 plants (Figure 6.6A). In addition, males suppress a few ARABIDOPSIS RESPONSE REGULATORS (ARR1 and 2) and upregulates transcriptional repressors of the NUCLEAR FACTOR Y family (e.g., HAP3) in SAP54 dependent manner. The former are implicated in SA signalling (Choi et al., 2010), whereas the latter – in flowering time control as well as root elongation and responses to draught stress (Nelson et al., 2007; Ballif et al., 2011).

To summarise, MTFs may potentially regulate several defence modules, including extensin and DUF26 receptors, NLRs, RAF kinases, SA pathway, glucosinolates and carotenoids, based on MTF-binding site predictions in gene promoter regions. In addition, MTF binding sites are found on range of other transcriptional regulators and chromatin remodelling factors which respond transcriptionally to insect attack in SAP54 plants. Many of the observed transcriptional changes in defence genes may be mediated by MTFs indirectly via their downstream effects on WRKY, AP2/EREBP, MYB, bHLH, or bZIP regulators which have ample binding sites in defence gene promoters and are likely to be part of positive or negative regulatory loops. Synthesis of glucosinolates, carotenoids and lignins may be controlled by GATA and DOF transcription factors which may not be directly regulated by MTFs.
6.2.6. SAP54 may interact with other targets than only MTFs

MTFs are the only known targets that are destabilised in planta by SAP54 (MacLean et al., 2014). Moreover, MTFs appear to be important for host plant selection by leafhoppers (section 6.2.2). Nevertheless, the role of other potential plant targets of SAP54 in generation of leaf-like flowers or insect oviposition preference have never been investigated. Here I gather results from a Y2H screen for SAP54 interactions with A. thaliana proteome library (Hybrigenics Services, France). In addition to MTFs, this revealed numerous other candidate protein-protein interactions between SAP54 and plant transcriptional regulators. I combined the interaction data with the RNA-seq data to reveal that many of the predicted strong interactors are significantly downregulated in SAP54 in sex specific manner (Figure 6.7).

**Figure 6.7.** Potential SAP54 interacting non-MTF plant targets show expression change in response to male and female insects in 35S:GFP and 35S:GFP-SAP54 plants. Log2(fold change) is displayed as measure of differential regulation of male or female insects.
female exposed plants compared to no-insect exposed plants. Red colour indicates upregulation and green - downregulation in the following treatments: (Lane 1) female vs no insect exposed GFP plants, (Lane 2) female vs no insect exposed SAP54 plants, (Lane 3) male vs no insect exposed GFP plants, (Lane 4) male vs no insect exposed SAP54 plants. The corresponding significance test for differential regulation is highlighted as YES or NO (p<0.05; q<0.05). Clustering based on Euclidean distance via centroid linkage (Cluster 3.0), viewed in TreeView. The strength of SAP54 interactions are graded from A (very high confidence for interaction) to D (moderate confidence for interaction); N/A (interaction confidence score cannot be calculated). Interactions with high or very high confidence are shaded blue. Number of the predicted CArG-box sites in gene promoters obtained via TAIR sequence motif search tool.

I discovered that numerous strong candidate SAP54 interactors are significantly downregulated in male exposed 35S:GFP-SAP54 plants but not in any other treatment. One of such interactors is RAD23C which is known to mediate MTF degradation via 26S proteasome and required for host plant selection by leafhoppers (MacLean et al., 2014). Other such candidates may play various roles in a range of cellular processes like autophagy, cell division, vacuolar traffic or transcriptional regulation. Furthermore, several SAP54 candidate interactors have CArG-box site in their promoter, suggesting a putative regulation by MTFs.

Some SAP54 interactors may have similar expression pattern to plant defence signalling genes. I clustered all genes showing high or very high interaction confidence from Figure 6.7 together with annotated defence signalling genes in Figure 5.15 based on their transcriptional response to male and female insects in 35S:GFP and 35S:GFP-SAP54 plants (Figure 6.8). I identified clusters that contained transcripts that were significantly differentially regulated in any of the insect treatments on SAP54 or control plants. Interestingly, cluster 1 in Figure 6.8 contained several candidate SAP54 interactors like serine peptidase TTP2 or chloroplast-localised gene of unknown function LETM1 which are upregulated only in female exposed 35S:GFP plants but downregulated in all other treatments similar to many RLKs, LRR-NBS proteins, MAP kinases and SA/JA signalling genes. Interestingly, KIP1-like kinase interacting protein clustered with LRR RLKs and a LRR-NBS receptors (cluster 2) which were significantly downregulated only by male insects in 35S:GFP-SAP54 plants (Figure 6.8). Other SAP54 interacting
targets like putative bHLH transcription factors are significantly upregulated in all insect exposed plants regardless of the presence of SAP54 (Cluster 3).

Figure 6.8
Figure 6.8. Candidate SAP54 interactors and plant defence genes demonstrate co-expression in response to insect attack in 35S:GFP and 35S:GFP-SAP54 plants. The A and B score interactors from Figure 6.7 are clustered together with 1062 defence genes annotated from figure 5.15. Each gene TAIR identifier was linked to the belonging of a functional group such as JA signalling or particular PRR family, as displayed in the clusters. Column 1 indicates transcript upregulation (red) or downregulation (green) in female exposed plants without SAP54; column 2 - male exposed plants without SAP54; column 3 - female exposed SAP54 expressing plants; column 4 - male exposed SAP54 expressing plants. Clustering based on Euclidean distance via complete linkage (Cluster 3.0), viewed in TreeView (colours depict log2(fold change)).

Taking together, SAP54 has other candidate interactors in addition to MTFs (Figure 6.7). These putative interactors are appear downregulated in male-exposed SAP54 plants together with numerous plant defence genes (Figure 6.7, 6.8). In contrast, SVP1, MAF5 which are involved in plant-insect interactions (Figure 6.3) are upregulated in male-exposed SAP54 plants (Figure 6.2).

6.4. Discussion

In this chapter I investigated how MTF expression is influenced by male and female insects in 35S:GFP and 35S:GFP-SAP54 plants. I demonstrated that a subset of MTFs is expressed in plant vegetative growth stage and is differentially regulated by male and female leafhoppers in SAP54-dependent manner (Figures 6.1, 6.2). Furthermore, MTFs play an important role in plant-insect interactions. Knock-out of MAF5 and SVP1 significantly enhanced leafhopper egg-laying preference in healthy plants but not during AY-WB infection when SAP54 is in the plant (Figure 6.3), suggesting that SAP54-dependent enhancement of insect oviposition preference could be MTF mediated. In contrast, AGL24 and FUL are required for insect oviposition on phytoplasma infected plants but not on healthy plants (Figure 6.3), suggesting that these MTFs may be required for the remodelling of MTF network in such a way that attracts insects for egg-laying. Male insects significantly downregulate several families of transcription and chromatin remodelling factors in SAP54 plants. Many of these regulators have predicted MTF binding sites in their promoters with probability higher than in random list of genes (Figure 6.6), suggesting that MTFs could mediate the transcriptional remodelling of male
colonised SAP54 plants. Furthermore, leaf-expressed MTFs have similar expression pattern to many plant receptor kinases and defence genes (Figure 6.4), suggesting a potential regulation of plant defences downstream MTFs. Although MTFs are shown to be destabilised in planta by SAP54 (MacLean et al., 2014) and have an effect on plant-insect interactions (Figure 6.3), SAP54 may have additional targets that could mediate the male-specific suppression of plant defences required for female egg laying (Figures 6.6 and 6.7).

Figure 6.9. Phytoplasma effector SAP54 may alter plant MTF network in AGL24- or FUL-dependent manner to suppress MAF5 or SVP1 functions. Such remodelling of MTF network may cascade to global reprogramming of plant transcription factors and changes in chromatin activity to suppress insect induced plant defence responses that are key in insect selection of plant hosts for oviposition. In addition to MTFs, SAP54 may have other targets that could be influenced by MTF network remodelling in insect specific manner and could contribute to the transcriptional remodelling of plants.

SAP54 interacts and destabilises type II MTFs, including AP1, SEP3 and SOC1 (MacLean et al., 2014) and is likely to interact with several other MTF targets in leaves (Figure 6.2). The known and potential SAP54 MTF targets lie at the heart of a complex and dynamic MTF interaction network. MTF proteins have multiple interactions with each other: they form homo- and hetero-dimers as well as higher order complexes, known as floral quartets, to control meristem identity and specify vegetative and reproductive organs (Davies et al., 1996; Egea-
Moreover, MTFs interact with nucleosome (chromatin) remodelling factors and form complexes with other transcription factor families to control flower development (Smaczniak et al., 2012b; Simonini et al., 2012). Therefore, interaction and destabilisation of MTFs may have a myriad of effects on other MTFs as well as other families of transcriptional regulators and chromatin remodelling factors (Figure 6.5). This could happen either via direct transcriptional regulation of other transcriptional regulators by binding to their promoters (Figure 6.6) or post-translationally by disrupting the transcription factor protein complexes and the existing regulatory feedback loops.

Changes in MTF network could result in up- or down-regulation of certain MTFs that are important in plant-insect interactions. Interference with MAF5 and SVP1 functions by SAP54 may be required to enhance female leafhopper oviposition preference. Moreover, other components like AGL24 and FUL could be required for such remodelling of MTF network. Since MAF5 and SVP1 are actually upregulated in male exposed 35S:GFP-SAP54 plants compared to downregulation in male exposed 35S:GFP plants, there is a possibility that SAP54 may, nevertheless, disrupt the protein complexes formed by MAF5 or SVP1 with their interactors, thus mimicking the effect of maf5 and svp1 mutants on insect oviposition choice (Figure 6.3). In such case expression levels of MTFs may not necessarily correlate with the protein levels and functions. Furthermore, in the current display of RNA-seq data, spliced variants of MTF proteins and other regulators were not considered. Neither MAF5, SVP1, AGL24 nor FUL directly interact with SAP54 (Figure 6.2). Nevertheless, the latter three are interactors of SOC1 – a direct target of SAP54. Moreover, SVP1 or AGL24 separately form complexes with both AP1 and SEP3 (Gregis et al., 2006, 2009), and FUL can interact with AGL24, based on Y2H studies (de Folter et al., 2005). Both AP1 and SEP3 are destabilised by SAP54 (MacLean et al., 2014). However, since AP1 and SEP3 are not expressed in the vegetative tissue (Figure 6.1), SVP1 or AGL24 may be interacting with another, yet unknown, MTF that may be targeted by SAP54. Interestingly, the soc1 and ap1 mutants did not show any significant effect on insect oviposition choice (Figures 3.6 and 6.3), indicating that remodelling of MTF network may be mediated via destabilisation of multiple MTFs. Furthermore, the role of the MAF5, SVP1, AGL24 nor FUL in plant-insect
interactions may be mediated through higher order complex formation among the leaf-expressed MTFs.

The interactions and resulting functions of MTF proteins in leaves could be very different from MADS role in orchestrating the floral transition by specifying inflorescence meristem and floral meristem identity. Most type II MTFs are known for their roles in flowering time regulation and floral organ development (Dornelas et al., 2011; Smaczniak et al., 2012a). For example, MAF5 and SVP1 are characterised as floral repressors, whereas AGL24 and FUL are positive flowering regulators. MAF5 and SVP1 negatively regulate floral signal integrators FT, SOC1 and FUL, thus suppressing shoot apical meristem from acquiring the inflorescence identity (Torti and Fornara, 2012; Shen et al., 2014a). AGL, FUL and SOC1 stimulate flowering by positively regulating inflorescence meristem identity and expression of floral meristem identity genes AP1 and LFY (Lee et al., 2008; Torti and Fornara, 2012). AGL24 is important in maintaining the inflorescence meristem and needs to be repressed by LFY/AP1 to activate floral homeotic genes and form determinate flowers with the characteristic organs (Yu et al., 2004). Although 35S:GFP-SAP54 plants are early flowering (Figure 3.7), like maf5 and svp1 mutants, and show indeterminate inflorescence growth characteristic to AGL24 function in floral meristems, the modulation of flowering time or meristem identity may not be the primary target of SAP54 to attract insects. First, experiments in chapter 3 demonstrate that SAP54 effect on insect egg laying is independent from plant being in vegetative, inflorescence or floral “state” (Figure 3.4). Second, lfy and ap1 mutants, arrested in the inflorescence state, did not affect leafhopper choice (Figure 3.6). This suggests that SAP54 may alter AGL24, FUL, SVP1 or MAF5 functions in vegetative tissue that are different from the functions of these proteins during floral transition and floral meristem specification.

AGL24 and SVP1 are known to have stage specific functions in plant reproductive growth. While being activator or repressor of inflorescence meristem at early stages of floral transition, later in floral meristems they form complex with AP1 and SEP3 to recruit LEU-SEU floral repressor of flower organ identity genes (Gregis et al., 2006, 2009). It may be possible that in the vegetative tissue they have completely different interactors and yet different functions from inflorescence meristems and floral meristems. Moreover, phytoplasmas may infect plants at different stages of development – before or after flowering.
Therefore, SAP54 may target multiple MTFs for robust modification of plant defences during vegetative and floral stages. I use Iceberg analogy to illustrate this idea (Figure 6.9). *maf5* and *svp1* mutants had significant role in insect choice during vegetative stage, as in the experiments performed in this chapter (Figure 6.3). However, different MTFs mutants may show the same response after bolting or during flowering. Such scenario implies that different subsets of MTFs may regulate plant defences during vegetative and reproductive growth. Further experiments need to be performed to test this.

Figure 6.10. Imagine a floating iceberg representing the plant genome and the submerged under-water part of the iceberg being the expressed genome. In vegetative organs a certain subset of MTFs may have diverse roles in leaf and root development as well as defence, whereas Fruit or flowering genes and their regulators are not expressed. During developmental time the water temperature rises and the underwater parts of the iceberg melt. Reduction of mass underwater makes icebergs flip over. This symbolises the transition from vegetative to flowering stages when a different subset of MTFs become active and regulate flowering time and plant reproduction. Certain functions like defence may be still regulated by MTFs but a different subset from the vegetative stage.

There is increasing evidence for MTF roles in other plant processes in addition to developmental regulation. For example, LFY is expressed during reproductive growth and is regulating biotic stress related genes, including receptor kinases, WRKY and MYB transcription factors to suppress plant defence response to *Pseudomonas* (*Winter et al.*, 2011). Chip-seq experiments have
identified that SVP1 has different set of targets in vegetative and reproductive growth stages. Jasmonate-signalling genes like COI1 and JAZ proteins were identified as potential direct targets of SVP1 in vegetative stage, and showed altered expression in *svp1* mutant or overexpression lines (Gregis *et al.*, 2013). SOC1 can target miRNA319 with known function in regulating TCP transcription factors and jasmonate synthesis (Immink *et al.*, 2012). Moreover, in addition to regulating each other and themselves, SOC1 and SVP1 have common transcription factor targets, indicating to the importance of the regulatory feedback loops in MTF network (Tao *et al.*, 2012). MTFs may regulate plant defence in growth-stage specific manner, depending on the presence of co-expressed interactors or (post-)translational modifications of MTFs. When all protein-DNA and protein-protein interactions are clustered in interaction-dense subnetworks, there remain substantial links between such subnetworks or modules in addition to dense interactions within each module (Boucher *et al.*, 2016). This suggests that, depending on the timing of expression or cellular co-localisation of genes in neighbouring modules, a given node in a pathway (corresponding to intra-connected module) may branch out to have numerous other effects on neighbouring pathways. Furthermore, a phenomena, known as protein moonlighting, occurs when spliced variants or small (post-translational) changes in protein (e.g., receptor kinases or transcription factors) determines its role in multiple cellular processes (Jeffery, 2014, 2016).

