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Arabidopsis thaliana-Spider Mite Interaction: Plant Perception, Signalling, and Response

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

ARABIDOPSIS THALIANA-SPIDER MITE INTERACTION: PLANT PERCEPTION, SIGNALLING, AND RESPONSE

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BY

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Abstract

The two-spotted spider mite, *Tetranychus urticae*, is a cell-content feeding chelicerate herbivore, feeding on over 1000 plant species, one of which is *Arabidopsis thaliana*. This research uses microarray data from two *A. thaliana* accessions that differ in susceptibility to spider mite feeding to identify how the plant defends itself against this herbivore. Mutant analysis of induced plant defense pathways and physiological assays of mite performance indicate that *A. thaliana* utilizes: a) damage associated molecular pattern receptors, PEPR1 and PEPR2, to aid in perception of attack; b) jasmonic acid as the key phytohormone involved in resistance signalling; and c) indole glucosinolates as effective secondary metabolites affecting mite performance and development. My findings provide insight into how *A. thaliana* defends itself against this class of arthropod herbivores using defences that have previously been associated with deterrence of insect herbivores, which are distantly related to chelicerates.

Keywords

Arabidopsis thaliana, *Tetranychus urticae*, herbivore-plant interaction, host plant resistance, damage associated molecular patterns, PEPR1, PEPR2, jasmonic acid, indole glucosinolates

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List of Abbreviations

μmol/m^2/s	MicroEinstein per second and square meter
aa	Amino acid
ABA	Abscisic acid
ABRC	Arabidopsis Biological Resource Center
Bla-2	*A. thaliana* accession resistant to spider mites
BRI1	Brassinosteroid Insensitive 1, a brassinosteroid receptor
cDNA	Complementary deoxyribonucleic acid
Ct	Cycle threshold
DAMPs	Damage associated molecular patterns
DEG	Differentially expressed gene
dpi	Dots per inch
DNA	Deoxyribonucleic Acid
ET	Ethylene
ETI	Effector triggered immunity
GO	Gene ontology
HAMPs	Herbivory associated molecular patterns
hpi	Hours post infestation
hr	Hours
HTI	Herbivore triggered immunity
IAA	indole-3-acetic acid
IG	Indole glucosinolate
JA	Jasmonic acid
Kon	Kondara, *A. thalaiana* accession susceptible to spider mites
LLR	Leucine rich repeat
MAMPs	Microbial associated molecular patterns
MAPK	Mitogen-activated protein kinase
n	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nucleotide binding
NRQ	Normalized relative quantity
OPDA	12-oxophytodienoic acid

OS	Oral secretions
PAMPs	Pathogen associated molecular patterns
PCA	Principal component analysis
Pep	Plant elicitor peptide
PROPEPs	Precursor proteins of Pep peptides
PRR	Pattern recognition receptor
PTI	Pathogen triggered immunity
RK	Receptor kinase
RLK	Receptor like kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SEM	Standard error of mean
T-DNA	T DNA
TF	Transcription factor
WAK1	Wall associated kinase 1
WIR	Wound induced response
WT	Wild type

Chapter One - Introduction

Natural processes and interactions among organisms can go unnoticed and unappreciated by us as we go about our daily lives. These processes and interactions often have a profound impact on our economy, our natural resources, even our personal health. Through studying the interactions between organisms that co-exist in the various ecosystems we cultivate and conserve, we can develop and deploy strategies in business, technology, and everyday life that will improve efficiency, productivity, and environmental sustainability. One such interaction between organisms, extensively studied and having an enormous and costly impact on the agricultural economies around the world, is the interaction between herbivorous pests and their host plants. By having a complete understanding of the molecular mechanisms of perception, signalling, and defence responses, we can engineer and breed crops better capable of defending themselves against known herbivores in such a way as to decrease or eliminate our dependence on pesticides when used in conjunction with other strategies in providing integrated pest management. Aside from the economic benefits of research into pest-plant interaction, such knowledge would also be beneficial as we continue to refine our impact on the natural world to establish a more harmonious relationship with it.

1.1 Plant defence

Being sessile in nature, plants cannot flee from an attacking herbivore. This is not to say, however, that they are defenceless. One strategy to combat the detrimental effects of arthropod herbivory is tolerance, which consists of a complex set of genetic traits that enable a plant to withstand or recover from damage through sequestration of limiting resources for regrowth (Kessler and Baldwin, 2002). This strategy does not adversely affect the growth or survival of the attacking arthropod (Smith and Clement, 2012). Another strategy is resistance, which consists of traits that negatively affect the herbivore. The term antixenosis describes an effect on herbivore behaviour in which the herbivore displays delayed acceptance or outright rejection of a plant host due to morphological or chemical plant features. Alternatively, plants can affect herbivore life history traits such as survival, development, and fecundity in what is termed antibiosis (Smith and Clement,

2012). Resistance mechanisms (defined here as the underlying chemical or morphological plant process that produce negative effects on the attacking herbivore) can come in the form of direct and indirect defence. Direct defences include chemicals that produce antifeedant, toxic, or repellent effects as well as physical barriers such as tissue toughness, plant pubescence, and trichomes (Smith and Clement, 2012). Defensive strategies can also act indirectly against the herbivore in the form of volatile organic compounds that are emitted by the plant to attract predators or parasitoids of attacking herbivores (Kessler and Baldwin, 2002).

Many defences are constitutive in nature, present regardless of the presence of attacking herbivore. Defences can also be induced upon the perception of attack. Direct and indirect defences are often inducible due to the cost associated with resistance traits. To continuously produce resistance traits would be detrimental to a plant if fitness-limiting resources (such as nitrogen) were invested in that response, or conversely if those traits happen to be toxic to the plant as well. Moreover, constant defences can interfere with beneficial interactions with pollinating insects (Kessler and Baldwin, 2002). On an evolutionary scale, constitutive defences may select for adaptation in herbivorous arthropods leading to their evasion of plant defence (Agrawal and Karban, 1999), so an inducible defence system would be of benefit to the plant. In addition, using 56 wild species of Solanaceae, Campbell and Kessler (2013) demonstrated that the transition from ancestral self-incompatibility (obligate outcrossing) to self-compatibility (increased inbreeding) leads to the evolution of an inducible as opposed to a constitutive strategy of resistance. Therefore, inducibility in self-compatible species may provide a means of creating variation in a defence response, diversifying it through time. Whereas, self-incompatible species have a means of increasing diversity of secondary metabolites through genetic diversity achieved through outcrossing (Campbell and Kessler, 2013). It should also be noted that there is a difference between induced resistance, which has an observable negative effect on the herbivore, versus induced defence, which has a measured increase in plant fitness. Although this thesis uses the terms defence and resistance interchangeably, plant fitness is not tested (though susceptibility/resistance is tested and can serve as a proxy for this), and therefore, strictly speaking, this thesis concerns induced resistance and not induced defence.

The ability to perceive and respond defensively to arthropod attack constitutes a form of immunity in plants and much of what we know about the mechanisms and evolutionary origins of immune recognition in plants derives from plant-pathogen interaction studies (Howe and Jander, 2007). Although pathogen infection and herbivore attack share many similarities, from pattern recognition receptors (PRRs) that recognise conserved molecular patterns associated with attackers, to phytohormones used to establish a signalling cascade ultimately leading to transcriptome reprograming and induction of a defence response, there are also many subtle differences that distinguish these two types of interactions. Defences against microbes can be highly effective on small spatial scales. For example, the hypersensitive response prevents the spread of biotrophic pathogens as the plant sacrifices cells surrounding an infection site and fills them with antimicrobial compounds. However, it is obvious that this type of response would be ineffective against non-sedentary herbivores (Kessler and Baldwin, 2002). Having the ability to distinguish between pathogen and herbivore attack would allow for a tailored defence response to each type of attacker in isolation or in concert and would give the plant a significant advantage.

1.2 Arthropod herbivory

Estimates report the number of insect species feeding on plants to be 45% of the approximately 1 million described insect species (Zheng and Dicke, 2008). Plants and arthropods (including insects and chelicerates) have coexisted for approximately 350 million years, and interactions between them have resulted in coevolution which has produced a large degree of variation in both the susceptibility of different plant species to various arthropod herbivores, as well as the differences in feeding strategies and preferred host plants by arthropods (Mithofer and Boland, 2008).

The variation in herbivore dietary choice is extreme. Some arthropod herbivores are polyphagous in nature, being generalists with ability to feed on many different plant families. Others are specialists, being monophagous or oligophagous and feed on a single or very few plant species belonging to the same family. The decision to feed on a host plant is determined in large part by the array of chemical secondary metabolites synthesized by the plant that act as deterrents or attractants to a particular herbivore

species. The suitability of a host plant is assessed in part by the use of contact chemoreceptors on the insects' mouthparts, antennae, and tarsi (feet) (Howe and Jander, 2007). Feeding strategies of arthropods also varies greatly. One feeding strategy involves causing damage with mouthparts evolved for chewing, tearing and snipping, such as seen in leaf-eating beetles (Coleoptera) or caterpillars (Lepidoptera) which comprise about two-thirds of all known insect herbivores (Schoonhoven *et al.*, 1998). Herbivores such as thrips and spider mites use tube-like stylets to pierce cells and suck up the liquid content, whereas leafminers develop and feed on the soft tissue between epidermal cell layers (Howe and Jander, 2007). Phloem feeders such as aphids, whiteflies and other Hemiptera, insert their stylets between cells and establish a feeding site in the phloem (Howe and Jander, 2007). Due to the extreme variability in the herbivore mode of feeding, it is not surprising that plant defence responses are also variable.

1.3 Perception of attack

Vertebrate animals use specialized, mobile cells that allow for acquired immunity, requiring immunoglobulin and T-cell receptor genes that are re-arranged in every individual throughout their lifetime to recognize and remove pathogens. This 'real-time' development of resistance is considered relatively recent in evolutionary terms (Boller and Felix, 2009). Vertebrate animals also have an innate immunity, considered evolutionarily ancient, utilizing PRRs that are fixed in the germ line. Plants must rely entirely on innate immunity, such that the ability of each cell to perceive well conserved 'danger' signals triggers an immune response locally and systemically to fend off attackers (Howe and Jander, 2007; Boller and Felix, 2009). These danger signals are perceived by PRRs that can bind molecular patterns associated with pathogen or herbivore attackers. Moreover, wound-associated molecular patterns endogenous to the plant are released upon tissue damage during attack, and their presence indicates damaged-self (Boller and Felix, 2009; Figure 1.1). Most of our current understanding about the mechanisms and evolutionary origins of the plant immune recognition system derives from plant-pathogen interaction studies (Jones and Dangl, 2006).

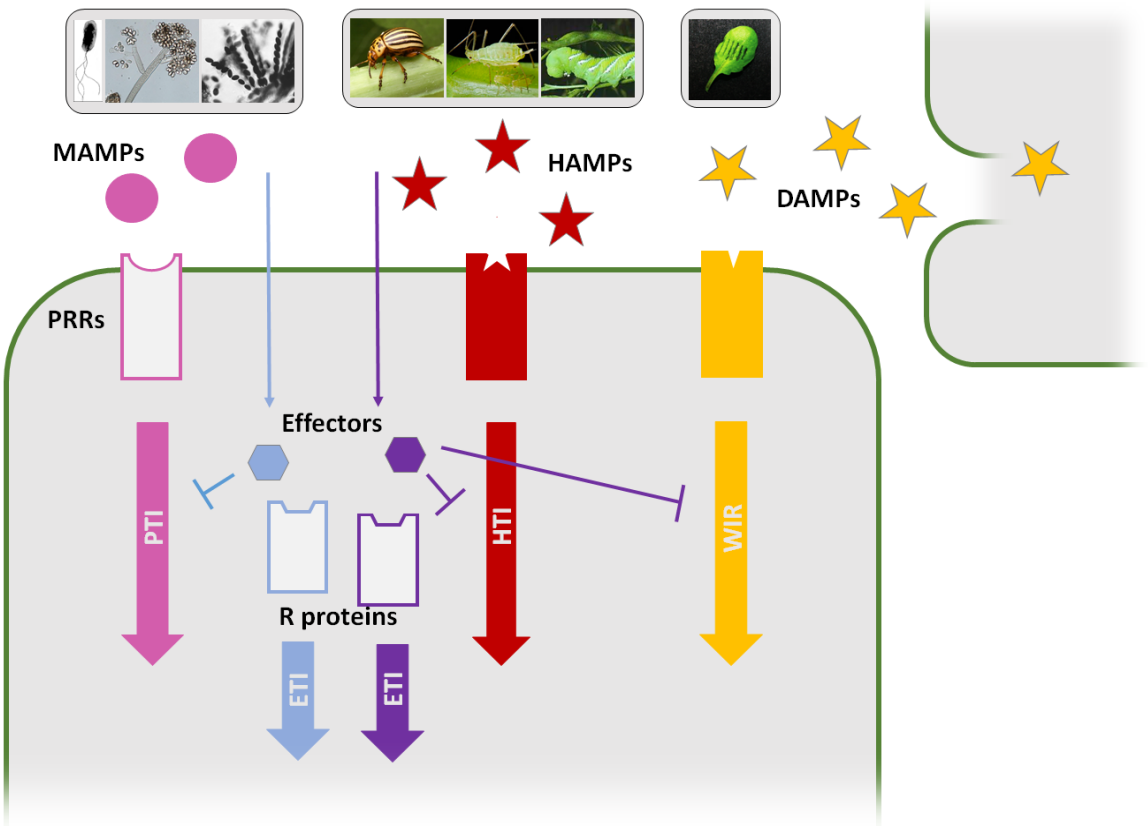


Figure 1.1 Schematic of plant perception of attack and induced defence. Studies of plant-pathogen interaction show defence responses are initiated by the recognition of conserved microbe/pathogen associated molecular patterns (M/PAMPs) by pattern recognition receptors (PRRs) within the plasma membrane. This induces PAMP-triggered immunity (PTI) that restricts the propagation of attacking pathogens. Some strains of pathogens have evolved effectors, introduced into the cell to suppress PTI leading to susceptibility. Recognition of pathogen effectors (or their activity) by plant resistance proteins (R proteins) leads to effector-triggered immunity (ETI) and plant resistance. Plants perceive herbivore attack through herbivore associated molecular patterns (HAMPs). HAMPs are elicitors originating from herbivore oral secretions and/or oviposition fluids. Plants can also perceive wounding associated with herbivory through damage associated molecular patterns (DAMPs). Recognition of herbivory through HAMPs and DAMPs triggers herbivore-triggered immunity (HTI) and wound-induced responses (WIR) resulting in the initiation hormone signalling pathways that are responsible for transcriptome and metabolic changes responsible for the production of secondary metabolites that may negatively affect the herbivore. Figure modified from Erb *et al.* (2012).

Interestingly, early responses to pathogens and herbivores following perception of attack are very similar and include ion fluxes across the plasma membrane, collapse of membrane integrity at the feeding site, initiation of kinase cascades, and generation of reactive oxygen species, all of which represent localized defences (Maffei *et al.*, 2007; Wu *et al.*, 2007). Production of phytohormones represents another similarity between pathogen and herbivore attack. These hormone signalling pathways ultimately lead to the induction of defence genes and the biosynthesis of secondary defensive compounds that can also occur systemically throughout the plant (Wu and Baldwin, 2009). The systemic accumulation of defensive compounds is important for resistance against non-sedentary arthropod herbivores.

As previously mentioned, danger signals can come from a variety of sources. If these danger signals are not originating from the plant, then the recognition of exogenous signals can occur directly by perception of herbivore-derived molecular patterns. In plant-pathogen interactions, danger signals are termed pathogen associated molecular patterns (PAMPs) or, more recently, microbe associated molecular patterns (MAMPs). These patterns are invariant bacterial surface molecules that are indispensable to the attacking microorganism and they do not exist in the host plant, which allows the plant to recognize them as foreign and to initiate an immune response (Postel and Kemmerling, 2009; Figure 1.1). MAMPs consist of diverse signals including carbohydrates, lipids, peptides, sterols, and (glycol)-proteins (Boller, 1995). One well-characterized MAMP/PRR pair is the conserved portion of the N terminus of bacterial flagellin (active epitope flg22) and its receptor FLAGELLIN-SENSING 2 (FLS2; Boller and Felix, 2009). Highly conserved orthologs of FLS2 are present in the genomes of many higher plants including *Vitis vinifera* (grape vine), *Populus trichocarpa* (California poplar), *Ricinus communis* (castor bean), *Arabidopsis thaliana* (thale cress), *Oryza sativa* (Asian rice), and *Zea mays* (corn) and indicate that the PRR, FLS2 for flg22 is probably evolutionarily ancient (Boller and Felix, 2009).

Danger signals originating from herbivores are termed herbivore associated molecular patterns (HAMPs) and represent a newly studied class of elicitors. It is hypothesized that plants have evolved the ability to perceive HAMPs to distinguish attack

by herbivores from those of other biotic agents (Felton and Tumlinson, 2008; Mithofer and Boland, 2008). HAMPs can be part of herbivore oral secretions (OS), oviposition fluids, and other fluids released/secreted by the herbivore (Mithofer and Boland, 2008; Wu and Baldwin, 2009; Figure 1.1). Although several HAMPs have been isolated and several receptors have been shown to be involved in herbivore defence, no HAMP/PRR pair has been identified thus far (Erb *et al.*, 2012; Smith and Clement, 2012). For example, an elicitor identified as a HAMP is β-glucosidase from OS of *Pieris brassicae* (white cabbage butterfly) larvae that elicits volatile production from cabbage plants (Mattiacci *et al.*, 1995). Interestingly, HAMPs can also be derived from proteins originating from the plant which are subsequently modified by the herbivore. For example, plant proteins can be proteolyzed by herbivores during feeding and the altered plant protein can then be recognized by the plant during continued feeding. One such HAMP isolated from *Spodoptera frugiperda* (fall armyworm) larval OS, is termed inceptin and promotes *Vigna unguiculata* (cowpea) ethylene production and increases in salicylic acid and jasmonic acid. Inceptins are proteolytic fragments of chloroplastic ATP synthase γ-subunit regions that mediate plant perception of herbivory through induction of volatile organic compounds, phenylpropanoids, and protease inhibitor (anti-digestive) defences (Schmelz *et al.,* 2006). HAMPs are also likely present in the mucus residue (‘slime trail’) of *Arion lusitanicus* (Spanish slug). Treating wounded leaves with this residue increased wound-induced jasmonic acid levels, shown to be effective in the defence of *Arabidops*is against molluscan herbivores (Falk *et al.,* 2013).

Danger signals originating from the plant are called damage associated molecular patterns (DAMPs). DAMPS are also evolutionarily conserved molecular signatures but differ from HAMPs in that they are endogenous to the host plant. They are released and subsequently perceived by PRRs as ‘damaged self’ markers upon initiation of herbivore feeding (Figure 1.1). DAMPs are generated at the site of damage. However, the signals generated following their recognition can be delivered to undamaged parts of the plant in a systemic manner (Tör *et al.*, 2009). During pathogen attack, DAMPs can be generated by lytic enzymes produced by pathogens that breach the structural barriers of plant tissues (Boller and Felix, 2009). For example, oligogalacturonides can act as endogenous elicitors with well-documented immune response activity. Though the mechanism of

perception of oligogalacturonides remains unconfirmed, the *Arabidopsis* wall-associated receptor like kinase (RLK) termed WAK1 has a high affinity to oligogalacturonides leading to the possibility that WAK1 or its homologs might act as part of the recognition mechanism for them (D'Ovidio *et al.*, 2004; Boller and Felix, 2009). Different plant species harbour different DAMPs. For example, well-known DAMPs found only in Solanaceae belong to the family of defence-related peptide hormones called systemins. In damaged tomato leafs, the 18-aa systemin peptide, derived from a 200-aa precursor protein, can travel to distal parts of the plant and activate defence responses systemically (Pearce *et al.*, 1991; Tör *et al.*, 2009). As the precursor of systemin is cytoplasmic, release of the active peptide is presumed to happen only upon cell damage. If this is the case, then it is likely that systemin acts as a DAMP for neighboring intact cells. A 160-kDa cell-surface receptor protein in membranes of *Lycopersicon peruvianum* (tomato) suspension cultured cells that possessed characteristics of a systemin receptor (Scheer and Ryan, 1999), was purified and identified as a leucine-rich repeat receptor kinase (LRR-RK) with high amino acid identity with the BRI1 receptor kinase from *Arabidopsis* (Scheer and Ryan, 2002). However, *bri1* mutant plants were found to be capable of initiating a systemin induced defence response (Holton *et al.*, 2007), indicating that additional systemin receptors exist, including SBP50 (systemin binding protein 50 kDa) (Schaller and Ryan, 1994).

A similar system exists in *A. thaliana*, where plant elicitor peptides (Peps) have been shown to act as DAMPs. *At*Pep1 (hereafter referred to as Pep1) is a 23-aa peptide first isolated from *A. thaliana* leaves based on its ability to induce an alkalinisation response in cell suspension cultures at subnanomolar concentrations (Huffaker *et al.*, 2006). Pep1 is derived from the C-terminal region of a small, presumably cytoplasmic precursor protein called *At*PROPEP1 (hereafter referred to as PROPEP1) that has six paralogs in the *Arabidopsis* genome, PROPEPs2-7. However, PROPEP7 is not expressed in seedlings or leaf tissue of *A. thaliana* (Yamaguchi *et al.*, 2010). *PROPEP* genes can be induced by their own peptides, MAMPs (such as flg22 and elf18), phytohormones such as jasmonic acid, salicylic acid, and ethylene, as well as wounding to various degrees (Huffaker and Ryan, 2007). Treatment of *A. thaliana* with Pep peptides induces defence gene transcription and overexpression of PROPEP1 confers added resistance to the

oomycete root pathogen *Pythium irregular* (Huffaker *et al.*, 2006). With respect to pathogen attack, it has been suggested that *PROPEP1*, *PROPEP2*, and *PROPEP3* take part in a positive feedback loop, amplifying the defence signalling pathways initiated by pathogens (Huffaker and Ryan, 2007). Photoaffinty labelling was used to identify a ~170 kDa receptor for Pep1 isolated from the surface of *Arabidopsis* suspension cultured cells. This receptor was identified as a LRR-RLK named PEPR1 (Pep Receptor 1) (Yamaguchi *et al.*, 2006). Later studies revealed it as a receptor for Peps1-6 (Yamaguchi *et al.*, 2010). A second receptor, PEPR2, was also identified as a LRR-RLK perceiving Pep1 and Pep2 (Yamaguchi *et al.*, 2010).

The first line of defence in plants consists of transmembrane receptors (PRRs) that perceive evolutionarily conserved molecular patterns. The terms associated with this first line of defence against attackers and the resulting induced immune responses are: PAMP triggered immunity (PTI) with respect to pathogens/microbes, HAMP triggered immunity (HTI) with respect to herbivores, and wound induced response (WIR), in terms of perception of endogenous DAMPs (Erb *et al.*, 2012; Figure 1.1). Some pathogens and herbivores have the ability to evade this first line of defence by using effectors that can avoid or suppress PTI/HTI/WIR (Jones and Dangl, 2006; Erb *et al.*, 2012). The second line of defence, acting mostly inside the cell, uses polymorphic nucleotide binding LRR (NB-LRR) receptors, often referred as R proteins. R genes encode proteins that specifically recognize effectors (or their activity) that were otherwise able to bypass/suppress PTI, HTI, or WIR, resulting in what is called effector-triggered immunity (ETI; Figure 1.1). When pathogen effectors are perceived by R proteins, the hypersensitive response (a form of programmed cell death) is usually initiated (Sanabria *et al.*, 2010; Erb *et al.*, 2012). The perception of attack by PRRs initiates signalling cascades leading to reprograming of plant transcriptomes and ultimately changes in their secondary metabolite profile in order for plants to defend themselves against herbivores.

1.4 Early and late induced responses

The recognition of MAMPs, HAMPs, and DAMPs by PRRs results in signal initiation and transduction, which leads to the activation or de-repression of defence-associated genes (Sanabria, *et al.*, 2010). The transcriptome changes resulting in the

metabolic activity required for herbivore defence is a direct result of the early induced responses upon perception of HAMPs and DAMPs (Erb *et al.*, 2012).

The earliest of these responses include ion fluxes leading to membrane depolarization (Boller and Felix, 2009; Wu and Baldwin, 2009). These ion fluxes include the influx of H^+ and Ca^{2+} and the simultaneous efflux of K+ and anions (particularly nitrate) (Boller and Felix, 2009). It has been speculated that Ca^{2+} may act as a secondary messenger, activating calcium-dependent protein kinases (Boller and Felix, 2009; Wu and Baldwin, 2009). Another early induced response is the increase in reactive oxygen species (ROS) production, which can act as antibiotic agents directly for resistance against pathogens or may contribute to defence indirectly by causing cell wall cross-linking and/or as stress signals inducing other defence responses (Boller and Felix, 2009). In addition, NADPH oxidases may be the main source for wounding and herbivory induced ROS (Wu and Baldwin, 2009). Another important early response is the activation of mitogen-activated protein kinase (MAPK) cascades (Boller and Felix, 2009). The MAPK cascade is conserved in eukaryotes and is involved in modulating a myriad of cellular responses to diverse stimuli (Wu and Baldwin, 2009). MAPKs transcriptionally regulate the WRKY family of transcription factors (TFs), which are important for modulating both developmental and defence responses (Wu and Baldwin, 2009). Ion flux, membrane depolarization, ROS production, and the activation of MAPK cascades represent the earliest responses to MAMP, HAMPs, and DAMPs, occurring within five minutes of perception of attack (Boller and Felix, 2009). Other early induced responses, occurring on the order of five to thirty minutes include: biosynthesis of the stress hormones as well activation of the signalling pathways associated with those hormones (and crosstalk between them; Boller and Felix, 2009; Wu and Baldwin, 2009). A significant result of hormone signalling is defence gene activation. There appears to be a similar gene activation response in reaction to known MAMPs and DAMPs, as shown by the pattern of gene regulation in response to various MAMPs, including flg22 and elf26, as well as DAMPs such as oligogalacturonides (Boller and Felix, 2009). Interestingly, RLKs are highly represented among induced genes, suggesting a positive feedback to increase PRR capabilities (Boller and Felix, 2009).

Late responses, on the order of hours or days, include seedling growth inhibition, representing a physiological change from a growth/development program to one of defence (Boller and Felix, 2009). Callose deposition can be considered an early or late response, depending on the plant species/accession in question. Callose is a β(1,3) glucan polymer used by the plant to strengthen and dam weak or compromised areas of cell walls at the site of pathogen attack (Clay *et al.*, 2009). Interestingly, callose deposition in response to spider mite feeding has been shown to occur as early as five minutes in the resistant accession of *A. thaliana* Blanes (Bla), but may take as long as an hour in the susceptible accession Kondara (Kon; Zhurov *et al.*, 2014).

1.5 Phytohormone signalling

While it has been known for some time that plant hormones play pivotal roles in the regulation of plant growth, development, and reproduction, it is also evident that defence programs in plants, against pathogens and herbivores, are orchestrated by a variety of phytohormones. These phytohormones consist of a group of structurally unrelated small molecules including, but not limited to, jasmonic (JA), salicylic (SA), and abscisic (ABA) acids as well as ethylene (ET; Erb and Glauser, 2010). Evidence in support of the idea that these compounds have major roles in plant stress responses includes increased concentrations of phytohormones following insect and pathogen attack (Erb *et al.*, 2009; Summermatter *et al.*, 1995; De Vos *et al.*, 2005), usually preceding other phenotypic adjustments. Furthermore, mutants that are compromised in their ability to synthesize or perceive certain phytohormones become more susceptible to pathogens and/or herbivores (Ferrari *et al.*, 2003; Bodenhausen and Reymond, 2007; Zhou *et al.*, 2009). Finally, application of these phytohormones mimics natural stress responses of plants (Ward *et al.*, 1991; Farmer *et al.*, 1992; Erb *et al.*, 2009). Following the perception of the attack by PRRs, plants use signalling cascades to reprogram their response in such a way as to deter or otherwise negatively affect the herbivore. Interestingly, PTI/ETI plant-pathogen interactions show that although recognition of pathogens can be highly specific (R gene resistance), plants have a common downstream signalling mechanism (Katagiri and Tsuda, 2010) that is initiated upon perception of a variety of attackers. This paradigm may hold true for plant-insect interactions (Erb *et al.*, 2012). The question then

arises, how this seemingly common signalling cascade results in different immune responses/metabolism reconfiguration. It is plausible that the magnitude and timing of hormone signals modulate transcriptome changes to specialize a plants defence metabolism in response to certain biotic stressors (Verhage *et al.*, 2010). SA, JA, and ET are well known key signals in the regulation of plant defence, with JA and its derivatives being of particular importance in regulating the response to herbivory (Farmer and Ryan, 1990; Wu and Baldwin, 2009; Verhage *et al.*, 2010).

The JA signalling cascade, including its derivative JA-Ile (a wound hormone), is widely considered to be a master regulator of plant resistance to arthropod herbivores (as well as necrotrophic pathogens). It plays a dominant role in regulating gene expression in response to mechanical wounding and herbivory as shown by microarray studies (De Vos *et al.*, 2005; Howe and Jander, 2007; Erb and Glauser, 2010). The importance of the JA pathway is supported by its involvement in the regulation of tritrophic interactions (indirect defence; Thaler, 1999), trichome-based defences (Boughton *et al.*, 2005), priming of direct and indirect defences (Engelberth, 2004), and the systemic transmission of defence signals (Schilmiller and Howe, 2005). JA also plays a pivotal role in switching the plant from a growth to defence program, allowing the plant to reallocate energy and resources (Pauwels *et al.*, 2009). In general, JA promotes defensive and reproductive processes while inhibiting the growth and photosynthetic output of vegetative tissues (Howe and Jander, 2007). Accumulation of JA at the site of wounding inflicted by chewing insects or mechanical damage occurs rapidly, within 30 minutes (Howe and Jander, 2007). JA is synthesized via the octadecanoid pathway in higher plants and nearly all of the genes encoding biosynthetic enzymes have been identified in *A. thaliana* (Schaller *et al.*, 2005; Howe and Jander, 2007) (Figure 1.2). Instead of seeing JA as a single phytohormone, it may be more appropriate to consider it to be a member of the phytohormone jasmonate family (Erb and Glauser, 2010). For example, in *A. thaliana*, the isoleucine conjugate, JA-Ile is more active than JA itself (Staswick and Tiryaki, 2004). Also, JA is restricted to plant cells and vascular tissues, whereas its methylated form (MeJA) as well as *cis*-jasmone are volatile and can easily move to other parts of the plant and even to other organisms (Birkett *et al.*, 2000).

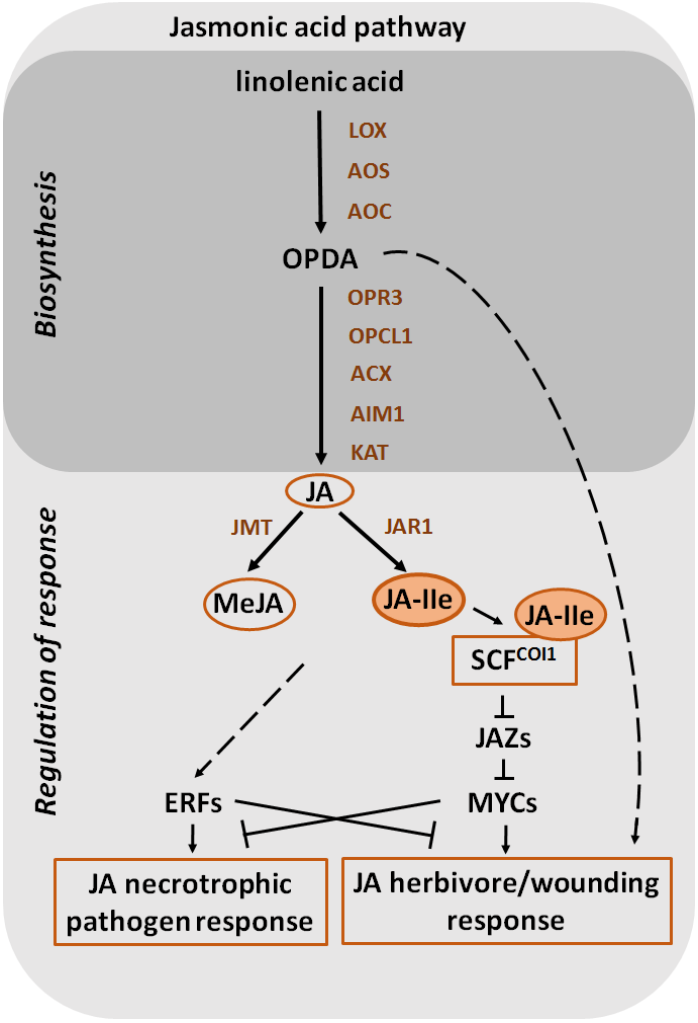


Figure 1.2 Simplified jasmonic acid (JA) biosynthetic and response pathway. Biosynthesis begins with the liberation of linolenic acid, 18:3, from membrane glycerolipids which are then converted to 13-hydroperoxylinoleic acid (13-HPOT) by 13-lipoxygenase (LOX). Allene oxide synthase (AOS) then produces 12,13-epoxyoctadecatrienoic acid, which is acted upon by allene oxide cyclase (AOC) to generate (9S,13S)-12-oxo-phytodienoic acid (OPDA). OPDA reductase (OPR3) then reduces (9S,13S) OPDA. This product is then converted to jasmonic acid (JA) after three cycles of β-oxidation. The JA pathway consists of at least two branches, including the ethylene response factor (ERF) branch, inducing defence responses to necrotrophic pathogens, and the MYC2 branch consisting of MYC transcription factors that activate transcription of genes associated with response to wounding and defence against herbivores.