Type I MTFs are key regulators in gametophyte, embryo and seed development (Masiero *et al.*, 2011). Despite few reports of MTF role in nutrient uptake, root elongation, lateral root development or stomatal patterning in leaves (reviewed in Smaczniak *et al.*, 2012a), the role of type I and II MTFs in vegetative organs is largely unknown.

The effects of MTFs on plant transcriptome could be mediated via nucleosome binding and chromatin remodelling factors (section 6.2.4). MTFs may regulate chromatin state of other MTFs or other transcription factor families. Interestingly, SET family chromatin remodelling factors SDG8 and SDG26 are positive regulators of MAF5 and MAF4 (Liu *et al.*, 2016) while Polycomb RING1 is a suppressor (Shen *et al.*, 2014b). The expression of these three chromatin remodelling factors is significantly suppressed in male exposed SAP54 plants (Figure 6.5), suggesting that SAP54 effect on MTFs may be mediated via chromatin remodelling. In addition, RAD23 showed significant male dependent
effects in SAP54 plants (Figure 6.7). In yeast, RAD4-RAD23 proteins are known to be key in chromatin remodelling during DNA repair (Gong et al., 2006). Thus, the observed male-dependent effects on chromatic state in SAP54 plants may have multiple mechanistic explanations.
"I am never content until I have constructed a mechanical model of the subject I am studying. If I succeed in making one, I understand; otherwise I do not."

“You can understand perfectly, if you give your mind to it”

Lord Kelvin (1904), *Baltimore Lectures on Molecular Dynamics and The Wave Theory of Light*

**Chapter 7**

**General Discussion on Ecological and Molecular Mechanisms in Plant-Microbe-Insect Interactions**

Part of this chapter is published in:

7.1. Summary and implications of the key findings

26S proteasome shuttle protein RAD23 is required for generation of phyllody in phytoplasma-infected plants (MacLean et al., 2014). In this thesis I discovered that AY-WB infection also enhances insect egg-laying preference in RAD23 dependent manner. This suggested that phytoplasma-induced leaf-like flower phenotype and enhancement of insect colonisation are genetically linked via RAD23. Phytoplasma effector SAP54 interacts with both RAD23 and MTFs, resulting in MTF degradation by plant 26S proteasome and induction of phyllody (MacLean et al., 2011, 2014). Furthermore, SAP54 significantly enhanced insect oviposition compared to control plants. These findings supported the hypothesis that phytoplasma may alter plant floral development to enhance insect vector colonisation of infected plants and thus aid phytoplasma spread in nature.

Surprisingly, I found that insect egg-laying preference for SAP54 plants is independent from the SAP54-induced developmental changes in plants and does not require plant transition from vegetative growth to bolting and flowering. Instead, SAP54 appears to modify processes in rosette leaves to promote leafhopper oviposition (Orlovskis and Hogenhout, 2016). Furthermore, I discovered that female leafhopper oviposition preference for SAP54 plants does not require female contact with males but depends on male insect simultaneous presence. This suggested that male insects induce plant responses that may be altered by SAP54 to attract females.

I analysed plant transcriptional responses to male and female leafhoppers on SAP54 plants and plants without SAP54. Surprisingly, male and female insects elicit sex-specific plant responses characteristic to PTI and ETI. Moreover, SAP54 suppresses insect induced plant responses in sex-specific manner by selectively downregulating male-induced defence and secondary metabolism pathways. Male colonized SAP54 plants demonstrate downregulation of plant hormone and defence responses to insects. This suggests that SAP54 may suppress plant priming to herbivores in insect sex-specific manner.

Furthermore, I identified four MTFs that are expressed in plant leaves and play important roles in egg-laying preferences by leafhoppers as well as demonstrate sex-specific regulation by SAP54. Firstly, this indicates that MTFs have additional roles in regulation of plant defence against herbivores. Secondly, it gives additional support to the model outlined in Figure 7.1. In this model SAP54
degrades MTFs to alter the function of MTF network in order to modulate male recognition by plant and male-induced plant responses. This stimulates female insect to choose male-exposed SAP54 plants for oviposition. Changes in flower development emerge as a result of remodelling MTF network. However, plant morphological changes are not required for female insect attraction to the plant.

Figure 7.1. Schematic model of the mechanism and role of phytoplasma effector SAP54 in enhancing insect vector colonisation of phytoplasma-infected host plants.

Key future work to verify or refine this mechanistic model would include 1) investigation of all (especially MTF) interactors of SAP54 in planta; 2) test whether plant exposure to male leafhoppers is required for female oviposition preference on MTF mutants that interact with SAP54 in planta and MTFs that are important for plant resistance to insects; 3) determine if MTF mutants demonstrate transcriptionally similar plant response to insects as SAP54.

Together, data in this thesis suggest that modulation of insect induced plant responses can be considered the extended phenotype of phytoplasma SAP54 gene. Increased insect vector egg-laying preference for infected plants expressing SAP54 may increase transmission of the pathogen in nature and thus benefit phytoplasma fitness.
7.2. Mechanisms of effector-mediated target destabilisation

Phytoplasma effector SAP54 degrades MTFs by using 26S proteasome shuttle protein RAD23 (MacLean et al., 2014). However, a detailed mechanism how MTFs are recruited to the proteasome complex and how SAP54 evade degradation by the proteasome remain to be elucidated in future experiments. MacLean and colleagues (2014) proposed several mechanistic hypotheses (Figure 7.2).

Figure 7.2. Models of SAP54-mediated degradation of MTFs. (A) SAP54 binds directly to both MTFs and RAD23. The latter takes the SAP54–MTF complex to the plant UPS where the MTFs are degraded. SAP54 may remain associated with RAD23 to prevent being degraded. (B) RAD23 and SAP54 do not interact directly, but via one or more ubiquitin moieties linked via lysine (K) residue(s) on SAP54. RAD23 takes the SAP54–MTF complex to the plant UPS (as in A). (C) An unknown pathway is involved in transportation of SAP54–MTF complexes to the host UPS, whereupon SAP54 interacts with RAD23 to evade degradation. RAD23 and SAP54 may interact directly (as in A) or via ubiquitin (as in B). Image taken from MacLean et al., 2014.
SAP54 has many conserved leucine, asparagine and glutamic acid residues along the peptide (Figure 2.6) and a predicted coiled-coil (CC) structure (Figure 7.3A). CC structure may be important for SAP54 interactions with the K-domain of MTFs (Figure 2.2). A proline residue at residue 53 may influence protein folding into two interaction CC helices which may be important for simultaneous interaction with MTFs and RAD23 in models A and B in Figure 7.2. I generated two SAP54 truncations by deleting either the N’- or the C’-terminal portion of the peptide up to the proline residue 53. This may generate two CC peptides as outlined in Figure 7.3A. I further generated transgenic *A. thaliana* lines to see if either of the two CC fragments of SAP54 could induce the leaf-like flower phenotype. Interestingly, both N’- and C’-terminal truncated fragments of SAP54 produced determinate flowers with normal floral organs (figure 7.3B). This indicates that residues in the both halves (or correct folding of the whole length SAP54) may be required for MTF degradation and induction of phyllody. Other truncations and single amino-acid mutations could be further analysed in a protein-protein interaction assay, such as yeast-two-hybrid, to elucidate the MTF and RAD23 interacting domains.

Cristal structure of SAP54 together with its MTF and/or RAD23 interactors would greatly enhance understanding about the structural motifs and residues that are involved in SAP54 interaction with MTF and RAD23 targets. It could be possible that the two predicted CC halves of a single SAP54 molecule simultaneously interacting with RAD23 or MTFs as in models A and B of Figure 7.2. Alternatively, the entire SAP54 may interact with either MTF or RAD23 and later form dimers to shuttle the complex to proteasome for degradation, consistent with model C in Figure 7.2. Interestingly, genomes of some phytoplasmas like *Ca. P. aurantifolia* strain SPLL encodes a tandem duplicate of SAP54 homolog (Al-Subhi, unpublished data), suggesting that SAP54 may be expressed as a dimer.

The exact mechanism how the other phytoplasma effector SAP11 destabilises its targets is unknown, too. There could be many possible mechanisms how phytoplasma effectors interfere with their target functions. Degradation of plant targets via 26S proteasome could be one mechanism, which is employed by other plant pathogenic bacteria as well (Nomura *et al.*, 2006). A review by Howden and Huitema (2012) discussed that bacterial effectors may also 1) exhibit a direct enzymatic activity in target phosphorylation, ubiquitination
or SUMOlation or 2) act as mimics of such signals, e.g., ubiquitin mimics, as well as 3) recruit plant enzymes that modulate the function of the target proteins.

**Figure 7.3.** Prediction of SAP54 structural domains that may be required for induction of leaf-like flower phenotype. (A) After the cleavage of the N'-terminal signal peptide (not shown), the remaining peptide of SAP54 is highly predicted to fold into two coiled-coil (CC) structures from residues 20-48 (red fragment) and 55-90 (red fragment) approximately, separated by a non-CC “bridge” (middle of blue fragment) around the proline residue at position 53. The first 20 residues at N'-terminal end of SAP54 are not predicted to form CC (green fragment). The two CC fragments may interact with each other, and the non-CC and part of the CC sequence in the middle of SAP54 may form a “bridge” between the two interacting CC helices. Two truncated forms of SAP54 – SAP54Δ1 and SAP54Δ2 were generated to transform *A.thaliana* with either of the two CC fragments. (B) 35S:GFP-SAP54Δ1 and 35S:GFP-SAP54Δ2 plants produce normal flowers.
7.3. *Microbial pathogens may use diverse strategies to increase their virulence and spread in nature*

Fitness of many microbial plant pathogens depends on the ability to evade recognition by their plant hosts and suppress pathogen-induced plant defence responses. In addition, pathogens which rely on vectors for transmission between plant hosts would benefit from increased host colonisation by the vectors. There are several potential strategies that plant microbial pathogens may employ to achieve these “goals”. One involves modulation of plant immunity (Boller and He, 2009) and the other may involve alteration of other plant processes, including development and tissue morphology (Mescher, 2012). I will briefly outline both in the following sections. I will discuss that in some cases the manipulation of development by itself may be adaptive to enhance the microbial colonisation or transmission. However, in other cases the evidence for the adaptive role of developmental changes is not so compelling. Instead, modulation of development may be a side-effect of targeting regulators with pleiotropic roles in development and plant immunity (Orlovskis *et al.*, 2016).

7.3.1. *Microbial pathogens evade plant immune recognition and suppress plant defences.*

Microbial plant pathogens show variations in molecules (MAMPs) on their surface or molecules inside their cells that would typically be recognised by plant Pathogen Recognition Receptors (PRRs). For example, modulation of bacterial cell wall chitins, peptidoglycans, lipopolysaccharides, flagellin or protein elongation factor Tu may evade recognition by plant receptors (Pel and Pieterse, 2013).

A complementary strategy involves delivery of bacterial effector molecules in the apoplast or inside plant cell to modulate pathogen recognition and downstream defence responses in plants. For example, some fungal pathogens secrete chitin binding proteins (de Jonge *et al.*, 2010) while some bacteria produce flagellin degrading peptidase (Bardoel *et al.*, 2011) to impede MAMP recognition by plant PRRs and molecules that can detoxify the apoplastic environment and inhibit host plant proteases (reviewed in Okmen and Doehlemann, 2014).
Pathogenic bacteria, like *Pseudomonas*, secrete a plethora of effector proteins inside the plant cell to suppress PTI (Boller and He, 2009). *P. syringae* effectors AvrPto and AvrPtoB can interfere with kinase functions of PRRs (Xiang et al., 2008) or complex formation between PRRs and their SERK co-receptors (Shan et al., 2008). Furthermore, HopA1 inactivates MAPKs dependent signals downstream of pathogen perception (Zhang et al., 2007). In addition, HopU1 targets several RNA-binding proteins such as GRP7 that interacts with transcripts of FLS2 and EFR – two important plant PRRs (Nicaise et al., 2013). Another *Pseudomonas* effector, HopM1, degrades MIN7, which may be involved in vesicle trafficking (Nomura et al., 2006) and perhaps transport of PRRs to the cell membrane. Bacterial effectors can interfere with plant hormone signalling as well. For example, HopI1 suppresses salicylic acid (SA) defences (Jelenska et al., 2007) but HopX1 upregulates jasmonic acid (JA) defences to potentially complement the effect of HopI1 on SA (Gimenez-Ibanez et al., 2014). In addition to *Pseudomonas* effectors mentioned above, there are many other effectors in *Ralstonia*, *Xanthomonas*, *Pantotea* interfering with plant targets which may function in modifying plant PTI (Block et al., 2008).

The strategies above illustrate that pathogens have specific adaptations to target plant immunity. Moreover, individual bacterial effector proteins may not function in isolation but rather act in concert to achieve robust modulation of induced plant immune responses.

### 7.3.2. Phytoplasma effectors may act in concert to alter plant immunity against insect vectors

Insect-vectored plant pathogens are known to modulate plant processes such as emission of volatile compounds or plant colour to attract their insect vectors (Mauck et al., 2016). However, whether insect vector attraction also occurs via suppression of insect-induced plant immune responses is yet elusive. Findings presented in this thesis (Chapters 5 and 6) suggest that phytoplasma effector transcriptionally remodels plant immune responses to insects, including PTI-like responses. Data suggest strong association between male-dependent female leafhopper oviposition preference for SAP54 plants and male-specific remodelling of plant responses to herbivores by SAP54 (Chapter 4). Thus, the SAP54-dependent alteration of plant transcriptional responses to insects may be
required for host plant selection by leafhoppers. Given that SAP11 suppresses JA pathway to increase leafhopper fecundity (Sugio et al., 2011), the two effectors – SAP11 and SAP54 – may act together to attract insect vectors to plants and increase the egg-laying on the insect-selected plants.

7.3.3. Parasites alter host plant vegetative and reproductive development

Prokaryotic and eukaryotic plant parasites have diverse effects on cellular structure as well as on organ and tissue morphology of their plant hosts. A detailed review by Le Fevre et al. (2014) discusses numerous examples of tissue proliferation and tissue or organ trans-differentiation by plant pathogenic bacteria, fungi and nematodes. In addition, insect herbivores such as galling wasps change plant morphology by producing galls that range from open pits on the surface of plant leaves to swollen enclosed structures with spikes or hairs (Stone and Schönrogge, 2003). Many of such morphological modifications may be caused by delivery of microbial nucleic acids (T-DNA, small RNAs) or protein effectors that pathogenic microbes or herbivores secrete into the plant (Evangelisti et al., 2014; Le Fevre et al., 2014). Alternatively, production of plant hormone (auxin, cytokinins, abscisic acid, salicylic acid) mimics by microbes or insects may be involved in remodelling plant tissues (Giron et al., 2013; Denancé et al., 2013; Le Fevre et al., 2014). In most cases, however, the molecules inducing the developmental alterations in plants and their targets are unknown.

Examples with phytoplasma effectors SAP54 and SAP11 targeting MTFs and TCPs, respectively, are not isolated incidents in nature where parasite effectors target transcriptional regulators with known function in development. For example, Xanthomonas effector AvrBs3 upregulates bHLH-domain transcription factor upa20, a master regulator of cell size (Kay et al., 2007). Similarly, anther-smut fungus Microbotryum violaceum differentially regulates homologs of floral homeotic genes SUPERMAN and PISTILLATA in dioecious plant Silene latifolia to induce anther genesis in female flowers (Kazama et al., 2005, 2009). Together with research on phytoplasma effectors, these studies also demonstrate the utility of pathogen infections and their effector proteins as tools for studying the genetic regulation of plant development.
7.3.4. Phytoplasma effectors may act in concert to alter plant development

When individual phytoplasma effectors are ectopically expressed in plants, they generate plant phenotypes that are reminiscent of certain developmental alterations in phytoplasma-infected plants. This is known for SAP11 and SAP54 and discussed previously. However, other phytoplasma effectors may also interfere with plant development. For example, ectopic expression of another phytoplasma candidate effector SAP05 in *A. thaliana* (Col-0) shows increased number of aerial rosette leaves from axillary meristems (AM) compared to wild-type Col-0 plants. SAP05 was found to interact with GATA transcription factors (A. MacLean, unpublished data). Furthermore, the number of rosette leaves from AM increases with the emergence of secondary stems from the AM (Figure 7.4). Interestingly, SOC1 and SOC1-like MTFs AGL42, AGL71 and AGL72 control induction of flowering stems as well as aerial rosette leaves from AM (Dorca-Fornell *et al.*, 2011). It is, therefore, possible that the downstream effects of several phytoplasma effectors may converge to certain families or transcription factors and demonstrate some degree of cross-talk.

![Figure 7.4. Induction of aerial rosette leaf number by SOC1-like genes and ectopic expression of phytoplasma effector SAP05.](image)

(a) Wild-type Col-0 plants usually develop single cauline leaf at axillary meristems (AM) that give rise to secondary branches. (b) *soc1* and *soc1 agl24 ami:agl71-72* mutants show increased number of aerial rosette leaves from AM. (d) 35S:SAP05 plants demonstrate numerous aerial rosette leaves from AM before emergence of secondary stems. (e) The number of aerial rosette leaves from AM in 35S:SAP05 plants increases after emergence of secondary stems. AM indicated by arrows in all photos. Scale bars are approximately 1 cm. Images (a-c) taken from Dorca-Fornell *et al.* (2011). Plants for images (d-e) generated by Dr Allyson MacLean.
Phytoplasma candidate effector SAP41 was found to interact with *A. thaliana* Basic Pentacysteine (BPC) transcription factors in a yeast-two-hybrid screen (A. Zwolińska, unpublished data). BPC facilitate MTF complex binding to their target genes, for example, binding of AP1-SVP1-SEU-LUG repressor complex to STK promoter (Simonini *et al.*, 2012).

Ectopic expression of candidate effector SAP44 in *A. thaliana* induced stunting (R. Wouters, unpublished data), and a yeast-two-hybrid screen revealed that SAP44 may interact with a plant NB-LRR (Nuclear Binding domain-Leucine Rich Repeat) protein (A. MacLean, unpublished data), suggesting that phytoplasma effectors may potentially be recognised by plant R-genes. Effector triggered immunity may slow plant growth (Denancé *et al.*, 2013) similar to the observed stunting in SAP44 expressing plants and phytoplasma-infected plants.

28 different (candidate) phytoplasma effectors are upregulated in plants, including SAP05, SAP11, SAP41, SAP44 and SAP54 (MacLean *et al.*, 2011). Thus, the phenotypic alterations in phytoplasma-infected plants may be the product of combined activity of multiple phytoplasma effectors.

### 7.3.5. Adaptive role of altering host development

Morphogenic alteration of plant tissue or tissue identity by itself can be adaptive to the parasitic life-cycle of microbes. For example, induction of pseudoflowers on plant leaves by rust fungi presents visual and olfactory cues that attract pollinating insects which disseminate fungal spores (Roy and Raguso, 1997). Changes in tissue coloration or volatile production from vegetative and floral organs may also play important role in attraction of arthropod vectors to infected plants (Mauck *et al.*, 2010; Shapiro *et al.*, 2012). Nevertheless, in many cases the adaptive significance of alteration of plant development by vector-borne plant pathogens is less clear. For example, *Mycobotrium* infected *Silene* flowers are actually less attractive to pollinator insect vectors of this fungus compared to healthy plants (Shykoff and Kaltz, 1998).

It is possible that developmental effects of phytoplasma effectors may have amplifying effects on the suppression of insect induced responses by other effectors. For example, rosette leaves of SAP54 plants are more attractive to leafhoppers than rosette leaves without SAP54. And leafhoppers select rosette leaves of phytoplasma-infected plants in preference to cauline leaves and leaf-
like floral tissue (Orlovskis and Hogenhout, 2016). If other phytoplasma effectors such as SAP05 generates aerial rosette leaves (i.e., more leaves with rosette leaf identity), this would amplify the effects of SAP54 on leafhopper host plant choice. Similarly, increased stem branching of SAP11 plants may aid leafhopper attraction. Such developmental effects may positively interact with SAP11 role in suppressing JA production and enhancing leafhopper fecundity (Sugio et al., 2011).

Developmental effects may also turn beneficial in certain stages of plant infection. For example, at late stages of infection, when xylem-inhabiting plant pathogen Xylella fastidiosa reaches high density, it forms biofilms and can induce xylem blockages associated with leaf blotches and yellowing (Chatterjee et al., 2008). Such morphological changes may encourage the sharpshooter vector of Xylella to leave infected plant and facilitate the dissemination of pathogen.

On the other hand, the developmental alterations in parasite infected hosts can also be viewed as side-effects of pathogen infection or effector activity. SAP54 may produce a myriad of developmental and physiological effects such as changes in leaf surface reflected light or plant volatiles in addition to generation of leaf-like flowers. Although I cannot completely exclude a potentially adaptive function of these altered plant phenotypes, these phenotypes neither appear to play a significant role in insect attraction and oviposition on SAP54 plants nor have a direct effect on plant longevity or phytoplasma replication within the plant (Chapters 3 and 4).

The potential reason for emergence of secondary developmental effects in infected plants may be the fact that certain microbial effectors target master regulators of various plant processes. Due to their pivotal regulatory roles in development and selection against accumulations of mutations, the genes of these master regulators are under evolutionary constraint to evade recognition by the effectors. SAP54 targets MTFs. MTFs are conserved developmental regulators but known to regulate defence-related targets like JAZ proteins (Gregis et al., 2013) or other transcription factors like TCPs. TCPs, also targeted by SAP11, are another family of conserved regulators for plant growth and organ formation. In addition, TCPs regulate a variety of microRNAs and the plant defence hormones jasmonic acid (JA) (Immink et al., 2012, Schommer et al., 2008) and salicylic acid (SA) (Wang et al., 2015). Plant development and immunity appear to be interlinked in a complex regulatory network. For example,
JAZ proteins, jasmonic acid pathway regulators, interact with many transcription factors in development such as NAC and GATA (Sen et al., 2016).

7.4. Phytoplasma effectors and genome organisation in agricultural context

It is important to consider how research on pathogen effector functions in laboratory can be translated to agricultural systems. Next I would like to describe recent work on single plant host (specialist) phytoplasma, called Maze Bushy Stunt Phytoplasma (MBSP), and the association between polymorphisms in MBSP genome and MBSP-induced disease symptoms in maize. Using MBSP as an example, I will discuss that phytoplasma effectors can be under strong selection and are likely to play an important role in disease symptom development in the field. In the following sections I will summarise findings from Orlovskis et al. (2017) and refer to figures of the full version of the paper in Appendix E.

7.4.1. Maize Bushy Stunt Phytoplasma (MBSP) induces diverse disease symptoms in its maize host

MBSP is predominantly transmitted by the maize specialist leafhopper, Dalbulus maidis (DeLong and Wolcott) (Hemiptera: Cicadellidae) (Nault, 1980; Oliveira et al., 2011). Both the pathogen and the vector are present throughout maize production zones in Central and South America (Oliveira et al., 2013; Van Nieuwenhove et al., 2015; Triplehorn and Nault, 1985) and are thought to have co-evolved with maize since its domestication from a teosinte ancestor (Nault and DeLong, 1980; Nault 1980; Doebley et al., 1997). Maize bushy stunt disease symptoms are characterised by leaf reddening, shortening of internodes, plant height reduction (stunting), lower grain yield and lateral shoot production (Nault, 1980). MBSP-infected maize plants show a diversity of symptoms, depending on maize genotype, weather conditions and perhaps also the MBSP isolate (Murral et al., 1996; Moya-Raygoza and Nault, 1998), and it is likely that MBSP is under strong selection for increased virulence and insect transmission on the maize genotypes that are widely grown in Brazil.

MBSP field isolates from two maize-growing regions in Brazil were collected, and several maize hybrids and lines were infected with these isolates (Orlovskis et al., 2017). Authors found that disease symptoms vary depending on
MBSP isolate and maize genotype. Variation in maize organ proliferation, in particular, lateral branching, was dependent on the collected MBSP isolates in all maize genotypes (Figures 2 and 3, Appendix E).

7.4.2. MBSP effectors and PMUs are under strong selection

SAP11 homolog was previously identified in a Mexican strain of MBSP (Sugio and Hogenhout, 2012). Since SAP11 from AY-WB induces lateral branching (Sugio et al., 2011), I hypothesized that the differential contributions of MBSP isolates to branching induction in infected maize genotypes may be due to variation in SAP11 sequences among the MBSP isolates. SAP11 gene and other genes previously identified to locate in the SAP11 PMU-like region in the Mexican strain were successfully amplified from all MBSP Brazilian isolates. Moreover, SAP11 sequence was identical among the MBSP isolates from Brazil and also with one MBSP isolate from Mexico (Figure S2, Appendix E). Thus, in contrast to the prior prediction, the variation in lateral branching symptoms among the MBSP isolates cannot be explained by sequence variations in the SAP11 and absence/presence of genes in the SAP11 PMU-like region.

To better understand MBSP genome organisation and effector repertoire, the MBSP Brazilian isolate which induced the greatest branching in maize was sequenced. The MBSP genome has an irregular GC-skew pattern (Figure 4, Appendix E) that is different from most prokaryotic genomes, which usually consist of two major shifts near the origin of replication and the terminus of replication (Guy and Roten, 2004). However, the AY-WB and OY-M genomes also have irregular GC-skew patterns (Figure 4, Appendix E) (Oshima et al., 2004; Bai et al., 2006) that is indicative high genomic plasticity, possibly caused by relatively recent recombination events of, for example, PMUs (Bai et al., 2006). The MBSP genome has 36 candidate effector genes (based on the presence of a signal peptide sequence and absence of predicted transmembrane domain beyond the signal peptide sequence, Bai et al., 2009) (Table S3, Appendix E). Interestingly, PMU-like genes, which were identified based on similarities to tra5, dnaB, dnaG and other genes that are present in AY-WB PMU1 (Bai et al., 2006), and effector genes co-localise between 0 and 250 kb on the genome map, but not in the 250-550-kb stretch of the MBSP genome (Figure 4, Appendix E). Similarly, in AY-WB genome, PMU-like and effector genes co-localise between
150 and 400 kb and less so in the other parts of the genome, whereas both groups of genes are distributed throughout the OY-M genome (Figure 4, Appendix E).

Ten of the 36 MBSP effectors have homologs in other phytoplasmas. These include tengu-su and SAP11 (Table S3, Appendix E). However, no SAP54 homolog was identified in the MBSP genome. Sixteen out of 36 MBSP candidate effector protein genes locate within or nearby five predicted PMU-like regions. Six putative effector genes encoding homologs lie within or adjacent to MBSP_PMU1 (Figure 7, Appendix E). SAP11 is also part of a PMU-like region in the MBSP isolate M3 genome (Table S4, Appendix E) and in a Mexican isolate of MBSP (Sugio and Hogenhout, 2012).

The whole genomes of several different MBSP Brazilian isolates were resequenced to identify changes in genome organisation or polymorphisms associated with the variation in MBSP disease symptoms in maize. Surprisingly, all sequenced genomes turned out to be nearly identical with a total of only 86 polymorphisms scattered evenly across the MBSP genome. The identified polymorphisms in MBSP Brazilian isolate genomes did not cluster to PMUs. The majority of these were synonymous single nucleotide polymorphisms (SNPs) rather than insertions or deletions (Table S5, Appendix E). Forty nine (49)% polymorphic sites were in non-coding intergenic regions, 45% in coding regions, 5% in pseudogenes and 1% in tRNA genes. Only one SNP was found to affect a candidate effector gene (locus tag c1710, Table S5, Appendix E). This effector gene lies within MBSP-PMU3 (Table S4, Appendix E) and is annotated as encoding a phase-variable surface lipoprotein. MBSP-PMU3 also contains sequences similar to candidate effector protein genes SAP21 and SAP27 of AY-WB phytoplasma (Figure 7A, Appendix E). The lipoprotein, SAP21 and SAP27 genes are about 100-kb apart in the AY-WB genome, but lie adjacently to pseudogenes that are found in PMU-like regions or that are present at high copy numbers in phytoplasma genomes (Bai et al., 2006). Homologs of the SAP21 and SAP27 genes are also found in the genomes of Peanut witches’ broom phytoplasma (PnWB) and Echinacea purpurea witches’ broom phytoplasmas, which belong to the 16Sr-II phytoplasma group and exchanged PMU elements via horizontal gene transfer with 16Sr-I phytoplasmas (Chung et al., 2013; Ku et al., 2013). Thus, MBSP genomes carry PMU-like pathogenicity islands, and a
candidate lipoprotein effector gene on MBSP-PMU3 is polymorphic among Brazilian MBSP isolates.

7.4.3. Effector variants are associated with symptom development in maize

Orlovskis et al. (in press) investigated which of the 86 polymorphisms are associated with the MBSP-isolate-dependent symptom differences of infected maize genotypes. Surprisingly, both candidate lipoprotein effector in MBSP_PMU3 and a lipoprotein ABC export protein were found to be associated with the observed variation in lateral branching phenotype during MBSP infection of maize.

MBSP Brazilian isolate M3 induced the strongest and most consistent lateral branching in all maize genotypes tested. The M3 allele of the polymorphic lipoprotein had a frame-shift mutation that would produce 2-times longer peptide than other MBSP alleles. Moreover, this lipoprotein is predicted to be exported by the phytoplasma. Two additional non-synonymous single nucleotide polymorphisms (SNPs) locate within a gene encoding a conserved ABC-family transporter, which has a lipoprotein transporter ATP-binding subunit LolD domain (Blastp E-value: 1.38e-82 against the non-redundant GenBank database). LolD mediates lipoprotein detachment from cytoplasmic membranes (Yakushi et al., 2000). Interestingly, two MBSP Brazilian isolates with the strongest induction of lateral branching also had a 2 bp insertion 47 bp downstream of the stop codon of lplA. It encodes a lipoate-protein ligase A which is conserved among phytoplasmas.