Furthermore, JA precursors, such as 12-oxophytodienoic acid (OPDA), that were once thought to be intermediates required for JA synthesis have been shown to have activity themselves (Stintzi *et al.* 2001).

The JA pathway serves as a core-signalling mechanism activated both by specific and non-specific PPRs following herbivore attack. Plants can fine tune the JA response to become more appropriate to the specific herbivore attacking in one of two ways. First, the plants may use other JA-independent phytohormone pathways to create a distinct response or it could be through the action of spatio-temporal modulators of the JA core response (Erb *et al.*, 2012). Evidence for JA-independent pathway stems from plants using SA-mediated signalling in response to hemipterans, which suggests that the SA pathway, independently of JA, is important in the resistance against phloem feeders like aphids and silverleaf whiteflies (Van Poecke, 2007; Wu and Baldwin, 2009; Erb *et al.*, 2012). On the other hand, most herbivores inflict more damage than phloem feeders, which activate the JA signalling pathway. Specificity of response can then be achieved through hormone cross-talk, most notably with SA and ET (Erb *et al.*, 2012). Generally, SA antagonizes JA-induced resistance. However, JA can also antagonize SA in certain plant species and strategy of attacker (Verhage *et al.*, 2010; Erb *et al.*, 2012). ET plays a modulating role, having both negative and positive effects on JA induced resistance (Verhage *et al.*, 2010; Erb *et al.*, 2012). The JA pathway has been described as having two branches in terms of defence gene activation. For example, when ET works in concert with JA, the responses activated are effective against necrotrophic pathogens (Vijayan *et al.*, 1998), and are coordinated through the activity of TFs encoded by ethylene response factor (*EFR*) genes. This branch of the JA pathway, effective against necrotrophic pathogens is described as the ET/JA pathway or the EFR branch (Verhage *et al.*, 2011; Figure 1.2). The other branch of the JA pathway is called the MYC2 branch, where JA-Ile is involved in activation of MYC TFs through degradation of JAZ transcriptional repressors that repress MYC and other JA defence genes (Chung, 2008). MYC2 represses many genes induced by the action of the ERF1 TF induced in response to necrotrophic pathogens, whereas EFR1 represses wound-responsive genes activated by MYC TFs, so these two branches of the JA defense signalling pathway are antagonistic to each other (Browse, 2009) (Figure 1.2).

There are several other phytohormones that play a role in modulating plant defence responses, as reviewed by Erb *et al.* (2012), including: abscisic acid, auxin (specifically indole-3-acetic acid, known as IAA), gibberellins, cytokinins, and brassinosteroids. While hormone cross-talk may provide the plant with a powerful regulatory potential to finely tune its defence, is also represents a target for plant attackers to manipulate the immune signalling network for their own benefit (Verhage *et al.*, 2010). This can be accomplished through the use of decoy molecules that mimic plant hormones to interfere in the signalling pathway or to induce expression of antagonistic hormones to suppress the correct response (Verhage *et al.*, 2010). It is also important to keep in mind that a plant may be stressed by various biotic and abiotic agents simultaneously in the field and this reinforces the need for a plant to be able to use phytohormones in modulating its responses to best combat stressors whilst conserving as much energy as possible for growth and reproduction.

1.6 Secondary defence compounds

Upon perception of herbivory, plants produce toxic secondary metabolites, defensive proteins, and volatile signalling compounds. They also initiate changes in morphology and growth patterns (Erb and Glauser, 2013). Defensive proteins can come in the form of proteinase inhibitors that affect herbivore digestion following ingestion of plant material. A well-studied class of plant secondary metabolites known for their insect repellent/deterrent properties, particularly in Brassicaceae, are glucosinolates (Wittstock and Gershenzon, 2002; Halkier and Gershenzon, 2006). Their basic structure consists of three structural groups including a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain (Mithen, 2001). There have been at least 120 different glucosinolates identified, found mostly in species of the Brassicaceae family (Fahey *et al.*, 2001). Glucosinolates are derived from amino acids and can be distinguished using the three major structural groups based on the amino acid precursor of the variable side chain. Indole glucosinolates (IGs) comprise 10% of known structures and are derived from tryptophan (Figure 1.3). Aliphatic glucosinolates (50%) are mainly derived from methionine (Figure 1.4), and aromatic glucosinolates (10%) are mainly derived from phenylalanine or tyrosine (Figure 1.5). The remaining 30% of known structures are either

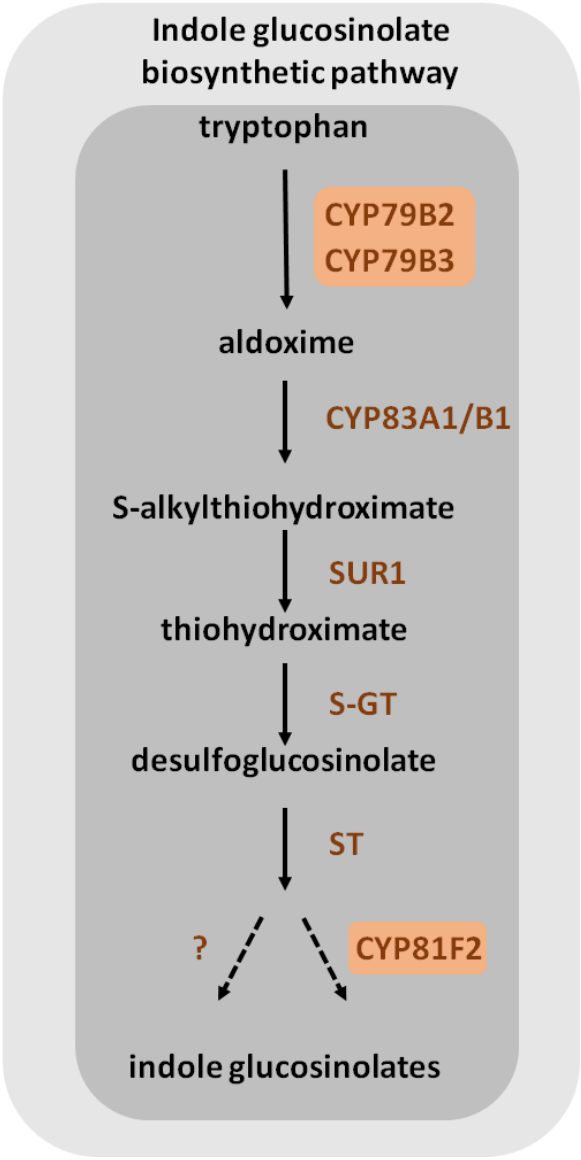


Figure 1.3 Simplified schematic of indole glucosinolate (IG) biosynthesis in *A. thaliana.* Indole glucosinolates are derived from the amino acid tryptophan. The first committing step in indole glucosinolate biosynthesis in *A. thaliana* is performed by cytochrome P450 (CYP) gene products CYP79B2 and CYP79B3 and involves the conversion of tryptophan to aldoxime. Aldoximes are then metabolized by CYP83A1 and/or CYP83B1 to form S-alkylthiohydroximates which are then cleaved by a C-S lyase, SUR1, into thiohydroximates. This is followed by glycosylation by S-glucosyltransferases (S-GT). The final sulfation step is catalyzed by sulfotransferases (ST). The CYP81F2 monooxygenase catalyzes the conversion of indole-3-yl-methyl to 4-methyl glucosinolates (4-OH-I3M and 4-MO-13M).

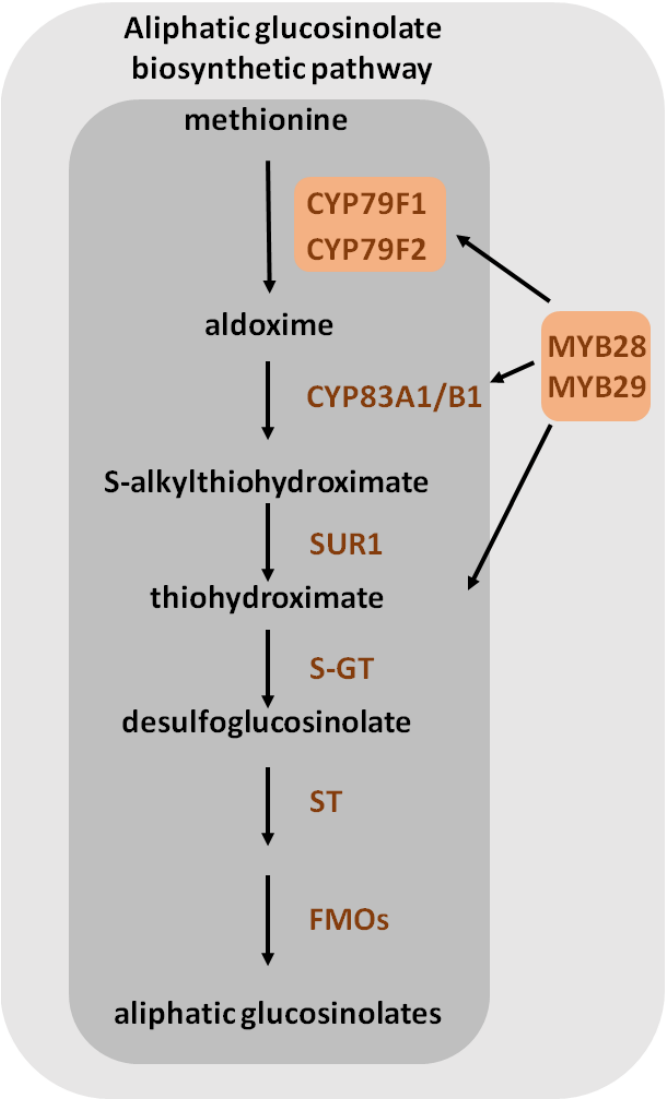


Figure 1.4 Simplified schematic of aliphatic glucosinolate biosynthesis in *A. thaliana*. Aliphatic glucosinolates are mostly derived from the amino acid methionine. The first committing step in aliphatic glucosinolate biosynthesis in *A. thaliana* is performed by cytochrome P450 (CYP) gene products CYP79F1 and CYP79F2 and involves the conversion of methionine to aldoxime. Aldoximes are then metabolized by CYP83A1 and/or CYP83B1 to form S-alkylthiohydroximates which are then cleaved by a C-S lyase, SUR1, into thiohydroximates. This is followed by glycosylation by S-glucosyltransferases (S-GT). The final sulfation step is catalyzed by sulfotransferases (ST). Flavin monooxygenases (FMOs) provide secondary modifications of aliphatic glucosinolates. Aliphatic glucosinolate biosynthesis is mediated by transcription factors MYB28 and MYB29.

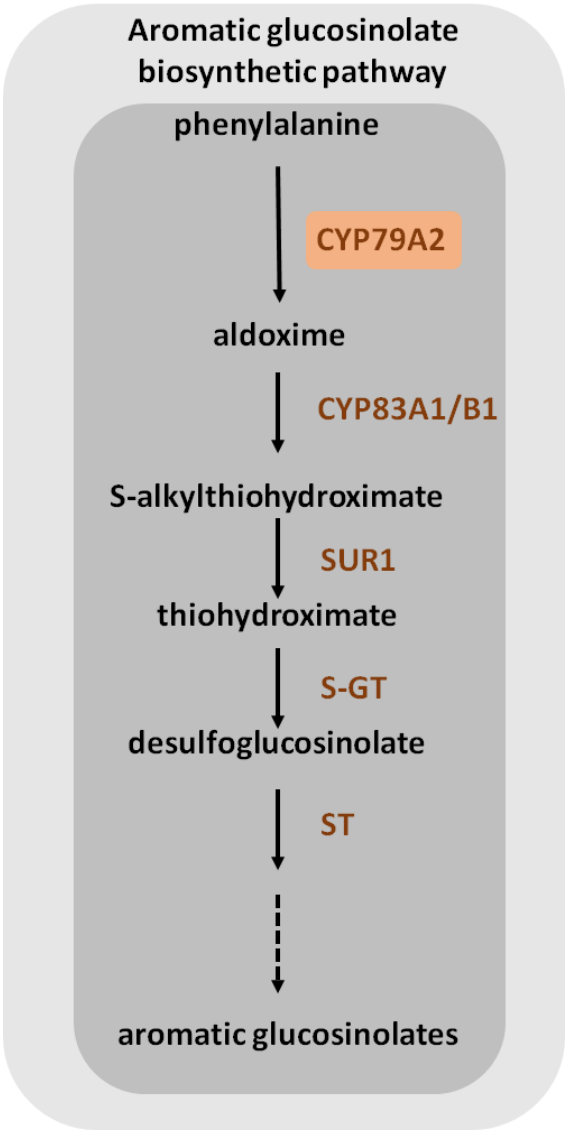


Figure 1.5 Simplified schematic of aromatic glucosinolate biosynthesis in *A. thaliana*. Aromatic glucosinolates are mostly derived from the amino acid phenylalanine. The first committing step in aliphatic glucosinolate biosynthesis in *A. thaliana* is performed by the cytochrome P450 (CYP) gene product CYP79A2 and involves the conversion of phenylalanine to aldoxime. Aldoximes are then metabolized by CYP83A1 and/or CYP83B1 to form S-alkylthiohydroximates which are then cleaved by a C-S lyase SUR1 into thiohydroximates. This is followed by glycosylation by S-glucosyltransferases (S-GT).

synthesized from other amino acids or it is unknown how they are generated (Fahey *et al.*, 2001; Mithen, 2001). Further structural variation occurs via chain elongation, oxidation, or hydroxylation of the side chain (Hopkins *et al.*, 2009).

Defensive properties of glucosinolates can be increased upon hydrolysis by the myrosinase enzyme (Hopkins *et al.*, 2009). However, there is evidence that myrosinase-independent IG activity occurs when aphids feed on *A. thaliana*. Specifically, *M. persicae* (green peach aphid) is affected by IG based on their post-ingestive breakdown and conjugation with other herbivory-induced metabolites (Kim and Jander, 2007; Kim *et al.*, 2008). Myrosinases are thioglucosidases stored in special myrosinase cells found throughout the plant in all organs (Rask *et al.*, 2000). The action of myrosinases is initiated upon plant tissue damage, for example by a chewing insect, upon which glucosinolates stored within the vacuole come into contact with myrosinase and as a result of the myrosinase activity, glucose and sulfate are released together with several toxic and pungent products (Hopkins *et al.*, 2009) including isothiocyanates, nitriles, and oxazolidinethiones (Bones and Rossiter, 2006; Wittstock and Halkier, 2002).

Interestingly, although plants use repellent or toxic secondary metabolites for protection against herbivores, some herbivore species have evolved counter-adaptations allowing them to feed on a host plant producing secondary metabolites that harm other herbivore species. These herbivores often become specialized feeders on a family or even individual plant species. For example, larvae of the specialist insect, *Pieris rapae* (cabbage white butterfly), have adapted to feed on host plants using the glucosinolate-myrosinase system. During this interaction, the hydrolysis reaction is redirected by *P. rapae* to favor the production of nitriles (less toxic product) instead of isothiocyanates by a gut protein (nitrile-specifier protein; Wittstock *et al.*, 2004).

The term host plant resistance is used to describe the sum of genetically inherited traits resulting in a plant of a certain species or cultivar being more resistant to an arthropod pest then a susceptible plant lacking those traits (Smith and Clement, 2012). The purpose of this study is to elucidate on host plant resistance of one species of plant with respect to one species of herbivore. This involves the evaluation of the interaction

between these two organisms, namely the plant species *Arabidopsis thaliana* which serves as a host to the herbivore *Tetranychus urticae*, commonly known as the two-spotted spider mite.

1.7 *Arabidopsis thaliana*

For 25 years *A. thaliana* has represented the plant model organism of choice for research in plant biology and has become the most widely studied species of flowering plants (Koornneef and Meinke, 2010). *Arabidopsis thaliana* was adopted as a model organism because of several useful features including a short generation time, small size, and prolific seed production through self-pollination. *A. thaliana* has a relatively small genome with five chromosomes (Koornneef and Meinke, 2010). During the last decade, *Arabidopsis thaliana* has been used in studies of plant-pest interactions with the hope to better understand the molecular mechanisms involved (Poecke, 2007). Due to the wide availability of genetic and genomic toolkits (Koorneef and Meinke, 2010), *A. thaliana* has been used as a host for studies involving insects in several feeding guilds (Reymond *et al.*, 2004; De Vos *et al.*, 2005; Kempema *et al.*, 2007). *A. thaliana* is the optimal choice of plant model organism for this study due to the wide array of mutants available. These mutant accessions are devoid of key aspects of defence and their use in this study will help determine what aspects of *A. thaliana* biology are involved in their response to spider mite herbivory, furthering the goal of understanding the molecular mechanisms behind host plant resistance.

1.8 *Tetranychus urticae*

Insects are the most diverse and abundant group of herbivores (Zheng and Dicke, 2008) and have been the subject of the majority of studies into plant-herbivore interaction. However, another class of herbivores in the Arthropod phylum also deserve similar attention, namely the chelicerates, including scorpions, horseshoe crabs, spiders, mites and ticks, given that these animals represent the second largest group of arthropods. The two-spotted spider mite, *Tetranychus urticae*, has been proposed as a good candidate for a chelicerate model organism (Grbic *et al.*, 2007). *Tetranychus urticae* has a small genome of 90Mbp, distributed on three holocentric chromosomes of equal size (Helle and

Bolland, 1967), which has been recently sequenced (Grbic *et al.*, 2011). Their sex determination is haplo-diploid, where fertilized eggs develop into diploid females and unfertilized, haploid eggs develop into males (Oliver, 1971). *Tetranychus urticae* can complete its life cycle, from egg to adult, in about seven days under favourable temperature (27°C) and humidity (55-60%) conditions. Furthermore, these arthropods can produce large numbers of offspring with many generations per year due to their short life cycle (Cranham and Helle, 1985). This species' life cycle begins as a deposited egg, hatching in as little as three days. The newly emerged larvae then feeds on a plant host before entering a quiescent stage, followed by molting into a protonymph. Following another period of feeding, the mite then undergoes another molting to become a deutonymph. Near the end of the deutonymphal stage, the mite enters its' final quiescent period before molting into an adult (Shih *et al.*, 1976).

Tetranychus urticae is a polyphagous herbivore feeding on more than 1,100 plant species spanning more than 140 different families and represents a major agricultural pest in annual field crops, horticulture crops, greenhouse crops (especially in *Solanaceae* and *Cucurbitaceae*) and ornamental greenhouse plants (Bolland *et al.*, 1998; Grbic *et al*, 2011). Field crop hosts include soybean, maize and cotton; horticultural host crops include apple, pear, peach and hops and greenhouse host plants include vegetables such as cucumbers, tomatoes, eggplants, peppers and zucchini. Ornamental crops at risk include roses, carnations and chrysanthemums. Perennial cultures affected by spider mites include strawberries, grapes, plums and alfalfa (Jeppson, Keifer and Baker, 1975; Migeon and Dorkeld, 2006-2013). Importantly, in laboratory settings, *T. urticae* feeds on *A. thaliana*, and has been observed on a number of related species in the Brassicaceae family (Migeon and Dorkeld, 2006-2013).

Determining the molecular mechanisms behind the interaction between *T. urticae* and host plants is important because, amongst arthropods, it has the highest incidence of pesticide resistance. This shows the need to develop agricultural models using new and environmentally sustainable techniques/technologies to manage this pest.

1.9 Natural variation in *A. thaliana* susceptibility to spider mite herbivory

Prior to beginning my project, several studies investigating the interaction between *A. thaliana* and spider mites were performed by previous lab members. These studies included determining the extent of variability in response to spider mites in natural *A. thaliana* accessions as well as microarray and meta-analysis of *A. thaliana* transcriptome response to spider mite feeding. These analysis as well as several aspects of this thesis were published in Zhurov *et al.,* (2014). Results presented here are derived from experiments I performed (unless explicitly stated otherwise); however for the purposes of placing these results in context or elaborating on their relevance, I sometimes reference results from other experiments described in Zhurov *et al.* (2014) when discussing them.

The variability in *A. thaliana* response to spider mite feeding was assessed by a former master's student, Cherise Ens, in 2007. Twenty-six different natural *A. thaliana* accessions of geographically and genetically diverse origin were assayed for plant damage following feeding of 10 adult female mites for 4 days (Figure 1.6A; Zhurov *et al*., 2014). Mite induced damage was quantified using the total area of chlorosis, a diagnostic feature commonly used to assess mite damage on crop plants (Zhurov *et al.*, 2014). Plant damage varied between accessions with a ~20-fold variation in chlorotic area. The accession designated Bla-2, showed the least amount of damage (2 mm^2) and is considered to be a resistant accession. The Kondara (Kon) accession incurred the most damage (40 mm^2) of total chlorotic area, and is considered a susceptible accession. Consistent with damage analysis data, spider mite larvae developed more slowly on Bla-2 detached leaves relative to Kon leaves and larval mortality was higher for larvae feeding on Bla-2 leaves relative to those feeding on Kon (Figure 1.6B; Zhurov *et al.*, 2014).

To further understand *A. thaliana* response to spider mite herbivory, transcriptional responses of Bla-2 and Kon accessions (being on opposite ends of the resistance spectrum) were assayed using microarray analysis in two experiments (Zhurov *et al.*, 2014).

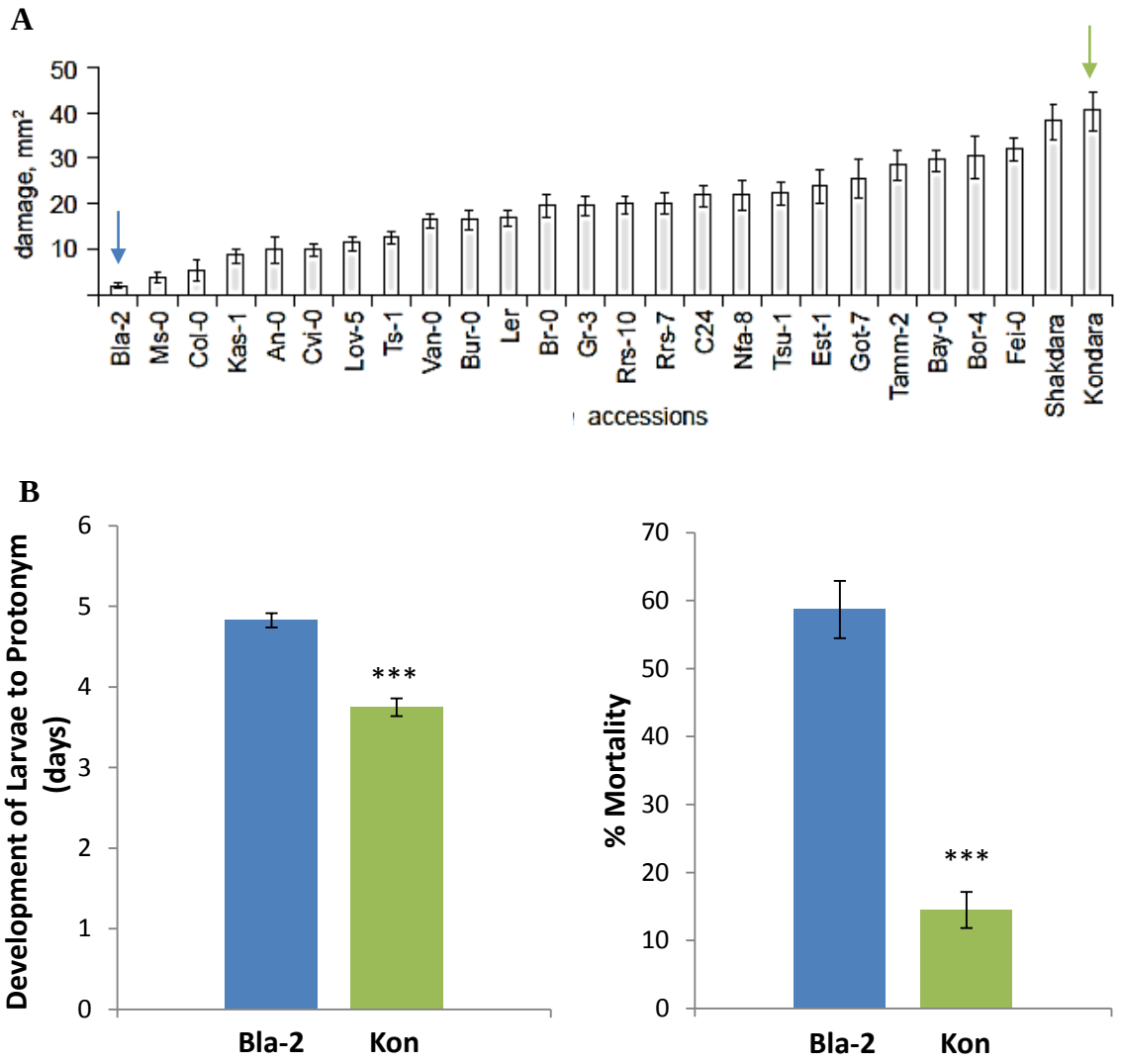


Figure 1.6 Variability in susceptibility of 26 *A. thaliana* accessions to spider mite feeding. **A** Damage assay using mean area of chlorotic spots. Plants were inoculated with 10 adult female mites for 4 days (n = 6 plants per accession). Shown are means ± standard errors of the means (SEM) **B** Spider mite larvae and developmental assays on detached leaves as assessed by mean day required to develop into protonymph and mean percent mortality respectively (n = 5 samples/accession, 50 - 60 larvae/sample). Replicated experiments of the same comparisons produced similar results. Error bars are ± 1 SEM. Asterisks represent significantly different comparisons (unpaired t-test, *** - $P < 0.001$). Published in Zhurov *et al.* (2014).

The first experiment was a feeding time course, where 10 mites were allowed to feed for 1, 3, 6, 12, and 24 h post infestation (hpi) after which shoot tissue was collected, and RNA was prepared and hybridized to the GeneChip Arabidopsis ATH1 expression microarray (Zhurov *et al.*, 2014). Ten mites were used in the time course experiment because, in field conditions, spider mites colonize new plants either by crawling to them or by drifting on wind currents (Zhurov *et al.*, 2014). The second experiment used a feeding site paradigm, where hundreds of mites were allowed to feed on the plant, completely covering the rosette leaves and the whole plant becomes a feeding site (Zhurov *et al.*, 2014). The feeding site microarray experiment was performed because early responses at the feeding site (local response) may be missed during the time-course experiment (only a small proportion of plant cells are damaged by 10 mites) (Zhurov *et al.*, 2014). After 1 hpi with hundreds of mites, plant shoot tissue was harvested, and RNA was isolated and hybridized to the ATH1 array (Zhurov *et al.*, 2014).

During the time course experiment, 841 differentially expressed genes (DEGs) were identified between non-infested control plants in at least one of the five time points in at least one accession (absolute fold change > 2, Benjamin-Hochberg false discovery rate adjusted p-value < 0.01 using the Bioconductor package limma (Smyth and Speed, 2003; Zhurov *et al.*, 2014). The feeding site data performed using hundreds of mites and the 1 h data obtained from samples treated with 10 mites were extremely similar, as shown with similar DEGs identified in the two data sets (Zhurov *et al.*, 2014). The magnitude of gene expression changes were higher in the feeding site data, so it was for analysis of early, local responses (Zhurov *et al.*, 2014). In the feeding site analysis, 660 DEGs were identified between non-infested controls and treated plants in at least one accession (Zhurov *et al.*, 2014).

Interestingly, despite the differences in plant damage and mite developmental assays, overall transcriptional responses to spider mite feeding in both accessions were similar and principal component analysis revealed that difference in accession accounted for more variation in gene expression than did treatment with spider mites (Zhurov *et al.*, 2014).

1.10 Objectives

The objective of my work is to elucidate plant responses to spider mite feeding using microarray data generated from both Bla-2 and Kon responses to spider mite feeding. From the plant's perspective, the interaction between *A. thaliana* and spider mites begins with the perception of attack by use of PRRs perceiving HAMPs and/or DAMPs associated with spider mite herbivory, through signalling via phytohormones resulting in transcriptional reprograming, and ultimately the production of defensive secondary metabolites. Gene ontology (GO) enrichment terms found exclusively in the feeding site data were associated with perception and defence signalling, revealing induction of DAMP receptors PEPR1, and PEPR2 and a suite of JA biosynthetic and response genes. GO terms found only in the 3 to 24 h samples revealed changes in metabolism and induction of defence through production of several secondary metabolites, glucosinolates being among them. The overall objective of this study is to test whether the molecular players identified by microarray analysis mentioned above and described in Zhurov *et al.*, (2014) are involved in the effective defence of *A. thaliana* against spider mites. The specific objectives of this study are:

1. **To determine if DAMP receptors PEPR1 and PEPR2 are involved in perception of DAMP ligands (Peps) following damage incurred by spider mite herbivory.** To test this, plant damage assays using wild type Columbia-0 (Col-0), and mutant *A. thaliana* lines lacking one or both of these receptors will be performed to assess plant performance following spider mite feeding. Spider mite larvae developmental and mortality assays will be used to assess mite performance on Col-0 and *pepr* mutant plants. It is hypothesized that plants lacking these receptors will incur more damage from spider mite herbivory and spider mite larvae will develop faster and/or have a lower mortality when feeding on *pepr* mutant plants compared to Col-0 controls. Gene expression analysis of *PROPEP* and *PEPR* genes will be analysed using qRT-PCR to determine the expression kinetics of elements in the Pep-PEPR WIR mechanism during a 24 h time course of spider mite feeding in Col-0 plants with the hypothesis that *PROPEPs* and *PEPRs* that are important to this

perception will be induced upon spider mite feeding as a result of the positive feedback loop identified in pathogen-PEPR studies.

2. **To determine if JA is the key phytohormone involved in defence signalling following perception of spider mite feeding.** To test this *A. thaliana* plants lacking genes encoding elements of the JA pathway (*aos* mutant for JA biosynthesis and *myc2myc3myc4* triple mutant for JA response) will be used for plant damage and spider mite larvae developmental and mortality assays to test plant and mite performance respectively. It is hypothesized that mutant plants lacking one of the aspects (biosynthesis or transcriptome regulation) of the JA pathway will be compromised in their ability to fend off spider mite attack, incurring more damage and allowing for faster mite development and lower mite mortality. Marker gene analysis using qRT-PCR will be used to determine if initiation of the JA signalling pathway is dependent on perception of attack through PEPR1 and PEPR2 using *AOS* and *MYC2* as marker genes with the hypothesis that these marker genes will be induced in Col-0 following spider mite herbivory and not in *pepr1pepr2*.

3. **To determine if IGs are effective secondary metabolites affecting spider mite performance and their ability to use *A. thaliana* as a host.** If IGs are effective as deterrents or have toxic properties to mites, their involvement would be apparent through plant damage and spider mite larvae development and mortality assays. It is hypothesized that mutants lacking genes encoding IG biosynthetic enzymes, *CYP79B2* and *CYP79B3* will suffer more damage following spider mite herbivory and spider mite larvae will develop faster and show lower mortality on *cyp79b2cyp79b3* double mutant plants, relative to Col-0 controls. Again, marker gene analysis using qRT-PCR will be used to determine if the induction of *CYP79B2* and *CYP79B2* is dependent on the presence of PEPR1 and PEPR2 and a functional JA pathway using *pepr1pepr2* and *aos* mutants. It is hypothesized that induction of *CYP79B2* and *CYP79B3* will be attenuated/absent in these mutants relative to induction in associated controls.

Chapter Two – Materials and methods

2.1 Plant material and growth conditions

Plant growth chambers were set at 22 °C with a relative humidity of 55 % and a short-day photoperiod (10 h light: 14 h dark) using cool-white fluorescent lights (PHILIPS very high output F96T12/CW/VHO/EW). Plants were grown from seed with a light intensity of 120 μE m-2 sec-1. *A. thaliana* accessions and mutant lines were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University), except for the *myc2 myc3 myc4* triple mutant, which was acquired from R. Solano (Universidad Autónoma de Madrid, Madrid, Spain) and the *cyp79b2cyp79b3* double mutant acquired from B. A. Halkier (University of Copenhagen, Denmark). Columbia-0 (Col-0) was used as the wild type for all mutant analyses, except for the analysis of the *aos* mutant that is in Columbia-6 (Col-6) background. All mutants used in this study are listed in Table 2.1 Seeds were stratified for three days at 4 °C in the dark before being sewn on autoclaved sand saturated with fertilized water. Fertilizer used was Plantex Poinsettia Plus (18-6-20), purchased from Plant Products® (Brampton, Ontario, Canada). Seeds sown on sand were placed in the growth chamber to germinate. Seedlings were allowed to grow for two weeks prior to transplantation. Sand was used to germinate seeds and generate seedlings due to the ease of transplantation from water saturated sand (minimal root damage). Following two weeks of growth on sand, seedlings were transplanted into 2.5 cm x 2.5 cm pots filled with moist autoclaved soil and left covered with a transparent lid for approximately 1 week before removal of the lid and regular watering.