Bacterial lipoproteins can have diverse virulence functions. They may be perceived as pathogen-associated molecular patterns (PAMPs) by host pattern-recognition receptors (PRRs) (Janeway and Medzhitov, 2002) such as extra- or intra-cellular TIR (Toll/Interleukin-1) domain or NOD family receptors to trigger immune responses in both plants and animals (Medzhitov, 2001). For example, mycoplasmal lipopeptide MALP2 is recognised by a TLR2 receptor (Takeuchi et al., 2000). Lipoproteins are also implicated in a wide range of invertebrate immune responses, including activation of anti-fungal and anti-bacterial responses (Whitten et al., 2004). These proteins have a role in cellular adhesion (Paredes et al., 2015) and the recruitment (transport) of host lipids (Herren et al., 2014). While in Gram-negative bacteria several ABC transporter subunits
(LoICDE) are required for lipoprotein detachment from the inner membrane (Yakushi et al., 2010), the single-membrane bounded phytoplasmas lipoprotein transport may coordinate by fewer ABC transporter subunits, such as LolD. Interestingly, in Pseudomonas aeruginosa a LolD-type ABC exporter and an exported lipoprotein promote the activity of the type VI secretion system (Casabona et al., 2013). Even though phytoplasmas lack components that are characteristic of Type III, IV and VI secretion systems, an intriguing possibility arises that the lipoprotein effector and ABC exporter are involved in the attachment of phytoplasma cells to host cells and the activation of the secretion of other candidate effector proteins, including perhaps MBSP SAP11 and tengu-su homologs.

7.4.4. Conclusions

MBSP genomes reveal highly dynamic organisation. Most of predicted MBSP effector proteins cluster within putative PMUs. Genes within these PMUs, including effectors, could be exchanged via horizontal gene transfer. PMUs are conserved among geographically distant MBSP isolates, indicating strong natural selection on PMU genes. This findings suggests that effectors like SAP11 may be important for phytoplasma virulence in plants or transmission by insect vectors in agricultural systems. Furthermore, phytoplasma effector function in host cells may depend on other genes that may regulate effector delivery into the host cell.

7.5. Role of phytoplasma effectors in disease epidemiology

Given the importance of phytoplasma effectors SAP54 and SAP11 in plant-insect interactions, phytoplasma effectors may be key factors in disease epidemiology in the field. Understanding effector contribution to the attraction, reproduction and dissemination of insect vectors would allow to mathematically describe the relationship between infection rate and effector function in pathogen acquisition and transmission.

Let me illustrate this with a theoretical example in two thought experiments. First, imagine a plant population where 10% of the total number of plants are originally infected with a single strain of phytoplasma. The number of plants in the population is constant. Whenever a healthy or infected plant dies from natural causes, a new healthy plant emerges. Phytoplasma can only be
transmitted from plant to plant via insect vectors. I assume that vectors do not reproduce or die during this thought experiment. So, the total number of insect vectors is fixed. Any insect vector that feeds on an infected plant, becomes infected itself. When infected vector feeds on a healthy plant, this plant becomes infected.

This is illustrated graphically in Figure 7A. Intuitively, I can predict that over time the number of infected insects and infected plants in the population will increase if there is a disease inoculum in the original plant population and insects freely move between plants. Increase of infected plants will be directly proportional to the number of infected vectors if insects are equally attracted to healthy and infected plants. But how would dynamics of disease spread over time change if phytoplasma infection (as result of effector function perhaps) would aid attraction of healthy vectors to infected plants? In other words, healthy insects would preferentially select infected over healthy hosts? Now it becomes helpful to describe the change in infected and healthy plant and insect populations mathematically to better appreciate how the effect of phytoplasma infection on plant-insect interactions would change disease spread (Figure 7B).

The speed at which healthy plants are turned into infected depends on the number of infected vectors (V) and proportion of healthy (or susceptible) plant hosts in the total plant population (S/(S+I)). In addition, speed of disease spread will also depend on the attraction of infected leafhoppers to the healthy plants compared to infected (p). Therefore, the change of infected plant number over time will depend on the term $V \times p \times \frac{S}{(1+S)}$ (equation 1). I assume that infected plants die at a constant rate $d$. As defined before, the number of healthy plants will change inversely proportional to infected plants (equation 2).

If infected plants are more attractive to leafhoppers, the number of infected vectors (V) would increase proportionally to available infected hosts in the population (I/(S+I)). Moreover, preference of healthy vectors to select infected plants over healthy plants can be described with parameter ($a$). Furthermore, the efficiency to acquire phytoplasma when healthy insects land on infected plant can attributed to parameter ($u$). Together, changes in infected vector number over time can be described by the term $\frac{1}{(1+S)} \times a \times u \times H$ (equation 3). The starting number of healthy insects (H) will logically decrease as more insects acquire phytoplasma from infected plants and become infected ($\Delta V\uparrow$) (equation 4).
Figure 7.5. Modelling how the effects of phytoplasma infection on plant-insect interactions affect phytoplasma disease spread in theoretical model. (A) Graphical representation of changes in number of infected plants as a result of phytoplasma transmission by its insect vector. (B) Mathematical relationships describing phytoplasma disease spread by its insect vector. Parameters in the model can be experimentally validated. Model is explained in the text.
In the model above, I can expect that the number of infected plants would increase faster than in a simple arithmetic progression if infected plants are more attractive to healthy vectors than healthy plants ($a>1$). All parameters in the model can be determined experimentally (Figure 7.5B). Parameter $u$ can be quantified by determining the proportion of healthy insects acquiring phytoplasma from infected plants. Parameters $a$ and $p$ could be quantified by giving either healthy or infected insects a choice between healthy and infected plants at different stages of infection. For example, during early stages of phytoplasma-infection plants may be more attractive to insect vectors than healthy ($a>1$), but at later stages, when insects have already acquired the pathogen, infected insect may orientate towards healthy plants ($p>1$), thus facilitating the dispersal of the pathogen (Mauck et al., 2010).

The thought experiment hitherto described how plant infection status would affect plant-insect interactions but did not take into account any underlying mechanism. How to predict the contribution of individual phytoplasma effectors to phytoplasma disease spread? To answer this, let’s consider the final thought experiment.

Again, the number of plants in the population is constant. Whenever a healthy or infected plant dies from natural causes, a new healthy plant emerges. However, this time, instead of single phytoplasma strain, there are two different hypothetical phytoplasma strains of that can infect healthy plants and are carried by the same insect vector. Once infected, each individual insect can carry only one of the two strains. Let’s assume that phytoplasma strain1 has an effector (e.g., SAP54) that better attracts the insect vector to the strain1-infected plant compared to strain2 which lacks this effector. In addition, strain1 has another effector (e.g., SAP11) that increases insect vector reproduction on the infected plant compared to strain 2 which lacks this effector. As a result, the rate of disease spread of the two phytoplasmas is expected to be different (Figure 7.6A).
\[
\begin{align*}
\Delta I_1 &= V_1 \times p \times \frac{S}{(l_1 + l_2 + S)} - dI_1 \\
\Delta I_2 &= V_2 \times p \times \frac{S}{(l_1 + l_2 + S)} - dI_2 \\
\Delta S &= d(l_1 + l_2) - (V_1 + V_2) \times p \times \frac{S}{(l_1 + l_2 + S)} \\
\Delta V_1 &= \frac{l_1}{(l_1 + l_2 + S)} \times a_1 \times u \times H + b_1 V_1 \frac{K}{(K-V_1 + V_2 + H)} \\
\Delta V_2 &= \frac{l_2}{(l_1 + l_2 + S)} \times a_2 \times u \times H + b_2 V_2 \frac{K}{(K-V_1 + V_2 + H)} \\
\Delta H &= H - \frac{(l_1 a_1 + l_2 a_2)}{(l_1 + l_2 + S)} \times u \times H + b_H H \frac{K}{(K-V_1 + V_2 + H)}
\end{align*}
\]

Figure 7.6. (continued next page)
In the current model (Figure 7.6), speed of increase in number of plants infected with strain1 ($I_1$) depends on the number of vectors that carry strain1 ($V_1$) as well as their attraction to healthy vs infected plants ($p$) and available healthy plants at any given time (equation 1). The same is true for plants infected with strain1 ($I_1$) (equation 2). Logically, number of healthy plants would change inversely proportional to the sum of plants infected with strain1 and strain2 ($V_1 + V_2$) (equation 3).

Equations 4 and 5 describe that changes in strain1- and strain2-infected vectors over time would depend on the availability of plants infected with one strain relative to sum of all infected and healthy plants multiplied by strain-specific (i.e., effector dependent) healthy vector attraction to infected plants ($a_1$ and $a_2$). If the acquisition efficiency of the two strains is also different (as a result of particular effector), the term $u$ can be specified for each strain. The specific birth rate ($b_1$) of infected insects on strain1-infected plants can be measured as the proportion
of insect reproduction on SAP11 plants from the total reproduction on SAP11 and control plants. Similarly, $b_2$ (vector reproduction on strain2-infected plants without SAP11) can be quantified in laboratory. Equations 4 and 5 also contain a density dependent term, which limits the increase of infected vector ($b_V$) beyond the carrying capacity ($K$) of the total insect population. The number of healthy vectors would decrease as healthy insects acquire strain1 or strain2 from infected plants and increase according to the specific birth rate of healthy insects ($b_{H}$) (equation 6).

Investigating the role of other phytoplasma candidate effectors in plants and insect may add new differential parameters and build even more complex (and perhaps more realistic) models. For example, effectors that function in insects may affect the preference of infected insects to locate healthy plants (parameter $p$). There are about 18 different AY-WB effectors upregulated in insect vectors (MacLean et al., 2011). Some of these effectors may influence the biology of infected insects. Similarly, the role of plant effectors can be incorporated in more complex way than shown in Figure 7.6. For example, if certain effector in the infected plants increase plant biomass, the total carrying capacity ($K$) of the plant population would depend on the number of plants infected with a particular strain of phytoplasma. Hence, $K$ can be expressed as a function of $I_1$ or $I_2$.

In summary, when new information about the function of phytoplasma effectors accumulate, this knowledge can be holistically integrated into more complex disease models. This would allow to better understand the relative contribution of individual phytoplasma effectors or combinations of effectors to disease spread. These models may inform field data collections and analysis of phytoplasma occurrence in agricultural crops, wild plants and insect vectors. Moreover, by combining the predictions of disease models with field data for effector repertoires and mechanistic knowledge about effector function scientists can test evolutionary hypotheses about selection on particular effector genes or PMUs in phytoplasma genome.
“An experiment is a question which science poses to Nature, and a measurement is the recording of Nature’s answer.”

Max Planck (1949), *Science* Vol 110, p 325

Chapter 8
Materials and Methods

Z. Orlovskis (2016) "Know Thyself."
8.1. Generation of plants for insect assays

Seed material

Generation of 35S:GFP-SAP54 and 35S:GFP transgenic *Arabidopsis* lines was done according to methods described in MacLean *et al.* (2011, 2014). The *rad23* mutant lines (used in Chapters 2 and 3) were provided by Richard Vierstra Lab and described in Vierstra (2009). The 35S:SVP line used in Chapter 3 was kindly supplied by Martin Kater Lab and described in Gregis *et al.* (2013). *ful-1* line was provided by Lars Ostergaard Lab. *soc-1* and *sep4-1* by Richard Immink Lab and described in Immink *et al.* (2012). *maf4-2* and *maf5-3* seeds were provided by Hao You Lab and described in Shen *et al.* (2014). *maf1* (known as *flm-3*) was provided by Claus Schwechheimer Lab and described in Lutz *et al.* (2015). Other MTF mutant lines used in Chapters 3 and 6 were obtained from The European Arabidopsis Stock Centre (NASC): *ap1-12* (N6232), *lfy-1* (N6228), *agl16-1* (N604701), *agl24* (N595007, SALK_095007). Transgenic plants with 35:GFP-SAP54Δ1 and 35:GFP-SAP54Δ2 (truncated variants of SAP54 from AY-WB strain phytoplasma) and 35S:GFP-SAP54 (full length SAP54 homologs from PnWB and Stolbur strains) were cloned as described in section 8.9 and transformed into *A. thaliana* identical to the methods in MacLean *et al.* (2011, 2014).

Plant growth conditions

Non-flowering plants for insect choice experiments were sown on insecticide-free F2 compost soil (Levington, UK) and grown at 22°C, short day photoperiod (10/14-h light/dark) for 8 weeks. In contract, flowering plants were grown at 22°C, long day photoperiod (16/8-h light/dark) for 6 weeks. 1 week after germination transgenic lines 35S:GFP-SAP54 and 35S:GFP were sprayed twice (one-week interval) with herbicide Harvest® (13.52% w/v glufosinate-amonium) following manufacturers recommendations (Bayer, Cambridge, UK). 4-weeks old plants were transplanted into 10x10x10 cm (H x W x D) plastic pots. Experiments involving pre-cut flowers, floral stems were removed by metal scissors 4 days prior to insect addition.
**Generation of infected plants**

To generate phytoplasma-infected plants, three-weeks old plants were infected with ‘Ca. Phytoplasma asteris’ strain Aster Yellows Witches Broom (AY-WB) by adding five AY-WB-carrying adult *Macrosteles quadrilineatus* Forbes (Hemiptera: Cicadellidae) to each plant in a transparent Perspex tube (10 cm high, diameter 4 cm) for 5 days. Two weeks after the removal of adult insects, three rosette leaves were collected for extraction of genomic DNA to confirm phytoplasma infection using AY-WB specific primers BF 5’ AGGATGGAACCCTTCAATGTC 3’ and BR 5’ GGAATCGCCTACAAAAATCC 3’ (MacLean et al., 2014).

**8.2. Rearing of insect colonies**

Phytoplasma-free colonies of *Macrosteles quadrilineatus* Forbes (Hemiptera: Cicadellidae) were maintained on pathogen-free oat plants (*Avena sativa*) in an aerated 50x50x50cm transparent plastic cage at 22°C, long day photoperiod (16/8-h light/dark), 48% humidity. Phytoplasma-infected colonies were reared on AY-WB-infected aster (*Aster amellus*) and Chinese cabbage (*Brassica rapa*) under the same conditions as healthy insect colonies.

To maintain the insect colonies male and female insects are reared together. Adult females used in all experiments described in this thesis may have already mated with males in the stock cage prior to experiments. Therefore, female leafhoppers may not require additional fertilization to lay eggs.

**8.3. Insect reproduction assays**

*Insect choice assays*

All insect choice experiments were performed in transparent polycarbonate cages 62cm x 30cm x 41cm (H x W x D) using the setup displayed in Figure 8.1. The opposite sides of the cage were fitted with white nylon mesh held by magnetic strips to the carcass of the cage for ventilation and access. Test and control plants were placed randomly diagonally opposite each other in the corners of a cage.