2.2 Spider mite rearing conditions

The spider mite colony used for experiments was generated from mites originally collected from apples near London, Ontario, Canada. The mite colony was raised on bean plants (*Phaseolus vulgaris*, cultivar “California Red Kidney”, Stokes, Thorold, Ontario, Canada), in growth chambers at 24 °C, 60 % relative humidity and with a 16 h light: 8 h dark photoperiod for more than 100 generations.

Table 2.1 List of *A. thaliana* mutants used in this study. All seeds were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University), except the *myc2myc3myc4* triple mutant that was acquired from R. Solano (Universidad Autónoma de Madrid, Spain) and the *cyp79b2cyp79b3* double mutant acquired from B. A. Halkier (University of Copenhagen, Denmark).

Mutant	Mutant description	Background genotype (WT)	Stock name (ARBC)	Mutation
pepr1	Lacks the leucine-rich repeat receptor kinase *PEPR1* gene. The PEPR1 receptor percieves engogenous Pep peptides, Pep1, 2, 3, 4, 5 and 6.	Col-0	SALK_059281 / CS800015	T-DNA insertion (knockout)
pepr2	Lacks the leucine-rich repeat receptor kinase *PEPR2* gene. The PEPR2 receptor percieves engogenous Pep peptides, Pep1 and Pep2.	Col-0	SALK_098161	T-DNA insertion (knockout)
pepr1/2	Lacks the leucine-rich repeat receptor kinase genes *PEPR1* and *PEPR2*. The PEPR receptor percieves engogenous Pep peptides.	Col-0	SALK_059281 x SALK_098161	cross of 2 T-DNA insertions (knockout)
aos	Lacks the allene oxide synthase (*AOS*) gene. This enzyme catalyzes dehydration of the hydroperoxide to an unstable allene oxide in the JA biosynthetic pathway (Figure 1.1).	Col-6	*aos* /CS6149	T-DNA insertion (knockout)
myc2myc3myc4	Lack MYC transcription factors: MYC2, MYC3 and MYC4. MYC2 branch of JA pathway inhibited. Seeds obtained from R. Solano (Universidad Autónoma de Madrid, Spain)	Col-0	N/A	
cyp79b2cyp79b3	lacks the cytochrome P450 enzymes responsible for the first commited step in IG biosynthesis, CYP79B2 and CYP79B3 Seeds obtained from B. A. Halkier (University of Copenhagen, Denmark)	Col-0	N/A	
cyp81f2	lacks the CYP82F2 enzyme that is responsible for generating significant amounts of IGs (4OH-I3M and 4MO-I3M) from I3M	Col-0	SALK_123882	T-DNA insertion (severe knockdown)

2.3 Spider mite isolation protocols

Spider mites were isolated from infested bean plants by washing 6 - 10 well infested bean plants in 0.001% Tween 20 - tap water solution at room temperature. The solution containing spider mites at various developmental stages was passed through a series of fine sieves. First a 500 μm mesh sieve was used to remove debris. A 300 μm sieve was used to isolate adult female mites for plant damage assays. Adult male mites pass through the 300 μm sieve because they are smaller than the females, and can be confused with deutonymphs. The mesh with female adult mites was washed with room temperature tap water to rinse off Tween 20 and evenly spread mites along the bottom of the sieve. The sieve was then gently dried with a paper towel and the mites were allowed to dry and recover before being placed on an experimental plant using a wet, thin paint brush of size 00. Spider mite eggs required for developmental and mortality assays were isolated by first passing the spider mite solution through a 150 μm sieve to remove all stages of mites except eggs. The solution was then passed through a 100 μm sieve to collect eggs. The eggs were washed in the sieve under room temperature tap water before being evenly deposited onto 1 cm x 1 cm filter paper squares to dry. Filter paper squares with ~150 eggs were used for developmental assays as described below (section 2.5). Protocols used for the isolation of spider mites at various stages can be found in Cazaux *et al.*, (2014).

2.4 Plant damage assay

A. thaliana plants were grown for four to five weeks before being infested. Plants of similar size at the same developmental stage (eight leaves) were used for experiments. On day zero, 10 adult female spider mites were placed on plants of control and mutant genotypes. The mites were allowed to feed for 3 days in an interaction chamber set to the same environmental conditions as the spider mite colony rearing chamber (24 °C, 60 % relative humidity and a 16 h light: 8 h dark photoperiod). Although the change in temperature, photoperiod and relative humidity could introduce stress to the plants and add a variable in addition to spider mite feeding, mutant and control plants were all treated equally with respect to growth conditions at all times. Therefore, whatever differences in damage between genotype seen could be attributed to differences in

genotype, or conceivably, differences in how genotypes respond to the change in abiotic conditions. It was observed that many of the mites originally placed on the plant had left the rosette by the conclusion of the experiment. On day three, the entire rosette was cut from the roots and scanned using a Canon® CanoScan 8600F model scanner at a resolution of 1200 dpi and a brightness setting of +25. Actual luminosity will vary depending on scanning instrument used; however, this is irrelevant as long as the parameters are kept constant for all plants to be compared following scanning. Scanned plants were saved as .jpg files for subsequent analysis. Adobe Photoshop 5 (Adobe Systems, San Jose, CA) was used for damage quantification using four steps. First, a new layer was overlaid on the picture of the scanned plant and a grid (0.25 mm x 0.25 mm) was added. The second step involved placing red dots of known pixel size (52 pixels) within grid units for which there was damage covering more than half of the grid unit (Figure 2.1). The next step, after all the damage had been covered by dots, was to calculate the number of dots from the total number of pixels (derived from the histogram tool) divided by the number of pixels per dot (52 pixels/dot). The last part of the process was to calculate area damaged by multiplying the number of dots by the area of one grid unit using the formula:

$$\text{Area damaged } (mm^2) = \text{number of dots} \times 0.25 \text{ mm} \times 0.25 \text{ mm}$$

Three replications of damage analysis, using different batches of plants, were performed for each comparison between *A. thaliana* genotypes. Upon completion of damage quantification, two-way ANOVAs followed by Tukey's Honestly Significant Difference tests were used to determine whether there were differences between the conditions of genotype and/or replication (separate batches of plants inoculated at different times) and whether there was an interaction between the experimental conditions. In cases where there was a significant difference between replications, then one-way ANOVA/un-paired t-tests were used to detect significant differences between genotypes within individual experiments. The plant damage assay was developed by Dr. Marie Navarro.

Figure 2.1 Scanned image of a plant before and after damage analysis. Using Adobe Photoshop 5, a new layer was first overlaid on the picture of the scanned plant. A 0.25 mm x 0.25 mm grid was then added. The second step involved placing red dots of known pixel size (52 pixels) within grid units for which there was damage covering more than half of the grid unit. After all the damage had been covered by dots, the total number of dots was calculated using the total number of pixels (derived from the histogram tool) divided by the number of pixels per dot (52 pixels/dot). The last part of the process was to calculate area damaged by multiplying the number of dots by the area of one grid unit (0.25 mm x 0.25 mm).

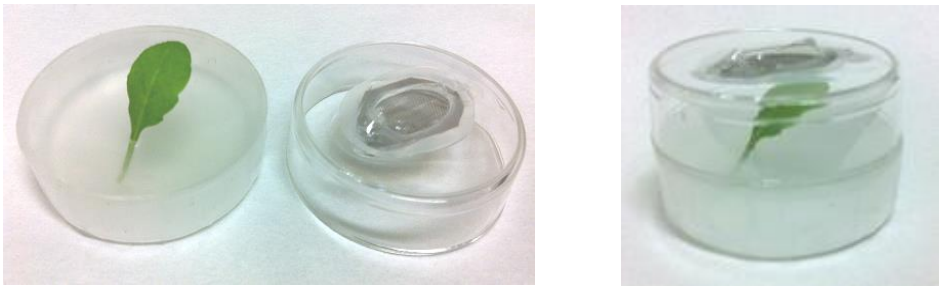
2.5 Spider mite larvae developmental/mortality assay

The spider mite larvae development and mortality assay was designed by myself and utilized small petri plates with a layer of Parafilm placed over one plate (bottom of set up) and filled with tap water so that no air is present under the Parafilm. The lid for the assay is also made from a petri dish bottom with a hole melted in the center of it. A 0.1 mm mesh sieve was glued over this hole such that spider mite larvae cannot escape, but air can pass through so as to reduce humidity within the plate (using non-toxic, odor free glue).

On day zero, a rosette leaf (7 - 8 emerged leaf of a 5 - 6 week old plant) was cut from a plant of desired genotype and the petiole was place through a hole in the Parafilm large enough to allow petiole to pass through without damaging it, but small enough to support the petiole at a ~45 ° angle and keep mites out of the water beneath the Parafilm. Also on day zero, a small square of filter paper (1 cm x 1 cm) with approximately 150 spider mite eggs newly isolated from bean leaves was placed beside the leaf on top of the Parafilm. The lid was applied and the setup was sealed using a strip of Parafilm such that the edges of the bottom petri dish and the top petri dish were flush and no mites ccould escape through the Parafilm seal. This represented a closed system where newly emerged larvae from the eggs could move to the leaf and start feeding (Figure 2.2A).

This experiment was synchronized ± 24 h during which time larvae emerged from eggs and moved around within the closed system, many feeding on the leaf, while others walked around on the Parafilm or plate lid. On day one, the total number of larvae on the leaf was counted and the filter paper with the remaining eggs was removed. The desired number of larvae on the leaf on day one was between 30 and 60 due to the time it takes to count them. The variable number of starting larvae on different individual samples may have introduced an additional effect of the density of mites on the leaf, potentially leading to different levels of defence induction in the detached leafs. Regardless, robust and reproducible differences between genotypes was observed. If the desired number of larvae were not present on the leaf, then larvae walking around on the Parafilm or lid were included in the assay to increase the sample size. Excess larvae were removed.

A



B

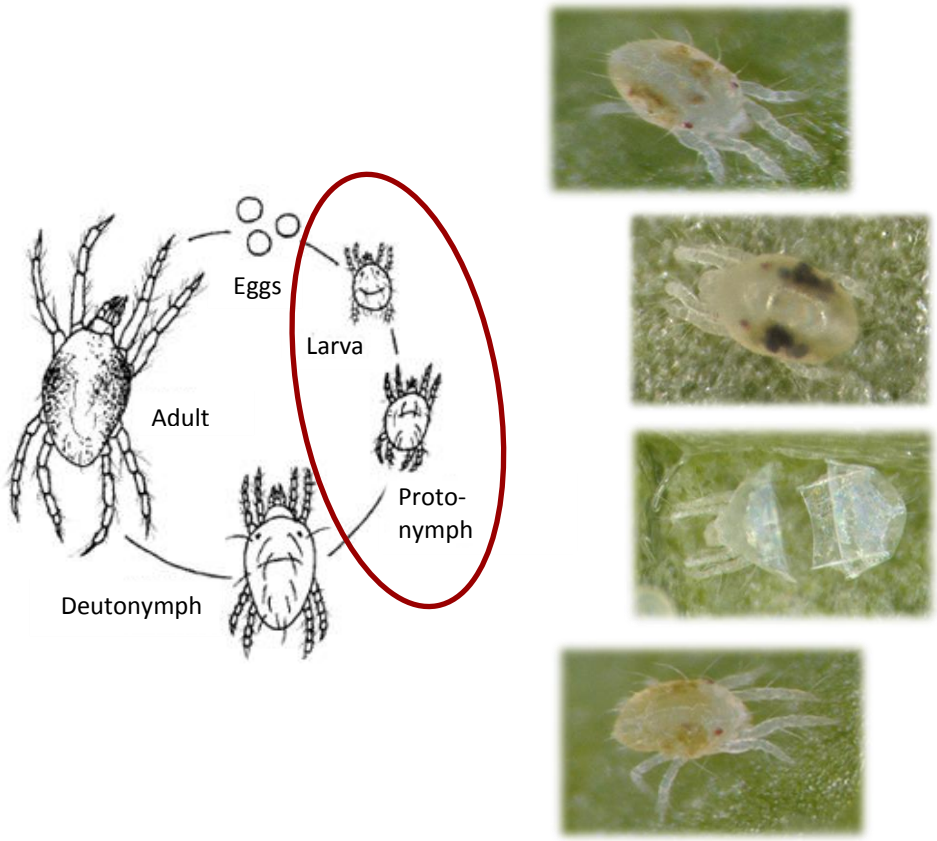


Figure 2.2 Development/mortality assay experimental set up. **A** Picture of experimental setup of detached leaf assay used in developmental/mortality assay. **B** Schematic of spider mite progression from larvae to protonymph during developmental/mortality assay.

A new leaf was added to the setup every other day (day 0, 2, 4…etc.) until all the larvae either molted into protonymphs or died. Every day during the experiment, the total number of surviving larvae was counted, as well as any larvae that had molted to protonymphs, and protonymphs were then removed (Figure 2.2B).

The developmental assay focused on the transition from newly emerged larvae to protonymph due to the easily observed addition of another pair of legs during this quiescent stage. Larval mortality was assessed at the conclusion of the experiment, where larvae that failed to develop into protonymphs died. The assay was conducted in an interaction chamber set to the same environmental conditions as the spider mite rearing chamber (24 °C, 60 % relative humidity and 16 h light: 8 h dark photoperiod). Therefore, if the response to the change in environment in detached leaves of different genotypes interacted with their response to spider mite larvae feeding, this could potentially introduce a confounding effect. However, as previously stated, robust, reproducible results were obtained from this assay.

Three replications of each development/mortality assay were performed for each comparison between *A. thaliana* genotypes, using different batches of plants. Upon completion of the experiments, two-way ANOVAs followed by Tukey's HSD tests were used to determine whether there were differences between the conditions of genotype and/or replication (separate batches of plants) and whether there was an interaction between the experimental conditions. In cases where there was a significant difference between replications, then one-way ANOVA/un-paired t-tests were used to detect significant differences between genotypes within individual experiments, followed by Tukey's HSD test when one-way ANOVAs were used.

2.6 Gene expression analysis by quantitative RT-PCR

Total RNA was extracted from approximately 100 µl of ground *A. thaliana* rosette tissue from four to five-week old plants of genotypes analyzed with and without spider mite treatments using the RNeasy Plant Mini Kit, including DNase treatment (Qiagen, Venlo, Limburg, Netherlands). Two µg of total RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit for qRT-PCR (Thermo Fisher

Scientific, Waltham, MA). Reactions were performed in triplicate for each biological replicate, using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA). The qRT-PCR was performed using an Agilent Mx3005P qPCR instrument (Agilent Technologies, Santa Clara, CA). Primer sequences and amplification efficiencies (E) are listed in Table 2.2. *PEX4* (*AT5G25760*), a ubiquitin conjugating enzyme, was used as a reference gene (Czechowski *et al.*, 2005) and was found to be transcribed at similar amounts in all samples as indicated by Ct values within ± 1cycle. Ct values of three technical replicates were averaged to generate a biological replicate Ct value. For plotting, expression values for each target gene (T) was normalized to the reference gene (R). Normalized relative quantity (NRQ) was calculated as follows (ER: efficiency of Reference gene, ET: efficiency of Target gene):

$$NRQ = \frac{(1+ER)^{CtR}}{(1+ET)^{CtT}}$$

NRQs were Log2-transformed and analyzed by means of a between-subjects two-way ANOVAs. The dependent variables were the Log2-transformed NRQs and the independent variables were genotype and spider mite treatment. ANOVAs were used to assess if there was a significance of the main effects (plant genotype and spider mite treatment) and the interaction plant genotype × spider mite herbivory (Rieu and Powers, 2009). The ANOVA results should be interpreted as follows: main significant effects of the plant genotype or spider mite herbivory means that these factors systematically affect a response variable, while a significant plant genotype × spider mite herbivory interaction indicates that genotypes respond in different ways to spider mite herbivory.

Table 2.2 List of primer sequences used in qRT-PCR and associated efficiencies.