A small population of male and female insects were taken from the stock cages and separated by sex. Ten male and 10 female adult *M. quadrilineatus*, which did not carry AY-WB phytoplasma, were released from a transparent...
Perspex tube (9cm high, diameter 3cm) in the center of the cage, at equal distance from each test plant. Adult insects were removed 5 days after addition to the cage. Plants were removed from the choice cage and contained individually in transparent perforated plastic bags at 22°C, long day photoperiod (16/8-hour light/dark). Nymphs were counted on each test plant 14 days after removal of adult insects from the cages. Data were expressed as proportion of total number of nymphs found on the test plants within each choice cage. Similar experiments were done with phytoplasma-infected and healthy plants or wild type and MTF-mutants plants.

For female-only oviposition choice experiments described in Figure 4.12 and Figure 4.15, five male leafhoppers were confined in two transparent 2cm diameter clip-cage placed on two fully expanded older rosette leaves of 8-week old 35S:GFP-SAP54 plants. Two empty clip-cages were placed on 35S:GFP control plants. Otherwise, female choice experiments were performed in identically to the method described above.

**Insect no-choice assay**

For the no-choice experiment (Figure 4) 5 female and 5 male non-infected adult *M. quadrilineatus* were added to individual plants surrounded by a transparent plastic cage. Plants were grown and insect progeny measured as in choice experiments.

**Single-leaf insect choice assays**

For the experiment in Figure 3D, single rosette leaves that remained attached to 35S:GFP and 35S:GFP-SAP54 transgenic plants were fitted opposite each other in a 2cm x 8cm x 12cm (H x W x D) transparent plastic cage fitted with nylon mesh-lined holes (4cm diam.) to allow for air circulation. Five male and 5 female adult *M. quadrilineatus* leafhoppers (which did not carry AY-WB) were introduced into the cage and allowed free access to both leaves. Eggs were dissected and counted under stereomicroscope (15x) five days after the first day of exposure to the insects.
Figure 8.1. Experimental setup for insect oviposition choice experiments. Photograph (top) illustrates the actual arrangement of one test and one control plant (A, D) in a choice cage. Several other choice cages are visible in the background with alternative positioning of the test plants. Diagramme (bottom) depicts all available positions for the test and control plant in the cage (A, B, C, D). Only two positions are occupied in any given cage, resulting from randomly placing the test plants in two out of the four available corners. Insects are introduced in the centre of the cage (equidistant from both plants) and released from a transparent plastic tube (E). Arrows indicate the physical dimensions of the cage.

8.4. Insect olfactory and visual choice assays

Measuring insect response to olfactory cues requires a reliable behavioural assay that is easy to use for measuring the behaviour of individual or a group of insects by comparing to a known positive attractant or negative deterrent stimuli. In addition, I wanted to use an assay that could simultaneously use either or both visual and olfactory stimuli to measure leafhopper settlement.
choice in adequately large space to do not restrict leafhopper flight, hopping and walking behaviour. To this end I tested already known insect olfactometer assays, adapted modifications to such devices as well as designed new experimental setups *de novo* (Figure 8.1).

![Figure 8.2. Design of various behavioural assays to measure insect response to olfactory stimuli.](#)

(A) Y-tube olfactometer. Insects are introduced either individually or as a group in the central column. Carbon- and water-filtered air passes through airtight chambers and brings test and control odours separately into the arms of the bi-directional choice tube (Y-tube). Air-flow was controlled via flow meters. (B) Y-tube in dark conditions under far-red light. (C) Modification of Y-tube into cylinder arena with each end presenting a different odour to insect(s) released in the centre. (D1) Choice test with test (plant in soil) and control (soil) odours in passive diffusion chamber with no added air-flow. (D2) Insects may respond to diffusing volatiles and enter a pitfall trap at either end of the chamber. (E) Odour source was placed in the centre of a plastic pot and covered with a dark perforated sticky film that permits passage of odours but blocks the visual stimuli from the odour source. (F) OVIC-box measures Olfactory or Visual Insect Choice. One end presents a visual cue competing with an olfactory stimulus at the opposite end for insect attraction. Insects are released in the centre of the box. Insects stick to a landing platform at both ends of the chamber.

The initial aim was to find a positive olfactory stimulus that attracts *Macrosteles quadrilineatus* or use another insect species – odour combination as a positive control to test the olfactometer designs depicted in Figure 8.1. With Y-tube olfactometer (Figure 8.1A) I observed positive attraction of *Drosophila*
suzukii to yeast, vinegar and banana odours but failed to detect *M. quadrilineatus* response to any plant (aster, China cabbage, tale cress, tobacco or oat) odours when compared to no-plant controls. I performed these trial experiments in both light and dark, with various levels of air-flow as well as using horizontal and vertical angles of the Y-tube to account for positive or negative photo- and gravikinetic behaviours of leafhoppers (Figure 8.1B). Since the Y-tube restricted leafhopper jumping behaviour I widened the choice chamber (Figure 8.1C) but failed to establish a positive attraction to a plant odour. In order to avoid any confounding effect of leafhopper response to air-draft, I tried a sealed chamber which is equally wide to allow for leafhopper jumping but relies on passive diffusion of odours as stimuli (Figure 8.1D1). Interestingly, leafhoppers moved towards the odour emitting compartments and were successfully trapped in the pitfalls (Figure 8.1D2). Nevertheless, this response can be explained by insect movement to any side with higher humidity (wet tissue or soil) irrespective of plant odours. Next I placed a test odorant (plant/banana/vinegar/yeast) into visually identical pots with perforated sticky cover to conceal the pot contents but permeate any odours and trap insects after their first landings (Figure 8.1E). Unfortunately, *M. quadrilineatus* did not show any landings onto the pots, and *D. suzukii* did not show the preference for yeast, vinegar or banana as in the previous assays, which were only suitable for presentation of volatile stimuli but not visual cues (Figure 8.1A-D). Because I aimed to compare the relative effect of visual and olfactory cues in plant selection by insects in the same setup, I designed a ventilated arena to simultaneously present insects with a visual and volatile stimuli (Figure 8.1F). Although leafhoppers were attracted to yellow colour, neither *M. quadrilineatus* nor *D. suzukii* responded to plant, yeast, vinegar or banana-skin emitted volatiles.

Finally, I decided to further modify the assay shown in Figure 8.1E to mimic the initial choice experiments with whole plants. I concealed the odour source in two plastic pots (Figure 8.2A) and were able to fit a sticky landing platforms of different colours around the odour sources (Figure 8.2B). Detailed assemblage of the assay is explained in Figure 8.2C, steps 1 to 4. Within this setup, *D. suzukii* demonstrated strong preference for vinegar and yeast extracts by entering the black pots and drowning in the test solutions. Leafhoppers were more passive to enter the pots, and only very few were detected on the plant or soil controls inside the pots (Figure 8.2D-E). Therefore, I fitted a colourless sticky landing platform
(OECO, Kimpton, UK) around each pot when measuring insect response to olfactory stimuli only. I added a coloured sticky landing platform to compare or complement visual and olfactory stimuli with each other.

Figure 8.3. Visual representation of experimental setup to measure leafhopper response to plant olfactory stimuli in presence or absence of visual cues. (A) Pots containing a single point source of a test and control odours are placed opposite each other in 62x30x41cm (HxWxD) transparent polycarbonate cages. (B) Colourless or coloured landing platforms with odour-less sticky surface are fitted on top of the odour sources. (C) Detailed setup of the pots with odour source: 1) required 10x11x11cm (HxWxD) and 9x8x8cm (HxWxD) black plastic pots, 50mL glass vial containing odour-emitting or odourless solution or equivalent odour source such as small pot with a plantlet, a sponge bun and 11x11cm landing platform covered with transparent sticky tape (Rollertrap, OECOS, Kimpton, UK); 2) place the sponge bun at the base of the bigger pot; 3) place the glass vial or the plantlet on top of the bun; 4) cover the setup with the smaller pot upside-down and finish with the landing platform. The perforated bottom of the smaller pot permits odour transmission. In absence of the landing platform few M. quadrilineatus were detected inside the smaller pot on cabbage (D) or tale cress (E) plant.
I released a mixed population of 20 male and 20 female insects in the centre of the arena (Figure 8.2A,B) under light or dark conditions for 6 hours at 22°C. During this period most insects had made their first landing choice and stuck to the landing platform. Insects were sexed and counted on the sticky traps as well as inside the pots. Majority of insects were found stuck on landing platforms. Only very few insects were ever found inside the pots. For each experiment I used new pots and landing platforms to avoid the residual semiochemicals left by insects from the previous experiment to bias the leafhopper choice in the next experiment.

Figure 8.4. Experimental setup of *Macrosteles quadrilineatus* visual choice experiment (A) Rosettes of 35S:GFP-SAP54 and 35S:GFP plants are contained within an air-tight transparent plastic cage (1x11x11cm; HxWxD) placed in opposite corners of ventilated 62x30x41cm (HxWxD) transparent polycarbonate cages. (B) Rosettes of 35S:GFP-SAP54 and 35S:GFP plants viewed side-by-side. Insects landed on the plants are permanently stuck to the landing surface. (C) Yellow colour rosette replica and 35S:GFP or SAP54 expressing plants were used as a positive control in our experiments. (D) Yellow (RGB 255, 250, 0) rosettes are more attractive to leafhoppers compared to either SAP54 or control plants.
In order to measure only insect visual choice based on plant colour in absence of plant olfactory stimuli, I modified the setup described in Figure 8.2 by cutting off plant rosettes and placing between two 11x11 cm transparent plastic sheets (Figure 8.3). I sealed the gap between the two plastic squares to prevent any plant odours from escaping and thus completely caging the plant rosettes. I covered the cage with a transparent sticky glue spray (OECO, Kimpton, UK; http://www.oecos.co.uk) to fix insects after the first landing. I used yellow (sRGB 255, 250, 0) rosette replicas as a positive control. Similar to the olfactory choice experiments above I released a mixed population of 20 male and 20 female insects in the centre of the arena under light conditions for 6 hours at 22°C.

8.5. Measurement of light reflectance and absorbance from leaf surface

Leaf reflectance was measured using VideometerLab3 19 channel (365-970 nm) multispectral imager and the associated software (Videometer A/S Copenhagen, Denmark) following the manufacturers user instructions. An image of the whole leaf was taken over the supplied blue background stage. After taking the image, the layer tool in the accompanying software was used to select the freeform of the entire leaf and measure the reflected light across the selected area. Measurements were taken and averaged from three oldest leaves of 8-week old 35S:GFP-SAP54 and 35S:GFP transgenic Arabidopsis thaliana plants. Average reflectance of 20 independent replicate plants per transgenic line were measured for each of the 19 channels. Leaf reflectance from 11 channels corresponding to the visual spectrum of insect vision (370-650 nm) were analysed. Since each channel is an independent reflectance measurement, data were analysed using two-tailed t-test for each channel and (Bonferroni) corrected for multiple comparisons.

SPAD-502Plus meter (Konica Minolta, Inc., USA) was used to measure the chlorophyll index of 8-week old 35S:GFP-SAP54 and 35S:GFP plant rosette leaves. Three oldest leaves were measured per plant by selecting an area of leaf blade with no intersection with midvein. The average absorbance from three technical replicates per plant was recorded for 36 independent plants.
8.6. Scanning Electron Microscopy (SEM) of leaf trichomes

For imaging hydrated surfaces of biological structures such as trichomes a cryo-SEM approach was used. Rosette leaves, cauline leaves or leaf-like flower petals of 8-week old 35S:GFP-SAP54 and 35S:GFP plants were placed on an aluminium (Al) platform using the Optimal Cutting Temperature (OTC) compound (Agar Scientific Ltd, Essex, UK) and immersed in nitrogen slush at -210°C for cryopreservation. The leaf samples were transferred to the cryostage of Alto 2500 cryotransfer system (Gatan, Oxford, UK) and subjected to sublimation for 3 min at 95°C. The sample surface was sputter-coated with platinum (Pt) for 2 min with 10 mA current at ≤-110 °C. Next, samples were transferred to a cryostage inside Zeiss Supra 55 VP field emission gun scanning electron microscope (Carl Zeiss Ltd, Germany) at -125°C. For visual comparison of trichome density between cauline leaves, leaf-like flowers and rosette leaves 6 images at 200x magnification were taken across leaf surface and 3 leaves per tissue type of 35S:GFP-SAP54 and 35S:GFP plants were imaged. Pictures of individual trichomes were taken at approximately 500x magnification.

8.7. Measurement of leafhopper probing and feeding on leaves

Quantification of insect excreted honeydew

Honeydew excretion was quantified as a proxy measure for leafhopper feeding preference for 35S:GFP-SAP54 and 35S:GFP plants. 5 ♀ and 5 ♂ adult Macrosteles quadrilineatus were introduced into 8x12 cm plastic cages containing filter paper (Watman, Buckinghamshire, UK) and 3 equal sized rosette leaves from opposite-facing 35S:GFP and 35S:GFP-SAP54 plants (Figure 8.4A). Filter paper was collected 5 days after the start of the feeding experiment and stained with 2% (w/v) ninhydrin solution in ethanol. The filter paper is heat-dried on a heating block (100°C) for few seconds to catalyse the reaction between ninhydrin and amino acids contained by honeydew and leaf exudates (Figure 8.4B). Photos of filter-papers were analysed using Fiji (ImageJ) analysis software by measuring the stained area for each test plant within single cage as outlined in Figure 8.4C. Colour images opened in Fiji were given scale by selecting the image of the ruler and equating the selected pixels with a known distance on the image (step 1). Next colour images are transformed to 8-bit monochromatic files (step 2). Contrast threshold was adjusted to highlight only the pixels representing
the stained area (step 3). Selected pixels were counted and expressed as stained area (step 4) according to the scale set in step 1. The stained area for each genotype was expressed as the proportion of the total stained area per cage (Figure 8.4D) and further analyses statistically with paired t-test.

Figure 8.5. Analysis of honeydew excretion by M. quadrilineatus. Leaves of 35S:GFP and 35S:GFP-SAP54 plants are arranged opposite each other above the filter paper in an enclosed cage. Foam buns cushion the petioles against the edges of a side opening in the cage. Leaves remain attached to the plant throughout the feeding experiment (A). Filter papers are removed and stained with ninhydrin (B). The step-to-step commands and appropriate tabs in Fiji software are executed to highlight the stained area (C). Data are analysed as the ratio between honeydew excretion around 35S:GFP and 35S:GFP-SAP54 plants (D).

Quantification of leafhopper probing and feeding sites in leaf tissue

M. quadrilineatus feeding/probing sites on Arabidopsis leaves were visualised using trypan blue staining. Leaves of 8-week old 35S:GFP-SAP54 and 35S:GFP plants were arranged identical to single leaf choice experiments described in section 8.3 and depicted in Figure 8.5. Instead of counting the number of eggs, the insect exposed rosette leaves were detached and submerged in the staining solution consisting of 0.7% (w/v) trypan blue, 33% (v/v)
lactic acid, 33% (v/v) phenol, and 33% (v/v) glycerol. To prepare the mixture, trypan blue is dissolved in boiling lactic acid and glycerol before carefully adding phenol and ceasing the boiling process. Leaves are directly immersed into the hot staining mixture and incubated for 5 min. Mixture must be hot to effectively bleach chlorophyll and other pigments from the leaves and facilitate the penetration of trypan blue. Samples were destained with 70% (v/v) chloral hydrate on a shaker until good contrast between stained and stain-free zones is obtained. The destained leaves were soaked in 80% (v/v) glycerol for 30 min and mounted on microscope slides for imaging under bright-field light microscope. Trypan blue stains dead plant cells around insect stylet punctures and tracks around probing and feeding sites (Figure 8.5). Four randomly chosen quadrants (1 cm²) adjacent to vascular tissue and four quadrants in mesophyll tissue were examined and the total amount of punctures and stylet tracks were counted together. Six older rosette leaves from 35S:GFP-SAP54 and 35S:GFP plants were analysed.

Figure 8.6. Experimental setup for feeding choice experiment and staining of *M. quadrilineatus* stylet punctures and penetration tracks (blue). Plant vascular tissue appear as darker lines contrasting the nearly transparent mesophyll cells. 5 male and 5 female insects were released in the choice arena for 5 days before staining the sample as described in the text.
8.8. **Measuring phytoplasma titer in infected plants**

Phytoplasma-infected plants were generated identical to methods described in the section 8.1. Since the replication of phytoplasma cells may vary between individual plant organs, the whole rosette and the whole inflorescence (stems and cauline leaves including) were collected separately in liquid nitrogen and grinded with pestle and mortar. Genomic DNA was extracted from 100 mg (wet weight) of the ground sample using QIAGEN DNeasy® Plant kit following the manufacturer’s protocol. DNA was eluted in 50 µL of distilled water.

I designed a probe-based multi-plex qPCR assay using a probe and a primer pair for AY-WB phytoplasma 16S rRNA gene as well as a probe and a primer pair for plant actin-2 gene as a reference. qPCR was performed in 25 µL final volume using 12.5 µL TaqMan Universal PCR Master 2X Mix (Roche Applied Biosystems, USA), 2 µL of 10 µM forward and reverse phytoplasma primers and 1 µL of 5 µM phytoplasma probe together with 1 µL of 5 µM forward and reverse actin primers and 0.5 µL of 5 µM actin probe, 5 µL extracted gDNA from AY-WB infected plants. qPCR was run on the CFX96 Real-Time System C1000 thermal cycler (Biorad) using the following settings: (1) 50°C, 2 min; (2) 95°C, 10 min; (3) [92°C, 15 sec; 60°C, 1 min] X 40 cycles; (4) 60 °C, 30sec. Ct value of AY-WB phytoplasma 16S ribosomal gene was compared to plant actin (reference gene) in each sample, and the relative value for phytoplasma titer obtained using the comparative cycle threshold method (2^{-ΔCt}). Each sample was amplified in 3 wells and average readings taken. 4 plant samples were analysed per tissue type.

The following primer and probe sequences were used:

- **Phytoplasma 16S forward primer**: 5’ CGTACGCAGTATGAAACTTAAA 3’
- **Phytoplasma 16S reverse primer**: 5’ CTTCGAATTAAACAACATGATCC 3’
- **Phytoplasma 16S probe**: 5’ [FAM]GACGGGACTCCGCACAAGCG[BHQ1] 3’
- **Plant actin forward AtACT2-primer**: 5’ GCTGAGAGATTCAGATGCCCA 3’
- **Plant actin reverse AtACT2-primer**: 5’GTGGATTCCAGCAGCTTCCAT 3’
- **AtACT2 probe**: 5’ [Cyanine5]AAGTCTTGTTCCAGCCCTCGTTTGTC[BHQ3] 3’
8.9. Sequence alignments and construction of phylogenetic trees

All sequence alignments were performed using ClustaW algorithm (default settings) in MEGA (Molecular Evolutionary Genetics Analysis) software (version 6; available from [www.megasoftware.net](http://www.megasoftware.net)). Phylogenetic trees were constructed in MEGA from the obtained alignments using Maximum likelihood Bootstrap (1000) method, setting gamma distribution and general time reversible model for nucleotide substitutions but Jones-Taylor-Thornton model for amino acid substitutions. Resulting trees were formatted and annotated in FigTree v1.4.2 ([www.tree.bio.ed.ac.uk/software/figtree/](http://www.tree.bio.ed.ac.uk/software/figtree/)).

To generate the tree in Figure 1.1, the following GenBank accession number for 16S rRNA gene sequences were used: NC000913 (Escherichia (E.) coli), NR044924 (Erwinia (Er.) tracheiphila), NR104928.1 (Pa. stewartii), NC004547 (Pectobacterium (Pe.) carotovorum), DQ508182 (Ca. A. triatominarum), NC004578 (Pseudomonas (P.) syringae), FJ494776.1 (Ralstonia (R.) solanacearum), NC007508 (Xanthomonas (Xa.) campestris), NC004556 (Xylella (X.) fastidiosa), NR074528 (Ca. Liberibacter (L.) asiaticus), NC003062 (Agrobacterium (Ag.) tumefaciens), NC007761 (Rhizobium (Rh.) etli), NC009964 (Bacillus (B.) subtilis), NC008533 (Streptococcus (S.) pneumoniae), NC007716 (Ca. Phytoplasma (Ph.) asteris), NC010163 (Acholeplasma (Ac.) laidlawii), X63781 (Spiroplasma (Sp.) citri), NC000908 (Mycoplasma (M.) genitalium), NC009480 (Clavibacter (C.) michiganensis), NC006087 (Leifsonia (Le.) xylí), AB026221 (Streptomyces (St.) turgidiscabies), AL645882 (Streptomyces (St.) coelicolor), AB211229 (Rhodococcus (Rho.) fascians), NC009525 (Mycobacterium (My.) tuberculosis) and NC002754 (Sulfolobus (Su.) solfataricus). Since no complete 16S rRNA sequence data are available for Ca. Arsenophonus (A) phytopathogenicus (*) and Pantoea (Pa.) ananatis (*) their relationships were based on the 16S RNA gene sequences of the related species ‘Ca. Arsenophonus triatominarum’ and the Pantoea species Pa. stewartii, respectively.

To obtain tuf gene sequences and produce Figure 2.4, genomic DNA from the collected field samples was extracted with DNeasy Plant kit (QIAGEN, US). Phytoplasma infection was assessed using universal primers R16R2 + R16F2n nested in P1 + P7 (Gundersen & Lee, 1996) and plant DNA quality checked using primers for EF1 gene. Phytoplasma-positive samples were further amplified for tuf gene as described in Makarova et al., 2012 and cloned via pGEM-easy TA
(Promega, US) system. Multiple colonies from each plant sample were sequenced for the \(tuf\) gene to detect potential infection with multiple phytoplasma strains. Obtained sequences were aligned with reference sequences from diverse phytoplasma groups and Maximum likelihood tree (bootstrap 1000) constructed using MEGA.

Table 8.1. Phytoplasma classification, strain names and GenBank accession numbers for sequences used to construct phylogenetic trees for SAP54 protein and \(tuf\) gene in Figure 2.4. SAP54 homolog from Rapeseed Phyllody Inducing Phytoplasma was sequenced recently and GenBank accession number is pending; classification in 16SrI-B group based on 16S rDNA genotyping. Tuf sequence from another Rapeseed phyllody strain belonging to 16SrI-B group was chosen (Macarova et al., 2012).

<table>
<thead>
<tr>
<th>Candidatus phytoplasma species</th>
<th>16Sr (sub)group</th>
<th>Phytoplasma strain containing SAP54 homolog</th>
<th>GenBank accession</th>
<th>Phytoplasma strain containing tuf homolog</th>
<th>GenBank accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co Phytoplasma asteris'</td>
<td>I-A</td>
<td>Aster Yellows Witches' Broom (AYWB)</td>
<td>CP000061.1</td>
<td>Aster Yellows Witches' Broom</td>
<td>CP000061.1</td>
</tr>
<tr>
<td>Co Phytoplasma asteris'</td>
<td>I-B (OY)</td>
<td>Onion Yellows-M (OY)</td>
<td>AP006628.2</td>
<td>Onion Yellows-M (OY)</td>
<td>AP006628.2</td>
</tr>
<tr>
<td>Co Phytoplasma asteris'</td>
<td>I-B (RPIP)</td>
<td>Rapeseed Phyllody Inducing Phytoplasma (RPIP)</td>
<td>ID pending</td>
<td>Rapeseed Phyllody</td>
<td>JQ824246</td>
</tr>
<tr>
<td>Co Phytoplasma asteris'</td>
<td>I-C</td>
<td>Leontodon Yellows</td>
<td>AB862484.1</td>
<td>Carrot Yellows</td>
<td>JQ824226</td>
</tr>
<tr>
<td>Co Phytoplasma asteris'</td>
<td>I-F</td>
<td>Apricot Aster Yellows Phytoplasma (AAYP)</td>
<td>AB862477.1</td>
<td>Apricot chlorotic leafroll (AAY)</td>
<td>JQ824251</td>
</tr>
<tr>
<td>Co Phytoplasma aurantrifolia'</td>
<td>II-A</td>
<td>Peanut Witches' Broom and Sweet Potato Little Leaf</td>
<td>WP_004994552.1</td>
<td>Sweet Potato Little Leaf</td>
<td>JQ824270</td>
</tr>
<tr>
<td>Co Phytoplasma pruni'</td>
<td>III</td>
<td>Spirea stunt</td>
<td>AB862489.1</td>
<td>Spirea stunt</td>
<td>JQ824281</td>
</tr>
<tr>
<td>Co Phytoplasma trifoli'</td>
<td>VI</td>
<td>Beetroot Leafhopper Transmitted Virescence Agent (BLTVA)</td>
<td>BAQ08267.1</td>
<td>Clover Phyllody</td>
<td>JQ824231</td>
</tr>
<tr>
<td>Co Phytoplasma phoenicium'</td>
<td>IX</td>
<td>Phoenicium Yellows</td>
<td>AB862490.1</td>
<td>Pichris echoides yellows (PEY)</td>
<td>JQ824256</td>
</tr>
<tr>
<td>Co Phytoplasma solani'</td>
<td>XII</td>
<td>Tomato Stolbur</td>
<td>CCP88386.1</td>
<td>Tomato Stolbur</td>
<td>JQ824280</td>
</tr>
</tbody>
</table>

To produce Figure 2.6, I obtained the sequences for SAP54 and elongation factor Tu (\(tuf\)) gene from phytoplasmas representing the same (sub)group of a single Candidatus phytoplasma species. SAP54 homologs were identified in reciprocal BLAST search using SAP54 peptide sequence from AY-WB phytoplasma as query. \(Tuf\) gene sequences were retrieved from GenBank using the accession numbers in Macarova et al. (2012). In most cases, the SAP54 and \(tuf\) sequence could be retrieved from the same phytoplasma strain. However, due to limited number of full phytoplasma genome sequences available in GenBank, obtaining the SAP54 and \(tuf\) gene sequences from the same
phytoplasma strain was not possible. Therefore, another strain within the same 16Sr subgroup was chosen for tuf gene sequence. The Identity and GenBank accession numbers for sequences used to construct phylogenetic trees for SAP54 and tuf genes are listed in Table 8.1.

### 8.10. Molecular cloning

Cloning of type I and type II MTFs and RAD23 isoforms is described in MacLean et al. (2014). SAP54 homologs from Stolbur and PnWB (SPLL) phytoplasma strains (Chapter 2) and the two truncated fragments of SAP54 homolog from AY-WB phytoplasma strain (Chapter 7) were amplified using the primer sequences in table 8.2 without including the predicted signal peptide.

#### Table 8.2. List of primers for Gateway cloning

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB1 adaptor</td>
<td>GGGGACAAAGTTGTTCGAAAAAAGCAGGCT</td>
</tr>
<tr>
<td>attB2 adaptor</td>
<td>GGGGACCACTTTGTACAGAAAGCTGGGTG</td>
</tr>
<tr>
<td>attB1 SAP54A1</td>
<td>AAAAAAGCAGGCTCCACCATGGATAAAGATATT</td>
</tr>
<tr>
<td>attB2 SAP54A1</td>
<td>AGAAAAAGCTGGGTGTATCTAGTTGATATTTGATATTGTTG</td>
</tr>
<tr>
<td>attB1 SAP54A2</td>
<td>AAAAAAGCAGGCTCCACCAGGAACCTAGAAAAATACTCTTTAACC</td>
</tr>
<tr>
<td>attB1 SAP54A2</td>
<td>AGAAAAAGCTGGGTGTATATTTTCTCATCAT</td>
</tr>
<tr>
<td>attB1 SAP54 (PnWB)</td>
<td>AAAAAAGCAGGCTCCACCATGGATGGCAGCGATCCAAAAC</td>
</tr>
<tr>
<td>attB2 SAP54 (PnWB)</td>
<td>AGAAAAAGCTGGGTGTATAGTTTTTCTCATCA</td>
</tr>
<tr>
<td>attB1 SAP54 (Stolb)</td>
<td>AAAAAAGCAGGCTCCACCATGGGATATTTGTAATAGATGAAGCTGC</td>
</tr>
<tr>
<td>attB2 SAP54 (Stolb)</td>
<td>AGAAAAAGCTGGGTGTATATTTAAGGATTTAAAGTG</td>
</tr>
<tr>
<td>Notes</td>
<td>attB1 primers contain part of attB1 adapter sequence (bold), followed by a Kozak sequence (italics), followed by gene specific sequence. attB2 primers contain part of attB1 adapter sequence (bold), followed by gene specific sequence.</td>
</tr>
</tbody>
</table>

After amplification of the target sequence by PCR, the original PCR product was amplified in a second round of PCR with a pair of full-length attB adapter primers. The product of the second PCR was run on EtBr-stained 1% agarose gel to cut out the correct size band before gel-purification using QIAquick Gel Extraction Kit (QUAGEN). The purified PCR product was cloned into Gateway-compatible donor vectors pDONR201 or pDONR207 using BP clonase II (Invitrogen) following manufacturer’s instructions. Reactions were transformed into electrocompetent Escherichia coli (DH5α) cells. Transformed bacterial colonies were identified by antibiotic selection and colony PCR using attB site specific primers. Plasmids from positive clones were purified using QIAprep Spin
Miniprep Kit (QIAGEN) and sequenced. Correct sequences were cloned from the donor-vectors into destination vectors using LR clonase II (Invitrogen) following manufacturer’s instructions. The destination vectors were pB7WGF2.0 for plant transformations and pDEST22 or pDEST32 for Y2H analysis.

8.11. Yeast-two-hybrid analysis for protein-protein interactions

Transformation of yeast

Saccharomyces cerevisiae yeast strain MaV203 (Invitrogen), auxotrophic for leucine (leu2) and tryptophan (trp1-901), was streaked on 2X YPDA (yeast extract-peptone-dextrose-adenine) agar media and grown at 28°C. A single yeast colony was inoculated in aliquots of 10mL 2X YPDA media and grown in shaking incubator (220 rpm) at 28°C to an OD_{600}=0.5, equivalent to a density of 5×10^6 yeast cells/mL. 1 mL of the culture was inoculated into pre-warmed aliquots of 100 mL 2X YPDA media and grown to final density of 2×10^7 cells/mL. Yeast cells were pelleted by centrifugation at 2500 g for 5 min at room (20-25°C) temperature to remove YPDA media and washed twice with sterile water. Finally yeast cells were resuspended in sterile water to final concentration of 1×10^9 yeast cells/mL.