Gene ID	Description	Forward primer	Reverse primer	Efficiency
AT5G64900	PROPEP1	CGGAACTTCGAAACAGCCGAAGG	CCCCCTTTGCTTTGCCTTGACC	1.000
AT5G64890	PROPEP2	TAACCAGCCGGAGGAACAAGAGG	GGCCAGGACGACCTGAACTAGG	1.000
AT5G64905	PROPEP3	ACGGTTCTTTGATCCCATTTACGTTCT	CGAGACCGGAACACTTCAAGAGAGG	0.962
AT5G09980	PROPEP4	TAGTTTTCCGGAGGATGGGGAGG	TTCTTGTTGGTGCCTCCCGGC	1.000
AT5G09990	PROPEP5	GGAGTCTCAACGTAATGAGGAAAGGG	ACTCCACCTCGTTTTCCCGAGC	0.939
AT2G22000	PROPEP6	AGAAGGAGACCAAGACCACCACC	AGATCCACACGGAGAGACACAAGC	0.911
AT1G73080	PEPR1	CGGCTACATTGCACCAGAAAACGC	TGTCCACCGCTCTCTTCCTCG	0.948
AT1G17750	PEPR2	GCACCAGAAAATGCGTACAAGACGG	ACCAGCTCGAGCAAAACAACTCC	0.959
AT5G42650	AOS	CGTTAGGAAGCTCCGTTAATTTCTC	TTCACGAAACTGGAACAAGAAAACA	0.984
AT1G32640	MYC2	TCGCTTACATCAACGAGCTTAAATC	TATCTTCACTTCAATCTCCATCCCC	0.900
AT4G39950	CYP79B2	GAAAAGAGGTTGTGCGGCTC	TCTCACTTCACCGTCGGGTA	0.994
AT2G22330	CYP79B3	TCTACCGATGCTTACGGGATTG	TACAAGTTCCTTAATGGTTGGTTTG	0.973
AT5G61420	MYB28	TGTCTGATTAGGGTTGAAACGGTG	CTATGACCGACCACTTGTTGCCA	1.000
AT5G07690	MYB29	TCCTACAACGGTCGTCTACCA	TTCTTCGGCAGTCCATGCTC	0.932
AT5G25760	PEX4	GCTCTTATCAAAGGACCTTCGG	CGAACTTGAGGAGGTTGCAAAG	0.992

Chapter Three – Results

3.1 Perception: Role of PEPRs in perception of spider mite herbivory through plant tissue damage

3.1.1 Plant damage and spider mite larvae developmental and mortality assays using *pepr* mutants

Potential PRRs for spider mite associated HAMPs and/or DAMPs would most likely be identified using the microarray data representing local responses, in the feeding site experiment with hundreds of mites feeding for one hour. Therefore, LRR-RLKs that could act as potential PRRs were searched for in the feeding site microarray data and two such receptors were identified as induced upon spider mite herbivory. These receptors were the homologous DAMP receptors PEPR1 and PEPR2 (Zhurov *et al.*, 2014). As mentioned previously, these membrane-bound PRRs are of the LRR-RLK family and have been implicated in amplification of a resistance response following pathogen attack (Huffaker and Ryan, 2007). To test whether *A. thaliana* plants lacking one or both of these receptors (as they have been shown to act redundantly) are more susceptible to spider mite herbivory, plant damage assays were performed in triplicate. Representative pictures of Col-0 and *pepr1pepr2* plants are shown in Figure 3.1A. Plant damage assays showed similar results (Figure 3.1B). Ten adult female spider mites were placed on 8 to 13 plants each of 4 genotypes: Col-0 (WT control), *pepr1*, *pepr2*, and *pepr1pepr2*. Although single mutants, *pepr1*, and *pepr2* showed no significant increase in damage relative to the Col-0 control, the double mutant, *pepr1pepr2*, displayed a mild increase in damage. The first experiment showed a 19% increase in plant damage (21 mm^2 damage in Col-0 compared to 25 mm^2 damage in *pepr1pepr2*). The second experiment showed no significant increase in damage in the double mutant, but a visible trend was observed. The third experiment revealed a 15% increase in damage in the *pepr1pepr2* double mutant (12 mm^2 damage in Col-0 compared to 14 mm^2 in *pepr1pepr2*; Figure 3.1B).

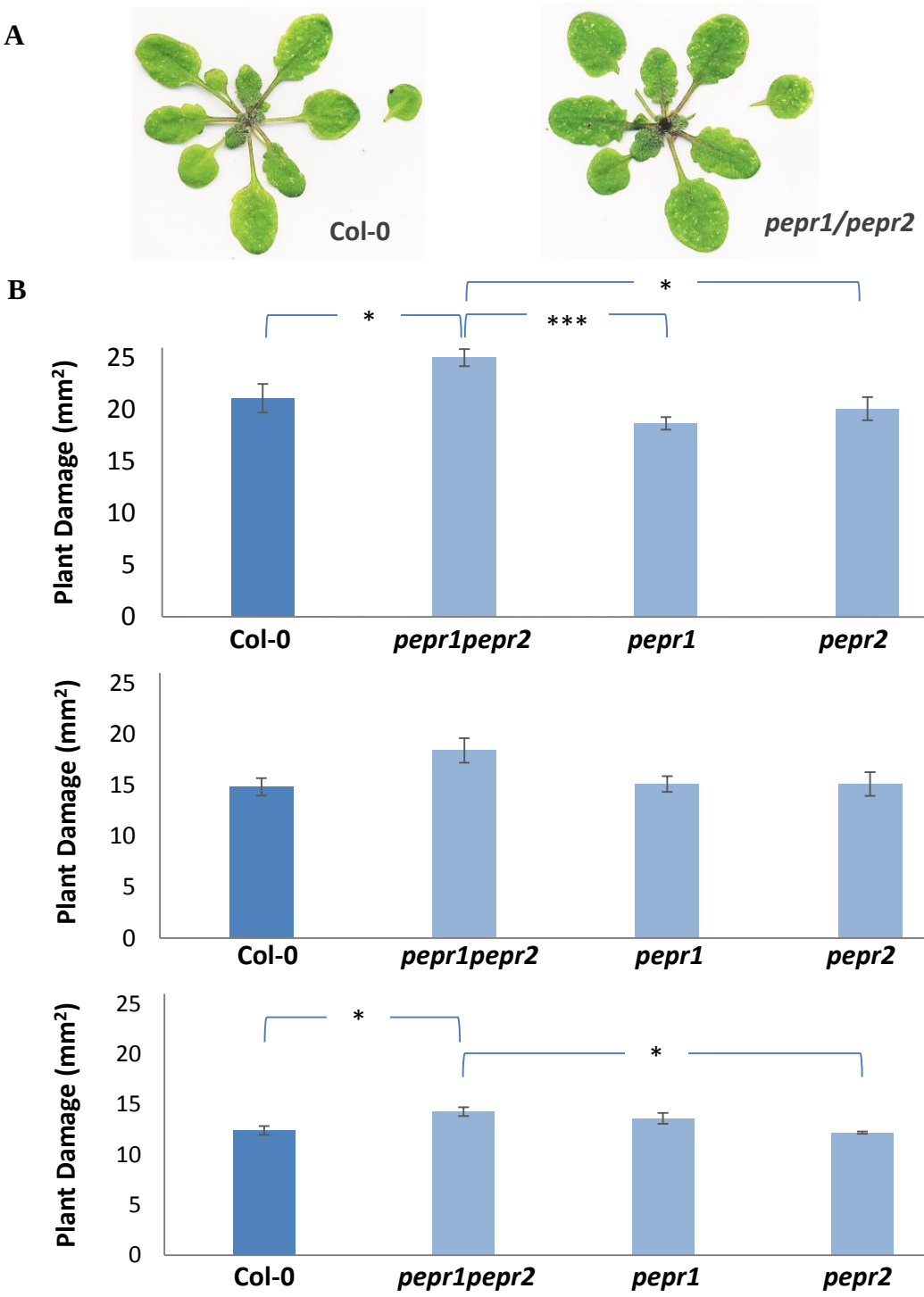


Figure 3.1 Plant damage assay of *pepr* double and single mutants compared to Col-0 WT controls. **A** Representative pictures of Col-0 control and *pepr1pepr2* double mutant following three days of female adult mite feeding. **B** Three replications of relative plant damage of plants inoculated with 10 adult female mites for 3 days (n = 8 to 13 plants per genotype). Error bars are ± 1 SEM, (Tukey HSD test, * - $p < 0.05$, *** - $p < 0.001$).

To test spider mite performance, larvae development/mortality assays were performed in triplicate. No significant differences in developmental timing were found for spider mite larvae to develop into protonymphs in any of the 3 experimental replications (Figure 3.2). A significant difference in spider mite mortality was observed in one of three experimental replications, where *pepr1* had a significantly lower spider mite larval mortality compared to Col-0 control fed larvae and a trend for decreased mortality in the *pepr1pepr2* mutant was observed for all experiments (Figure 3.3).

3.1.2 PROPEP and PEPR gene expression time course following spider mite feeding

To better understand the kinetics of *PEPR* and *PROPEP* transcript expression, a time course analysis was performed where 10 adult female mites were allowed to feed on Col-0 (WT) plants for 1, 3, 6, 12, and 24 h. At 24 h, control plants that were not treated with mites were collected at the same time as all other samples (the experiment was synchronized so that all samples were collected at 24 h). This experiment was conducted and RNA was extracted by Dr. Marie Navarro. cDNA synthesis through qRT-PCR data analysis was performed by myself. Genes analysed for differential transcript expression upon mite feeding included both Pep receptors, *PEPR1*, and *PEPR2*, and all of the genes encoding functional PROPEP peptides: *PROPEP1* to *PROPEP6*.

Interestingly, although *PEPR1* showed no detectable change in expression following spider mite feeding (Figure 3.4A), *PEPR2* moderately increases in expression during the 12 to 24 h period (Figure 3.4B). *PROPEP1* transcription appeared to be repressed upon perception of spider mite herbivory during the first 6 h of attack before returning to basal levels by 12 h (Figure 3.4C). *PROPEP2* showed a trend for increased expression during the 3 to 6 h period (Figure 3.4D). *PROPEP3* showed varying expression upon spider mite herbivory, increasing in expression slightly at 3 h and falling to basal levels again until 24 h where it increased again (Figure 3.4E). Similar to *PEPR2*, *PROPEP4* increased in expression late in the time course experiment, rising above basal levels only at 12 h and 24 h (Figure 3.4F). *PROPEP5* and *PROPEP6* did not show any transcription induction during spider mite herbivory throughout the course of 24 h (Figures 3.4G and H).

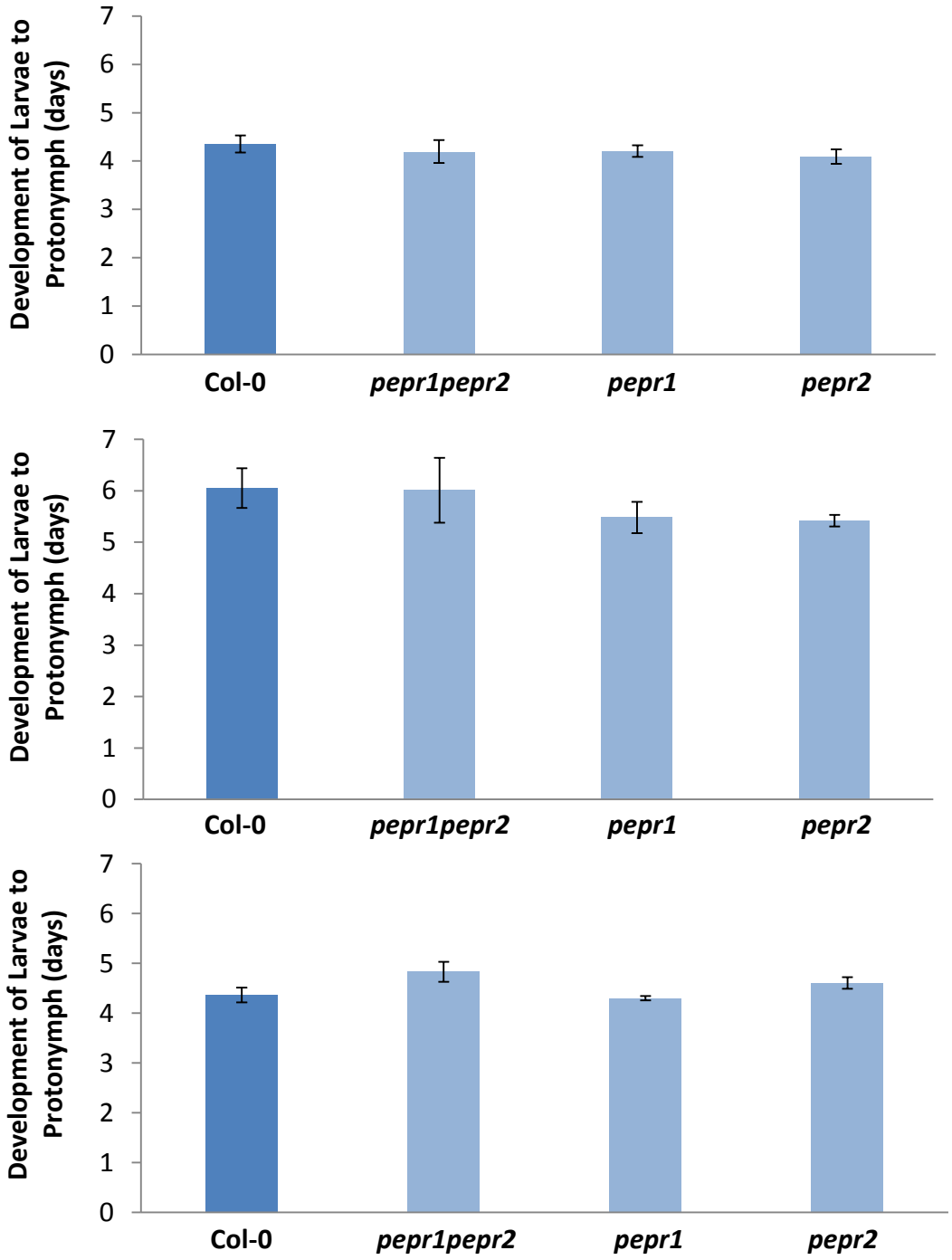


Figure 3.2 Spider mite larvae developmental assay using *pepr* double and single mutants compared to Col-0 WT controls. Three replications of larval development assayed by mean number of days for larvae to develop into protonymphs. Three to five detached leaves/genotype inoculated with 30 to 60 newly emerged spider mite larvae assayed for day they developed into protonymphs. Error bars are ± 1 SEM.