100 µL of the yeast suspension was added to 360 µL of transformation mixture consisting of 240 µL 50% (w/v) polyethylene glycol (PEG), 36 µL 1M lithium acetate (LiOAc), 50 µL of 2mg/µL boiled single-strand carrier DNA (Salmo salar sperm DNA, Invitrogen) and 1 µg of both pDEST22 and pDEST32 plasmid carrying the cloned gene for interacting proteins. The mixture was vortexed thoroughly and incubated at 42°C for 40-60 min before centrifugation at ≤10 000 g for 30 sec to remove supernatant and resuspended in 500 µL sterile water.

Yeast transformation and subsequent screening for interactions was repeated three times independently. For repeating the transformations, frozen competent yeast cells were made following the protocol from Gietz and Schiestl (2007). After washing off the YPDA medium, yeasts were resuspended in filtersterilised mixture of 5% v/v glycerol and 10%v/v DMSO and stored in 100 µL aliquots at -80°C. To use cells again, cells were thawed at room temperature and resuspended in 100 µL sterile water and proceeded directly to transformation again.
**Screening for yeast transformants**

The transformed yeast suspension was plated on synthetic defined (SD) minimal agar medium lacking tryptophan (-W) and leucine (-L) and incubated at 28°C for 3 to 5 days till formation of individual yeast colonies. Plant RAD23 and MTFs were cloned into yeast vectors pDEST22 (complementing W auxotroph) but SAP54 homologs - in pDEST32 (complementing L auxotroph). Upon uptake of both plasmids a yeast cell is able to grow on the SD-L-W selective media.

**Screening for protein-protein interactions via yeast-two-hybrid**

Three individual colonies with yeasts transformed for both constructs were suspended in 1 mL of sterile water as well as 1:10 and 1:100 serial dilutions. Thus each interaction was replicated 3 times for each dilution. 10 µL yeast suspension was spotted on SD minimal agar medium lacking tryptophan (-W) and leucine (-L) and histidine (-H) as well as 10, 30 and 60 mM 3-amino-1,2,4-triazol (3-AT). The pDEST22 encoded a fusion protein of GAL4 gene activation domain (AD) with plant RAD23 and MTF proteins but pDEST32 - a fusion protein of GAL4 gene Binding domain (AD) with SAP54. Upon interaction of SAP54 and plant target proteins, the AD and BD trigger expression of yeast histidine synthesis gene (HIS3) complementing the histidine auxotroph yeast and allowing to grow on SD-L-W-H selective media. 3-AT prevents the (auto-)activation of HIS3 gene. Increasing the concentration of 3-AT selects for stronger interactions between proteins.

The expression of plant RAD23 and MTFs and phytoplasma SAP54 proteins in yeast was confirmed by protein extraction, SDS-PAGE and immunoblotting with antibodies for AD and BD following the exact methods (Kushnirov, 2000).

**8.12. Other protein methods**

**Agrobacterium-mediated transient protein expression in N. benthamiana**

Cultures of Agrobacterium tumefaciens strain GV3101 previously transformed with expression vectors containing plant type-I MTF genes or SAP54 were inoculated in liquid LB-medium with appropriate antibiotic selection and incubated overnight at 220rpm, 28°C. Cultures were pelleted at 2300g for 10 min.
and resuspended in infiltration buffer consisting of 10mM MgCl$_2$, 10mM 2-(N-Morpholino)ethanesulfonic acid (MES) at pH=5.6 to OD$_{600}$=0.6 for SAP54 and OD$_{600}$=1.0 for MTFs. Acetosyringone was added to each culture to final concentration of 100 µM and incubated for 1h at room temperature to enhance the expression of A. tumefaciens virulence genes and thus facilitate the transformation. Two youngest fully expanded leaves of four-week old Nicotiana benthamiana were co-infiltrated with equal volumes of two cultures using a 1 mL needleless syringe. After 72 hours two leaf disks (1 cm diam.) from the infiltrated site were collected in liquid nitrogen.

**SDS-PAGE and Western-blotting**

Collected plant samples were grounded in liquid nitrogen, mixed in 40 µL of 10mM DTT and 1x NuPAGE LDS sample buffer (Invitrogen) and boiled for 10 min. For detecting yeast protein levels, cells were pelleted by centrifugation, resuspended in 0.1M final conc. NaOH, incubated for 10 min, pelleted and resuspended in 40 µL 1x NuPAGE LDS sample buffer before boiling. 12.5% SDS-polyacrylamide gels were loaded with the denaturated protein sample (3 µL for flag-tagged proteins; 15 µL for 10xmyc-tagged proteins; 10 µL for proteins with GAL4-AD and GAL4-BD) and 10 µL pre-stained protein Marker (Broad Range P7708s, BioLab). The protein gel was run at 150V in Mini PROTEAN III tanks (Bio-Rad) with 1xTris-glycine SDS running buffer until the samples reach the lower half of the gel. Proteins were transferred to 0.45 µm Protran BA85 nitrocellulose membrane (Watman) using electroblotting at 250mA for 90 min in 1xTris-glycine 20% methanol transfer buffer.

**Immunodetection of tagged-proteins**

The nitrocellulose membrane containing immobilised, denaturated proteins was incubated on gentle shaker at room temperature for 40 min with blocking buffer (5% (w/v) milk powder in 1x Phosphate buffered saline (PBS) and 0.1%(v/v) detergent Tween-20 (Sigma-Aldrich)). The membrane was then incubated with the blocking buffer containing the primary antibody for 1 hour at room temperature or 4°C overnight. Anti-flag (monoclonal mouse, Sigma Aldrich) and anti-myc (polyclonal, rabbit, Sigma Aldrich) were used at 1:10000 and 1:6667 dilution in
blocking buffer respectively. Anti-GAL4-AD and anti- GAL4-BD (polyclonal, rabbit, Sigma Aldrich) were used at 1:10000 dilution. After washing the primary antibody with the blocking buffer, the membrane was incubated with secondary antibody (same conditions as primary antibodies). Then membrane is washed with 1x PBS and 0.1% (v/v) Tween-20. Bound antibodies were detected by Immobilon Western Chemiluminiscent Horseraddish Peroxidase substrate (Milipore, UK) when exposed to Super RX film (Fujifilm, Germany) and developed.

Protein structure predictions

Bacterial effector signal peptides were predicted using SignalP 3.0 using both Neural networks and Hidden Markov models (available at http://www.cbs.dtu.dk/services/SignalP-3.0/). Candidate lipoprotein effector signal peptides were analysed using PRED-LIPO tool (available at http://bioinformatics.biol.uoa.gr/PRED-LIPO/). Presence of putative transmembrane domains was performed using TMHMM Server 2.0 (available at http://www.cbs.dtu.dk/services/TMHMM/). The coiled-coil structural predictions were calculated with PRABI coiled-coil prediction tool (available at https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_lupas.html).

8.13. Analysis of plant transcriptional response to insects

Generation of plants for RNA-sequencing

The experiment used 8-weeks old Arabidopsis thaliana (Col-0) plants ectopically expressing Aster Yellows phytoplasma strain Withes’ Broom effector SAP54 (35S:GFP-SAP54) or a control construct (35S:GFP). Plants were grown at short day photoperiod (10h/14h day/night) prior and during the experiment. Plants were selected for presence of the transgene by herbicide selection 2 weeks after germination. Single fully expanded leaf of each plant was exposed to either 5 male or 5 female insects by placing them in a transparent 2cm diameter clip-cage. An empty clip-cage was placed for no-insect control. Plant tissue samples were collected 48h after exposure to insects. Insect number and exposure time was previously experimentally optimised by measuring number of feeding sites and eggs laid per clip-cage.
RNA extraction for RNA-seq and quality control

Leaf tissue was collected from the leaf area enclosed by the clip-cage and stored at -80°C for subsequent RNA extraction using QIAGEN Plant RNeasy kit (following manufacturer instructions). RNA integrity was assessed by gel electrophoresis (1% Agarose) visualisation of ribosomal bands in extracted dsRNA and ssRNA (65°C denaturation and immediate transfer on ice to prevent hybridisation). RNA concentration and quality was assessed using Nanodrop (Thermofisher). Total ≥2µg of each RNA sample at ≥50ng/µL concentration, 260/280 ratio between 1.9 and 2.1, and 260/230 ratio between 1.5 and 2.0 was submitted for RNA-sequencing.

RNA sequencing

Library preparation and sequencing was performed in The Genome Analysis Centre (TGAC, Norwich Research Park). Total of 24 RNA samples were submitted for IlluminaTruSeq cDNA library construction and sequencing on Illumina HiSeq 2000 platform pooling 4 libraries per lane, with 50bp single-end reads and 25M read coverage per sample. The Raw reads were assessed for their quality and processed for mapping onto Arabidopsis reference genome (available from The Arabidopsis Information Resource; TAIR 10).

RNA-seq read alignment and differential expression analysis

TGAC performed data quality control using FastQC (fastqc-0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to check for the basic metric of quality control in the raw data, it gives a quick impression of whether the data is of good quality before doing further analysis. An in-house contamination-screening pipeline called Kontamination (not published yet) was used to check for any obvious contamination in the raw reads. Since the data quality was good, there was no trimming done on the raw reads.

Alignment of RNA-seq reads to transcriptome reference (Arabidopsis thaliana TAIR10 was done using TopHat (Tophat v2.1.1, http://ccb.jhu.edu/software/tophat/manual.shtml) with --min-anchor-length 12. TopHat will report junctions spanned by reads with at least 12 bases on each side of the junction. Every junction involved in spliced alignments is supported by at
least 8 reads with 12 bases on each side. This must be at least 3 and the default is 8. The read alignment files (bam and bam index files) for each sample were visualised with IGA.

Transcript reconstruction and differential expression analysis was done using Cufflinks (Cufflinks-2.2.1, http://cole-trapnell-lab.github.io/cufflinks). Normalized FPKM (fragments per kilobase per million reads) were used for differential gene expression analysis. Statistically significant expression changes were identified based on p and q metrics. P-values reflect the magnitude (fold change of treatment relative to control) and variation among biological replicates, whereas q-values account for false discovery rate based on library size.

**Functional representation of differentially regulated transcripts**

Graphical visualisation for functional categorisation of differentially regulated transcripts was performed using MapMan 3.5.1R2 functional annotation tool (Thimm et al., 2004). The tool, supporting resources and annotation database (based on TAIR9 annotation) can be downloaded from http://mapman.gabipd.org/web/guest/home. Statistical analysis on enrichment of differentially changed pathways was performed using Wilcoxon rank test and Benjamini-Hochberg (BH) p-value correction (Usadel, 2005). Methods for manual creation of new pathways and categorical filtering of expression data were adopted from (Usadel et al., 2009) and explained in sections below.

I custom made the graphical overview of pathway of interest such as defence signalling cascades in Figure 5.11 and loaded as separate pathway file into MapMan. Next I loaded a mapping file containing list of gene identifiers and descriptions manually assigned to new functional bins previously absent from the MapMan annotation. To generate the mapping file, I analysed recently published literature and public TAIR database (www.arabidopsis.org/browse/genefamily) for all membrane-located receptor-like kinases and their classes (Shiu and Bleecker, 2001), cytoplasmic receptors such as NLR proteins (Hofberger and Jones, 2014; Kroj et al., 2016; Sarris et al., 2016), CDPK-SnRK superfamily (Hrabak et al., 2003), MAP kinases cascade (Asai et al., 2002; Jonak et al., 2002) as well as SA, JA and ET biosynthesis and signalling genes (Verk, 2010). Expression data were supplemented with categorical variables for filtering different custom set attributes such as significance (previously determined in DE
To predict (TF) binding sites, I searched for known TF binding motifs in Arabidopsis transcription factor database ([www.arabidopsis.med.ohio-state.edu/AtTFDB/](http://www.arabidopsis.med.ohio-state.edu/AtTFDB/)) and queried Upstream Gene Sequences (-3000bp) in TAIR Statistical Motif Analysis tool for presence of the respective motifs ([www.arabidopsis.org/tools/bulk/motiffinder](http://www.arabidopsis.org/tools/bulk/motiffinder)).

**Iterative Group Analysis (IGV) for functional pathway enrichment**

I ranked all transcripts based on the fold change values for any comparison between two experimental treatments. The ranked lists of most upregulated and downregulated transcripts were submitted for enrichment analysis of various functional classes based on published gene ontologies using Iterative Group Analysis (IGA) tool. This is an open-source command-line run package available to download from supplementary materials in Breitling *et al.* (2004). The IGA was run first on a randomised list of transcripts to determine the threshold for false gene enrichment. Functional pathways with large number of genes can be overrepresented in the experimental sample by chance, therefore, the false discovery threshold was further applied to the ranked gene lists. The command line used for gene family enrichment in chapter 5:

```
inga --i_name.txt --ot_name.txt -agene-anot.txt -ggene-names.txt -t0.1
```

where `i` – input file with ranked gene list; `o` – name of output file; `a` – annotation file; `g` – gene list in pathways; `t` – threshold [0;1]. Threshold for similar stress responses was 0.2 and cis-element enrichment 0.9.

**Clustering analysis**

Clustering genes with most similar differential regulation across the treatments in RNA-seq data was done in Cluster 3.0 ([available at http://bonsai.hgc.jp/~mdehoon/software/cluster/](http://bonsai.hgc.jp/~mdehoon/software/cluster/)). I used Euclidean distance to calculate the gene similarity matrix that would take into account both the magnitude of fold change in any given treatment as well as relative proportions of fold change between pairs of treatments (i.e., relative up- or down-regulation with respect to different treatments) and is suitable for log-transformed expression data (D'haeseler, 2005). The calculated similarity matrix was then used in agglomerative hierarchical clustering by adding nearest neighbour (the
next most similar gene) to any given other observation. The size of each cluster was based on centroid or complete linkage. Centroid linkage delimits clusters based on the distance between the average similarity scores for all observations in the cluster. This method was chosen for datasets including few genes such as MTF family proteins. Complete linkage delimits clusters based on the furthest distance between any two observations in the clusters and tends to generate more equal-size clusters (http://support.minitab.com/en-us/minitab/17/topic-library/modeling-statistics/multivariate/item-and-cluster-analyses/linkage-methods/). For this reason, complete linkage method was chosen for larger datasets such as MTF clustering with plant defence genes. The clustering files were visualised with TreeView (available at http://jtreeview.sourceforge.net/).

**RNA extraction for rt-qPCR**

Total RNA was extracted using Tri-Reagent (Sigma-Aldrich) following manufacturer’s instructions and applying DNaseI treatment after the extraction (RQ1 DNase set; Promega, Madison, WI, USA). Resulting RNA was analysed for purity and yield using EtBR staining in agarose gel electrophoresis and spectrophotometer (NanoDrop 2000 Thermo Scientific, Loughborough, Leicestershire, UK). RNA samples with A260/A280 ratios between 1.9 and 2.1 were diluted to similar concentrations before proceeding to cDNA synthesis.

**cDNA synthesis**

cDNA was synthesised from 1 μg RNA using the M-MLV-RT Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions and primed using oligo-dT.

**Real-time quantitative PCR (rt-qPCR) for defence gene expression**

Resulting cDNA was diluted 1:10 with distilled H2O before using for rt-qPCR. Each PCR reaction contained 25 ng of cDNA and 0.5 μg of each primer added to SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in a final volume of 20 μL. Three technical replicates of each sample were used per experiment. Reactions were loaded in 96-well format white ABgene PCR plate (Thermo Scientific) and run in a CFX96 Real-Time System with a C1000 Thermal Cycler.
(Bio-Rad, Hemel Hempstead, Hertfordshire, UK) with the following settings: 3 min at 95°C, with 40 cycles of 30 s at 95°C, 30 s at 60 °C, 30 s at 72°C, followed by melt curve analysis: 30 s at 50°C (65-95°C at 0.5°C increments, 5 s for each).

Primers for 5’ region of actin (AT2G37620) and 3’ region of GAPDH (AT1G13440) was used as a reference. The geometric mean of the reference gene expression was calculated for each well. Relative expression values were calculated following the comparative ∆Ct method (Schmittgen and Livak, 2008). The mean Ct value was calculated between the three technical replicates. The relative change in the expression of a gene was calculated as ∆Ct = (mean Ct gene of interest - mean Ct reference gene). The fold-change between treatments was calculated as (∆Ct treatment- ∆Ct control)/ ∆Ct control. This method ensures consistency with DE calculations for RNA-seq data.


De novo transcription factor binding site predictions

I aimed to create a network demonstrating protein-protein (PPI) as well as transcription factor-target gene (TFI) interactions. Many databases for TF binding sites such as AtcisDB (http://arabidopsis.med.ohio-state.edu/AtcisDB/), TRANSFAC (http://gene-regulation.com/pub/databases.html) contain predictive binding motifs for certain classes (families) of transcription factors (TFs). In contrast, databases like ORegAnno (http://oreganno.org/) or PAZAR (http://pazar.info/) contain experimentally verified information for specific pairs of gene target-TF interactions. In order to bypass the shortcomings of the both types of databases, I aimed to do de novo TF-target binding predictions using only experimentally validated A.thaliana TF binding motifs from JASPAR CORE Plantae database (http://jaspar.genereg.net/). I obtained binding site matrices for 192 different TFs. I obtained -5000 bp upstream DNA sequences (no overlap with neighbouring CDS) for every gene coding sequence in A. thaliana genome (Regulatory Sequence Analysis Tools RSAT PLANTS http://www.rsat.eu/) and scanned all 192 TF binding matrices across all regulatory sequences to identify pattern match with high degree of stringency (Marcov order 2; p<5E-07 to minimize false discovery). Each match between the experimentally obtained TF binding motif and target gene was recorded as an interacting edge and combined with PPI.
**Acquiring protein-protein interaction data**

All experimentally verified and predicted PPI scores were obtained from STRING v10.0 database (fully licenced version download from [http://string-db.org/](http://string-db.org/)), integrating data from other databases like BioGRID, InterAct and containing a total of 1,048,575 *A. thaliana* PPI (Szklarczyk *et al.*, 2015). I considered only confirmed PPI as well as strong PPI prediction (i.e., PPI with the following scores coexpression >500; exp_transfer>900; database transfer>900; textmining>500; overall score>500). Furthermore, I filtered the resulting list of genes, leaving only the transcripts which are expressed in our dataset.

Next I combined the filtered PPI layer with TFI layer to give 183,835 interactions among different 15,430 genes. Interestingly, this list of genes is highly redundant (89.9%) with the expressed list of 17,153 genes from our RNA-seq data, and therefore, captures most of the potential interactions.

**Visualisation of defence gene interaction network**

The gene list obtained above was further queried for genes that are involved in defence (identical to the MapMan defence map described earlier) and their first level interactors (Chapter 5, figure 5.15). Interactions were visualised using Cytoscape v3.4 ([http://www.cytoscape.org/](http://www.cytoscape.org/)). I imported a separate annotation table as edge (interaction) attributes to colour code the predicted PPI, confirmed PPI and TFI. Similarly, annotations for node (gene) fold change and functional categorisation such as identity of JA, MAPK etc. pathway (Figure 5.11) were imported. Network layout was based on the imported node attributes.

**Functional enrichment analysis within the interaction network of all defence-related genes**

BINGO application ([http://apps.cytoscape.org/apps/bingo](http://apps.cytoscape.org/apps/bingo)) was downloaded and installed into Cytoscape. BINGO (Maere *et al.*, 2005) calculates overrepresented GO terms in the network and display them as a network of significant GO terms using a hypergeometric test and Benjamini-Hochberg (FDR) correction.
8.15. Statistical analysis

Statistical analysis was performed in Minitab16 and R-studio. Insect oviposition data were analysed using paired t-test, two-tailed t-test or GLM. Assumptions of the statistical tests – normal distribution and equal variance – were checked with the Anderson–Darling and the Levene’s tests, respectively. Principal Components Analysis and hierarchical clustering of RNA-seq libraries was done with R-based CummeRbund package (available at Bioconductor.org together with user manuals). For this the Cufflinks data were read in CummeRbund and analysed with MAplot and csDendro functions, respectively.

Statistical tests for enrichment analysis of gene functions was performed using MapMan, BINGO or IGA software packages, as described in previous sections. Additional hypergeometric tests for evaluating the enrichment of transcription factor binding sites was performed in Microsoft Excel2013 (hypergeometric distribution, cumulative probability).
Philosophical Epilogue
Morality in Modern Science and Society

“Truth is the only daughter of time,” attributed to Leo da Vinci\(^1\), encapsulates the ideal, pure reality, which, described by Plato, exists since the beginning of universe and is attainable by the reason only. The pioneering experimentalist Aristotle used empirical observations to attempt to describe the Platonic reality\(^2\). He was criticised by many to be deceived by his senses. “Science is but an image of truth,” noted Francis Bacon\(^3\), father of the scientific method, that inclines under the centuries-old schools of scepticism from Greek sophists to David Hume\(^2\). And yet, scientific discoveries have been at the heart of many transformations of society. French botanist Leo Errera said\(^4\): “Truth is on a curve whose asymptote our spirit follows eternally.” Given the premise that reality, as it is, may be beyond description of the scientific method, many scientists describe the instrumental reality, as it appears\(^5,6\). Equipped with such understanding about nature, how should scientists better communicate the implications and influence the decisions about the applications of their theories in morally just manner?

I would like to discuss the strengths and limitations of the scientific method as a paramount to social constructivism, political decision-making as well as ethical foundations of morality. I will argue that the latter is the most crucial aspect in the path from enlightenment to engagement and empowerment by scientific knowledge.

Pursuing the truth is one of the main goals of all philosophical (including pre-historic mythological and religious) teachings in all civilisations of all times\(^2\). Like scientific investigation is empirical, the others are rational methods to analyse and conceive the reality. “A theory can be proved by experiment; but no path leads from experiment to the birth of a theory,” said Albert Einstein\(^7\). Through scientific induction we can make generalisations – theories, hypothesis or thought experiments – and conceive the ideal Platonic reality. Through scientific deduction we test such theories using observation or experimentation. Thus, the scientific process is inherently rational and empirical. As is any other philosophical process, given it is complemented with tools and experimental systems to verify the ideas. However, within the experimentation itself resides the limitation of scientific conclusions. Karl Popper elaborates\(^8\): “The method of science depends on our attempts to describe the world with simple theories:
theories that are complex may become untestable, even if they happen to be true. Science may be described as the art of systematic over-simplification—the art of discerning what we may with advantage omit.” Reduction of a complex problem to a testable hypothesis is instrumental to scientific deduction and key to the remarkable success of science in development of new technologies. Notably, this has also a great impact on normative ethics by creating the “utilitarian selectionism” where everything that improves the condition (of majority) is desired and apprized. Nevertheless, the utilitarian value of scientific discoveries inherently lies within the framework of the simplified theory originally considered. Natural systems and human existence as their part is more complex than the simplified models tested by scientists and implemented in socio-economic processes. English weather is inherently chaotic system; so is potato yield more to the national economy and gastronomic dinner than ten mega-pennies of revenue per hectare.

“To measure is to know,” a strong belief of Lord Kelvin, may be a splendid illustration of scientism – the over-esteemed confidence that any problem is tractable by the empirical aspect of scientific process. Moreover, scientism is at the heart of “scientific socialism” where application of scientific data to solving social issues and making political decisions is under strict utilitarian logics and pre-defined simplified templates of society. For example, what will be the price of kilo rice tomorrow? Or, substitution of human-labour with machine-work guided by the company revenue. Such considerations have been at the heart of post-modernist era criticism on science. Social constructivism has influenced both the theoretical implications and application of science. In his thought-provoking book “The Structure of Scientific Revolutions” Thomas Kuhn wrote: “As in political revolutions, so in paradigm choice — there is no standard higher than the assent of the relevant community... this issue of paradigm choice can never be unequivocally settled by logic and experiment alone.” There are undisputable advantages of working within a framework of a given theory agreed by community to understand the mechanistic basis of many natural or social phenomena. Identification of a “turning-point” when new data propose alternative theory is fundamental to paradigm shift and scientific revolutions. While this principle is in general concurrence among scientists for how we make objective conclusions and implications from experiments, the adoption of this principle to how we decide
on applications of scientific theories is rather more dogmatic and less open to alternative views.

The reason for this is that decision making process on application of scientific theories is largely detached from scientific discovery process itself. J. Robert Oppenheimer was a key figure in the Manhattan Project and said\textsuperscript{11}: "It is a profound and necessary truth that the deep things in science are not found because they are useful; they are found because it was possible to find them."

Although I hereby lay no contra-arguments to the stated fact that many discoveries are made in serendipity, I argue, however, that a scientist should not be blind to the conflict of interest by the paymasters of the research. While significant proportion of science is funded by governments and, ever increasingly, public crowd-funding\textsuperscript{12}, a significant proportion of funding comes from industry - corporations with private interests\textsuperscript{13}. Moreover, most of government research spending concern military R&D\textsuperscript{14}. Although "It is open to every man to choose the direction of his striving; and also every man may draw comfort from Lessing's fine saying, that the search for truth is more precious than its possession," as noted Einstein\textsuperscript{15}, I repute more to what was reasoned by British scientist and novelist C.P. Snow\textsuperscript{16}. "A scientist has to be neutral in his search for the truth, but he cannot be neutral as to the use of that truth when found. If you know more than other people, you have more responsibility, rather than less."

I clarify that hitherto I made no argument in favour of applied versus fundamental sciences. I conveyed that whatever discoveries derive from the two alike, are subject to decisions for its utility by scientist as the member of public. Role of science is enlightenment, engagement and empowerment\textsuperscript{17}. Jean-Baptiste Lamark signified\textsuperscript{18}: "It is not enough to discover and prove a useful truth previously unknown, but that it is necessary also to be able to propagate it and get it recognized." Imanuel Kant's "Doctrine of Virtue" and David Anscombe's "Modern Moral Philosophy" recognise enlightened individuals as leaders of society\textsuperscript{19,20}. They engage public and communicate their philosophy and discoveries which are attained by reason and experiments alike. Furthermore, possession and recognition of knowledge and know-how is foundation for empowerment of society. Therefore, virtuous individuals or, better, virtuous society, should consider the limitations of scientific process and all conflicts of
interest before utilising the knowledge they possess already or invest in acquisition of such knowledge.

With reference to Kuhn, many of these decisions are unattainable by experiments and utilitarian logics alone\textsuperscript{10}. Given the limitation by any one methodological approach in attaining the truth, scientific method can only complement other philosophical methods to address the questions about morality in modern society and thus enable just and objective path from enlightenment to empowerment of scientific and philosophic ideas. John Paul II addressed the Pontifical Academy of Sciences in his speech\textsuperscript{21}: “\textit{Every scientist, through personal study and research, completes himself and his own humanity. […] Scientific research constitutes for you, as it does for many, the way for the personal encounter with truth, and perhaps the privileged place for the encounter itself with God, the Creator of heaven and earth. Science shines forth in all its value as a good capable of motivating our existence, as a great experience of freedom for truth, as a fundamental work of service. Through research each scientist grows as a human being and helps others to do likewise.”} The mission of a scientist is to pursue the truth, inform about his findings and guide others in their path to humanity by contextualising the discoveries of science in ethical framework of moral philosophy.

Notes and References of the Epilogue

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\textsuperscript{2} Bryan Magee (1998), The story of philosophy

\textsuperscript{3} James Wood (1893), Dictionary of Quotations from Ancient and Modern, English and Foreign Sources, 383:3.

\textsuperscript{4} John Arthur Thomson, Introduction to Science (1911), p57


\textsuperscript{7} The Sunday Times, 18 July, 1976

\textsuperscript{8} Karl Raimund Popper and William Warren Bartley (1991), The Open Universe: an Argument for Indeterminism, p44

\textsuperscript{9} Presidential Address to the British Association for the Advancement of Science, Edinburgh, 1871
10 Thomas S. Kuhn (1962), *The Structure of Scientific Revolutions*, p93


16 J. Robert Moskin (1966), *Morality in America*, p61

17 Coined during personal communication with Dr Padhmanand Sudhakar (Earlham Institute, NRP, UK).


20 David Anscombe (1958), “Modern Moral Philosophy”

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Appendix A

Appendix B

Appendix C

Supplemental Figures 1-3
Supplemental Figure 1 (for Table 2.1, Chapter 2, page 78).
SAP54 homologs from AY-WB, PnWB and Stolbur phytoplasmas demonstrate conserved protein-protein interactions with plant MTFs and RAD23 proteins in yeast-two-hybrid experiment. SAP54 homologs were cloned as prey in pDEST32 vector containing Binding Domain of GAL4 gene. Plant MTF and RAD23 target proteins were cloned as bait in pDEST22 vector containing the Binding Domain of GAL4 gene. Successful transformants with both bait and pray plasmid are selected on SD media lacking Leu/Trp (–L-W). Upon interaction of the prey and the bait proteins, expression of marker genes allows yeast growth on selective SD media lacking Leu/Trp/His (–L-W-H).
Supplemental Figure 2.

SAP54 and GFP plants have similar trichome density on leaf abaxial and adaxial sides before and after exposure to *Macrososteles quadrilineatus* feeding and egg-laying. The total number of trichomes was counted over entire leaf blade on the adaxial and abaxial sides of *A.thaliana* leaves that were un-exposed to *M. quadrilineatus*. Trichome number was normalised by total area of the leaf as a measure of trichome density per cm$^2$. Trichome density was determined on another group of SAP54 and GFP plants that were exposed to 10 male and 10 female adult *M. quadrilineatus* feeding and oviposition for 5 days in a small choice cage (Figure 8.6; materials & methods).
Supplemental Figure 3.

Macrosteles quadrilineatus oviposition sites on Arabidopsis thaliana leaves.
Leafhopper M. quadrilineatus females deposit their eggs in four possible locations on the abaxial side of A. thaliana leaves: (1) inter-vein region where eggs are protruding out from the leaf surface; (2) leaf blade margin where eggs are embedded beneath epidermal tissues and often packaged parallel to each other; (3) embedded within or deposited right next to the midvein; (4) within leaf pedicel. Black triangles are pointing to the location of individual eggs. Pictures depict eggs of different ages after deposition. The quantity of eggs in each location is not representative and varies from leaf to leaf. Bars are approximately 1mm in all pictures.
Appendix D

Appendix E