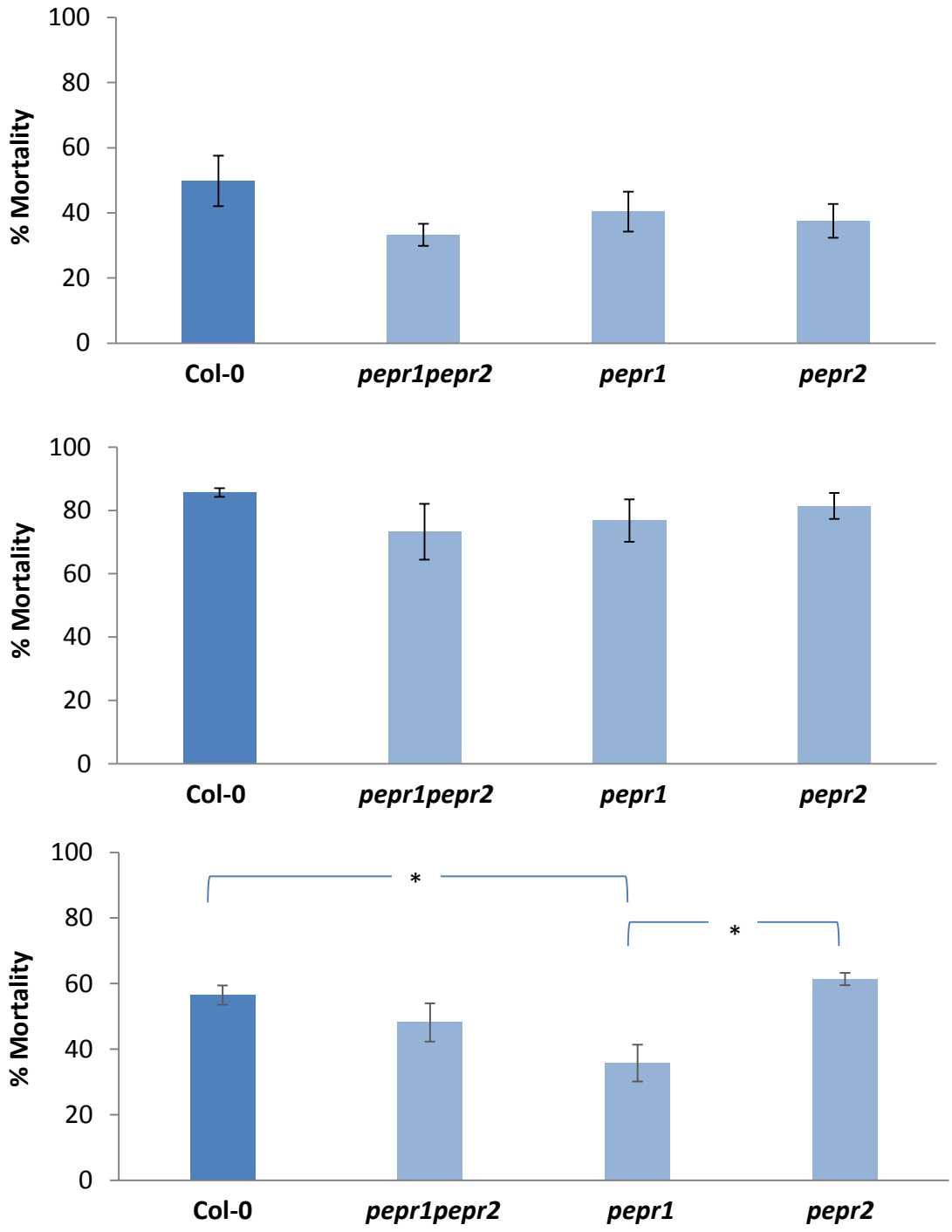
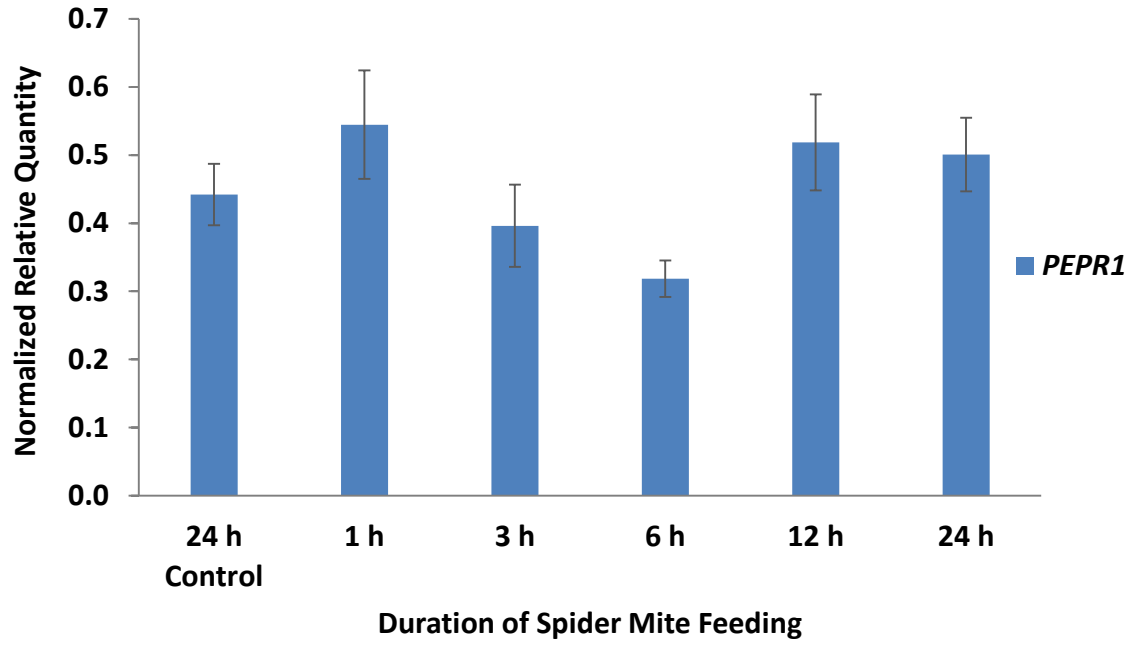
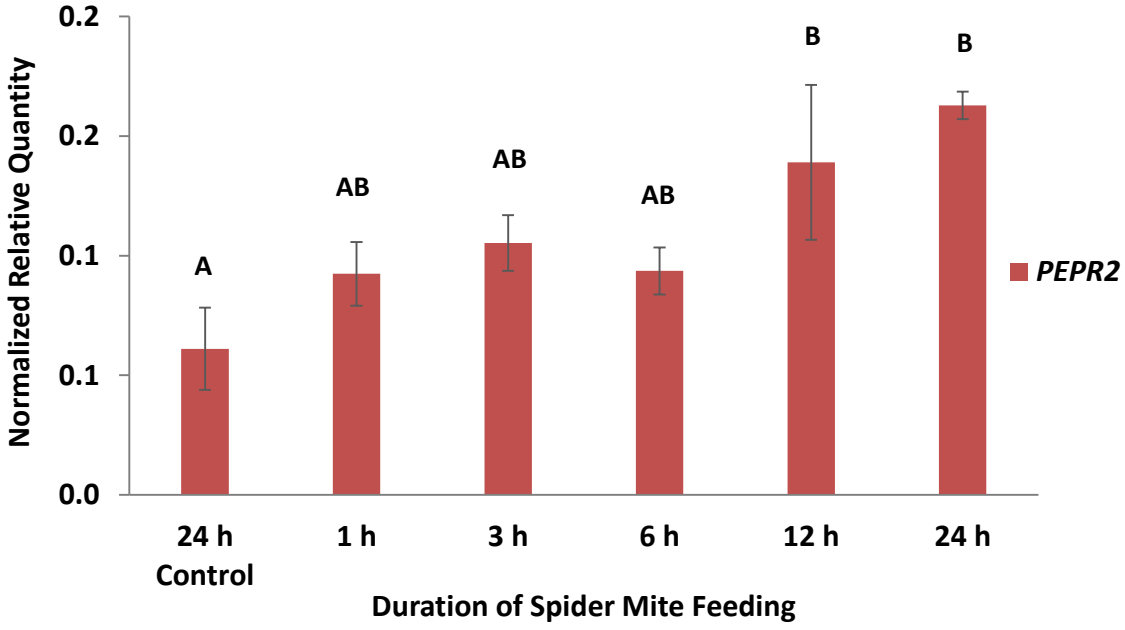


Figure 3.3 Spider mite larvae mortality assay using *pepr* double and single mutants compared to Col-0 WT controls. Three to five detached leaves were inoculated with 30 to 60 newly emerged spider mite larvae. Larval mortality determined at the conclusion of the experiment (larvae that did not develop into protonymphs died). Error bars are ± 1 SEM, (Tukey HSD test, * - $p < 0.05$).

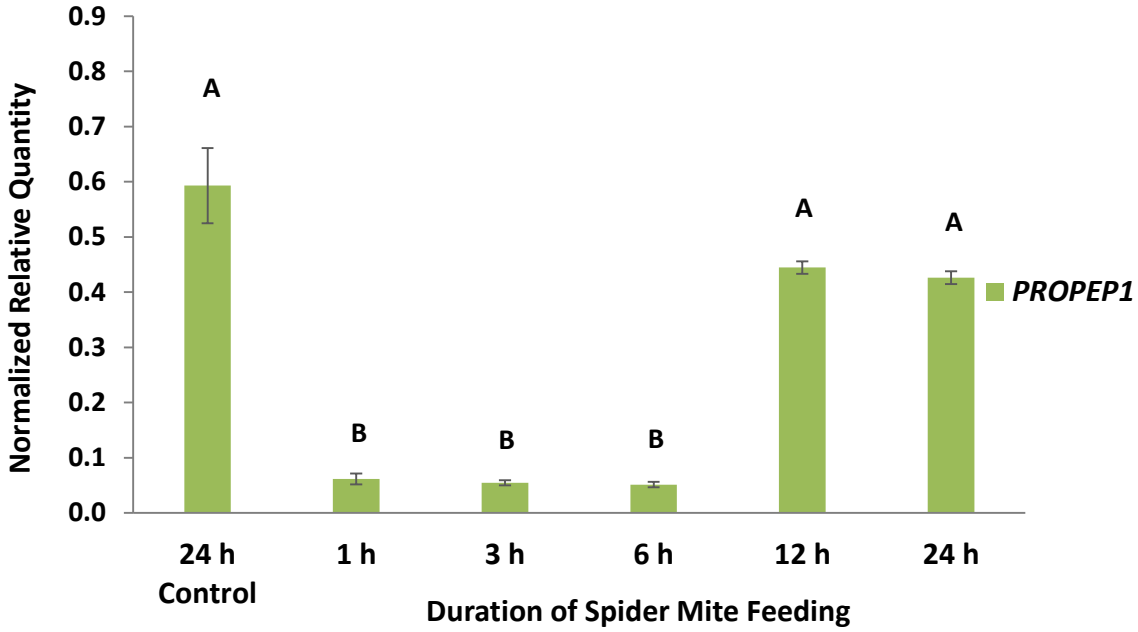
A


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Normalized Relative Quantity
24 h Control
1 h
3 h
6 h
12 h
24 h
Duration of Spider Mite Feeding
PEPR1

B


0.2
0.2
0.1
0.1
0.0
Normalized Relative Quantity
A
AB
AB
AB
B
B
24 h Control
1 h
3 h
6 h
12 h
24 h
Duration of Spider Mite Feeding
PEPR2

C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0.5
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0.2
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Normalized Relative Quantity
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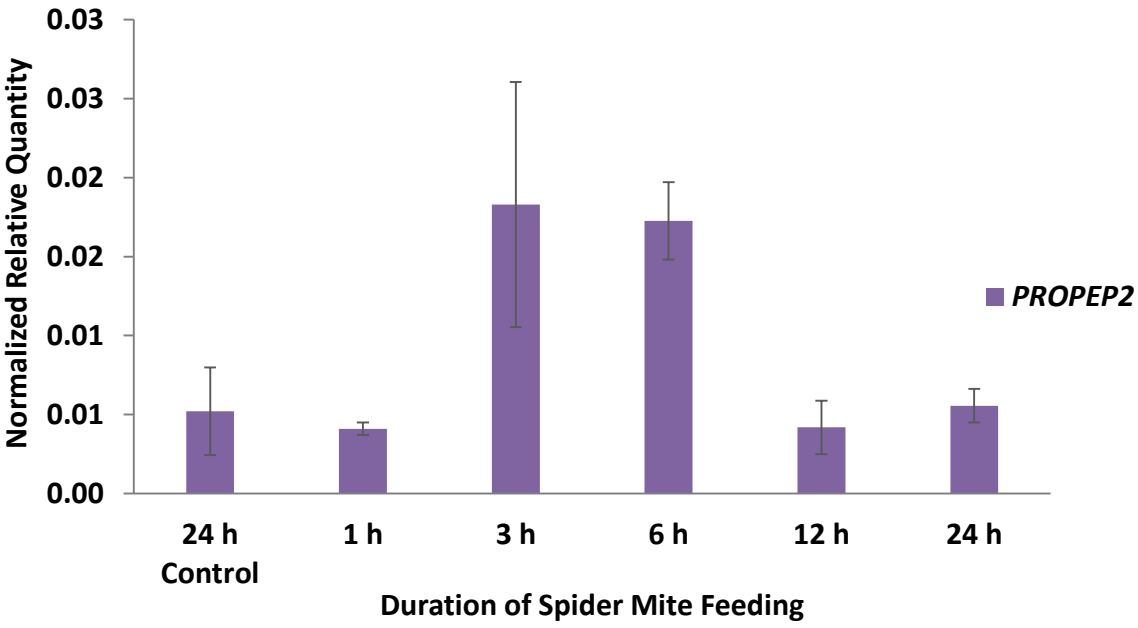
B
B
B
A
A
PROPEP1
24 h Control
1 h
3 h
6 h
12 h
24 h
Duration of Spider Mite Feeding

D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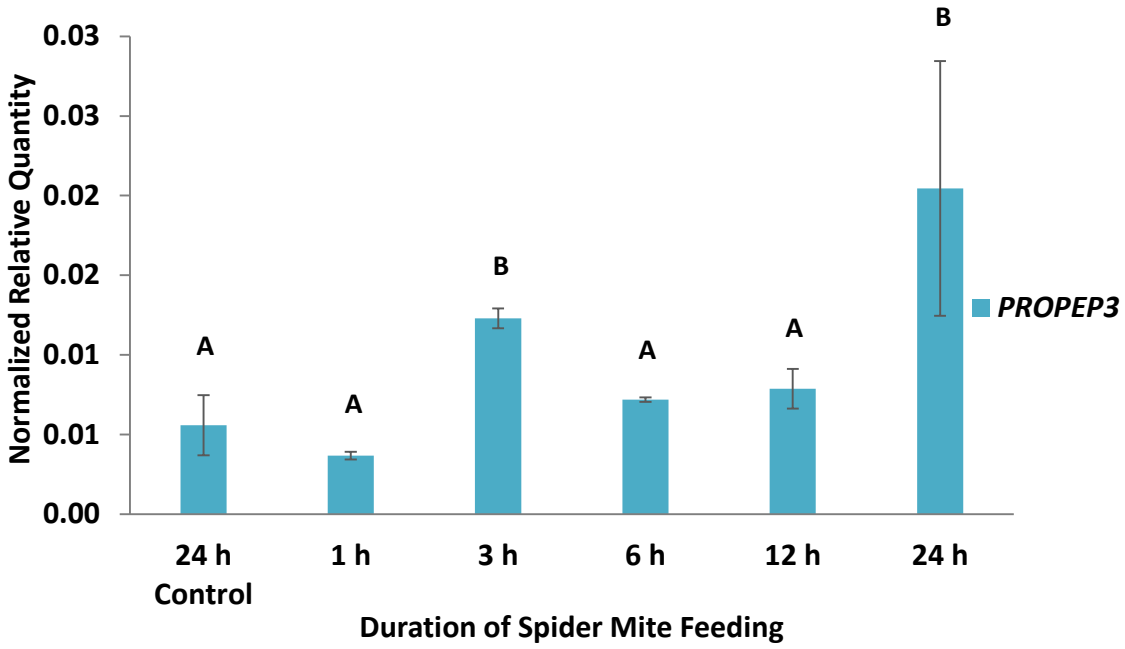
12 h
24 h
Duration of Spider Mite Feeding

E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A
B
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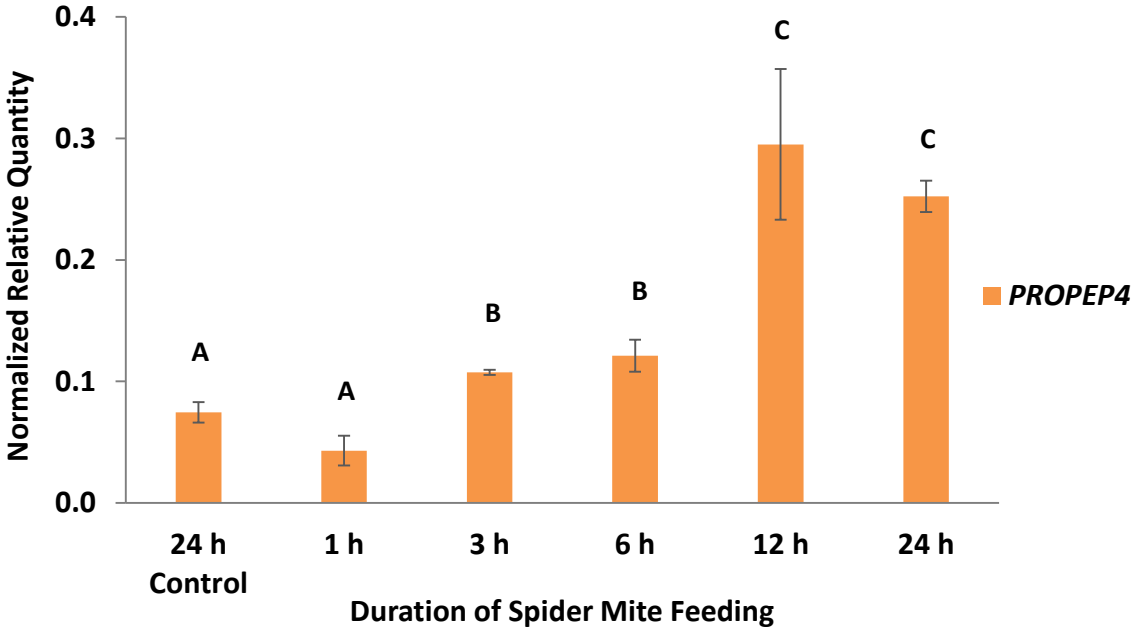
3 h
6 h
12 h
24 h
Duration of Spider Mite Feeding

F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Normalized Relative Quantity
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B
C
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6 h
12 h
24 h
Duration of Spider Mite Feeding

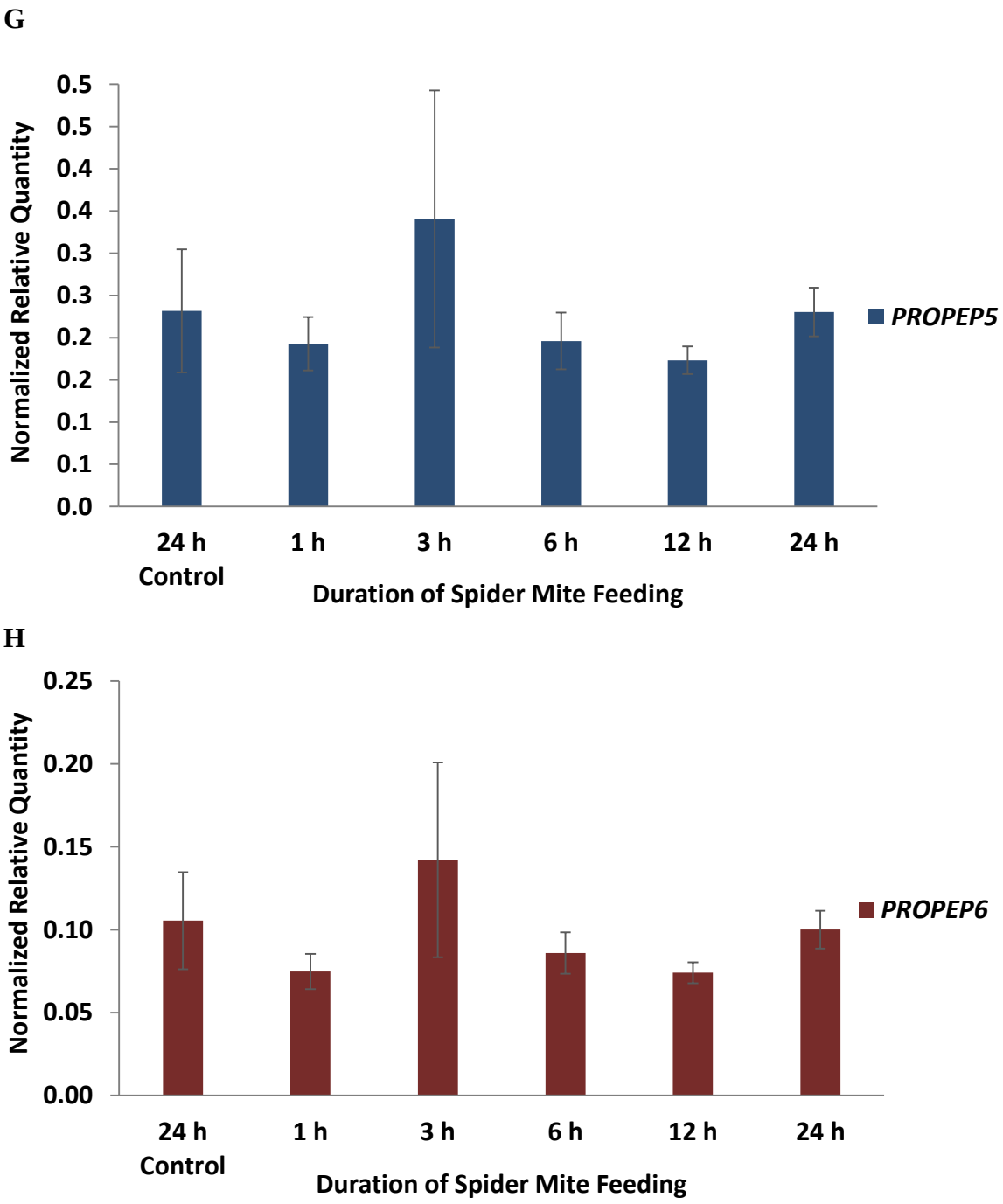

Figure 3.4 Gene expression of *PEPRs* and *PROPEP* genes during a time course experiment in Col-0. A-H Normalized relative quantity of transcripts of labelled genes following treatment with 10 female adult spider mites for 1, 3, 6, 12, and 24 h. Mean ± 1 SEM fold changes of expression levels detected by qRT-PCR in Col-0 (n = 3 biological replicates consisting of 3 pooled plants per replicate). Different letters represent significant differences (Tukey HSD test, $p < 0.05$).

3.2 Signalling: The Role of Jasmonic Acid in the Response to Spider Mite Feeding

3.2.1 Plant damage and spider mite larvae developmental and mortality assays using *aos* and *myc2myc3myc4* mutants

GO categories for DEGs up-regulated in the Bla-2 resistant accession implicated JA and SA biosynthetic processes in response to spider mite feeding. Meta-analysis of the expression profiles of spider mite induced responses compared to responses to different hormones revealed that only MeJA/OPDA triggered responses clustered together with mite-triggered responses (Zhurov *et al.*, 2014). This suggests that JA is the major hormone involved in the signalling associated with *A. thaliana* response to spider mite feeding. To test if the JA pathway is indeed responsible for orchestrating the resistance response, plant damage and spider mite larvae development and mortality assays were conducted using two mutants on opposite ends of the JA pathway. The *aos* mutant lacks the allene oxide synthase (AOS) enzyme that is among the enzymes responsible for the conversion of linolenic acid to OPDA (Figure 1.1). The *aos* mutant is devoid of its ability to synthesize JA, therefore all responses requiring the JA pathway will be disrupted in this mutant.

Plant damage assays revealed a severely susceptible phenotype in *aos* plants compared to Col-6 controls (almost 5 fold increase in damage in *aos*, Figure 3.5A). Spider mite larvae performance on *aos* mutants was better relative to controls, consistent with plant damage assay. Spider mite larvae developed about two time faster on *aos* mutants and larvae suffered almost no mortality compared to Col-6 (Figure 3.5B and C).

Described previously, the JA pathway diverges into two branches in its signalling of defence responses in *A. thaliana*, often termed the MYC2 branch and the ERF branch (Figure 1.2; Verhage *et al.*, 2011). The MYC2 branch is prioritized over the EFR branch during insect feeding (when not manipulated by insect effectors in OS) (Verhage *et al.*, 2011). The MYC2, MYC3, and MYC4 TFs are considered key regulators of many JA responsive genes (Schweizer *et al.*, 2013).

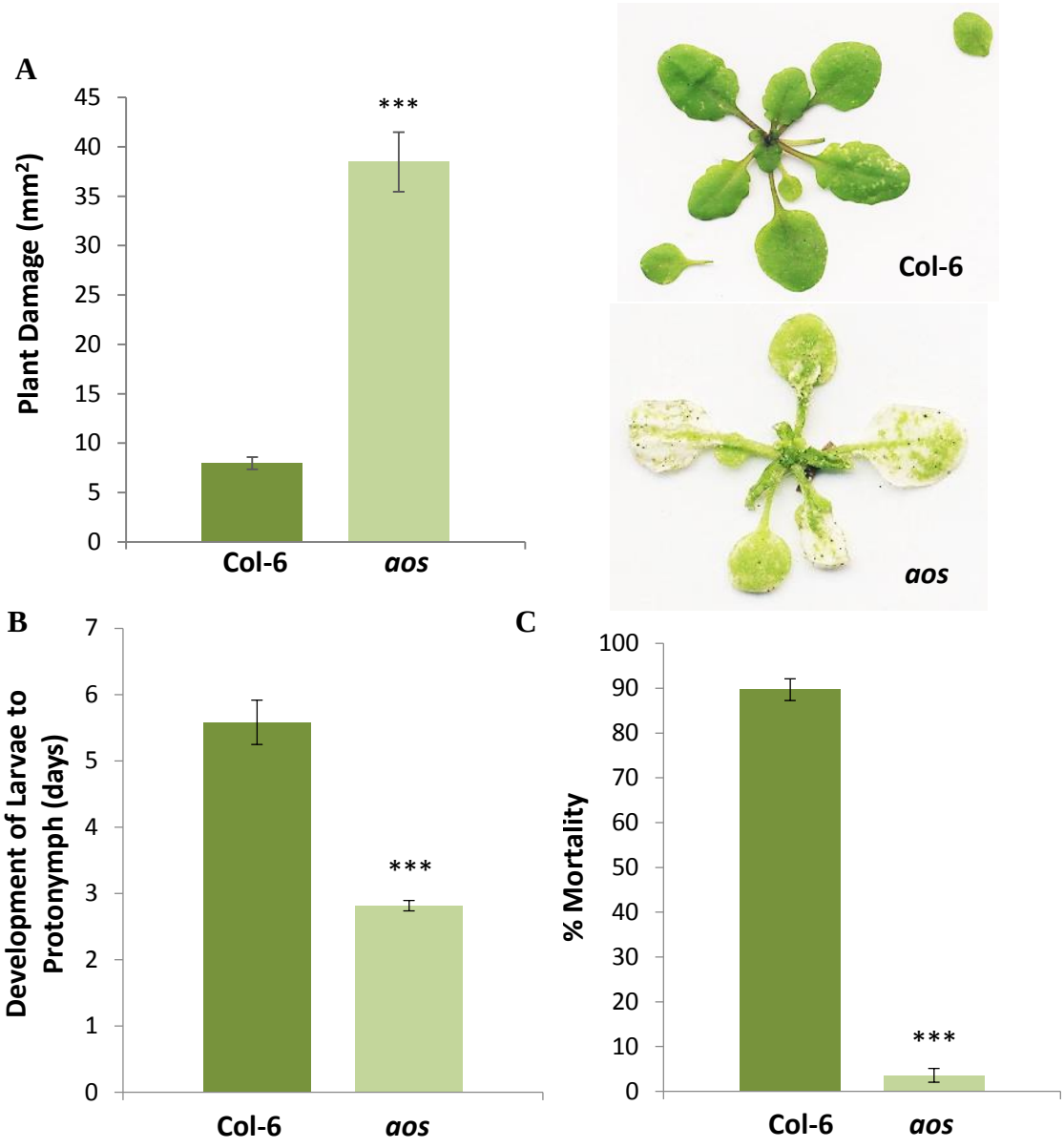

Figure 3.5 Importance of JA biosynthesis in *A. thaliana* defence response to spider mites. **A** Relative plant damage of Col-6 (WT) and *aos* mutants as assayed by mean chlorotic spot area following feeding by 10 spider mites for 3 days (n = 9 to 10 plants per genotype). Pictures of representative plants from each genotype shown on the right. **B** Spider mite larval performance assayed by average number of days required for larvae to become protonymphs. **C** Mean percentage of larval mortality after feeding on Col-6 or *aos* detached leaves. Error bars are ± 1 SEM (n = 5 replicates with 30-60 larvae each; unpaired t-test, *** - $p < 0.001$). Published in Zhurov *et al.* (2014).

Thus the *myc2myc3myc4* triple mutant, lacking the three major TFs involved in JA signalling through the MYC2 branch, though still capable of synthesizing JA, was used to determine if downstream signalling is required for spider mite resistance. Results from plant damage and spider mite performance using the *myc2myc3myc4* mutant are similar to those gathered using the *aos* mutant, showing a marked increase in plant damage (Figure 3.6A) and increase in spider mite larvae performance as assayed by developmental timing and mortality using the *myc2myc3myc4* mutant (Figure 3.6B and C). This indicates that a functional JA pathway, from biosynthesis through signalling and transcriptional reprograming via the MYC2 branch is required for an effective resistance response of *A. thaliana* to spider mites.

3.2.2 JA marker gene analysis in *pepr1pepr2* mutant

To determine if the observed requirement of the JA pathway in signalling the resistance response is dependent or associated with the perception of damage through DAMP receptors PEPR1 and PEPR2, marker gene analysis was performed using *AOS* and *MYC2* as maker genes of the JA pathway, that are induced upon spider mite herbivory. The *pepr1pepr2* double mutant was assayed for marker gene induction and compared to the level of induction of marker genes in Col-0 control following 1 h of feeding by 10 adult female spider mites. I hypothesized that if PEPRs are involved in the perception of spider mite herbivory and are required for the associated induction of JA signalling, then this response should be reduced in the *pepr1pepr2* double mutant relative to the Col-0 control.

Both *AOS* and *MYC2* are induced upon spider mite herbivory in both Col-0 and *pepr1pepr2* plants (Figure 3.7A and B). Therefore, the data indicate that neither JA biosynthesis nor signalling require spider mite perception through PEPRs. As there were no significant differences in transcript levels within treatment type and between genotypes, I can conclude that the level of induction is comparable in Col-0 and *pepr1pepr2* plants. Importantly, basal levels of expression of *AOS* and *MYC2* in Col-0 and the *pepr* double showed no difference, therefore I can conclude that a lack of PEPRs does not alter constitutive JA signalling (Figure 3.7A and B).

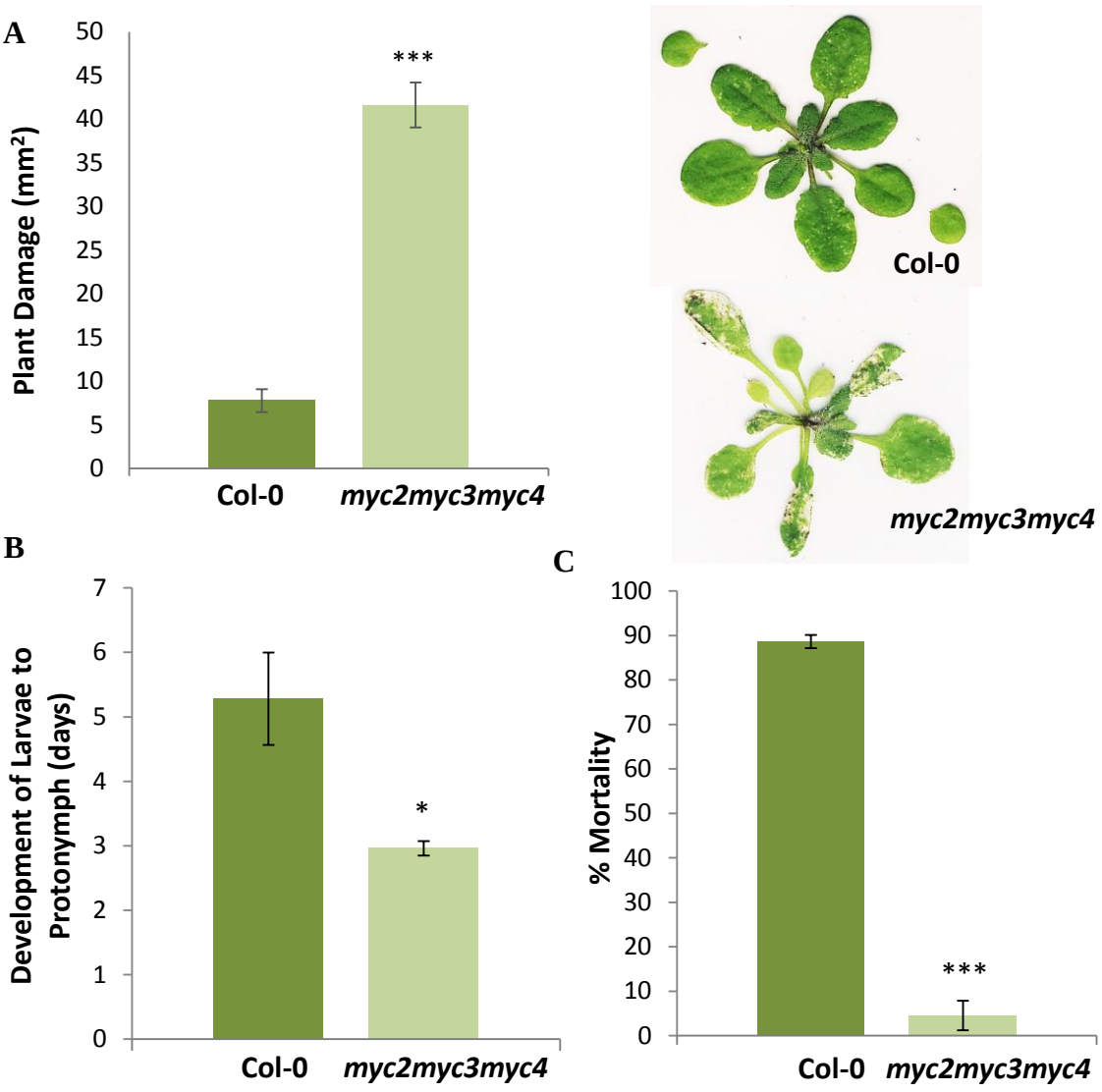

Figure 3.6 Importance of the MYC2 branch of the JA pathway in *A. thaliana* defence response to spider mites. **A** Relative plant damage of Col-0 (WT) and *myc2myc3myc4* mutants as assayed by mean chlorotic spot area following feeding by 10 spider mites for 3 days (n = 12 plants per genotype). Pictures of representative plants from each genotype shown on the right. **B** Spider mite larval performance assayed by average number of days required for larvae to become protonymphs. **C** Mean percentage of larval mortality after feeding on Col-0 or *myc2myc3myc4* detached leaves. Error bars are ± 1 SEM (n = 5 replicates with 30-60 larvae each; unpaired t-test, * - $p < 0.05$, *** - $p < 0.001$). Published in Zhurov *et al.* (2014).

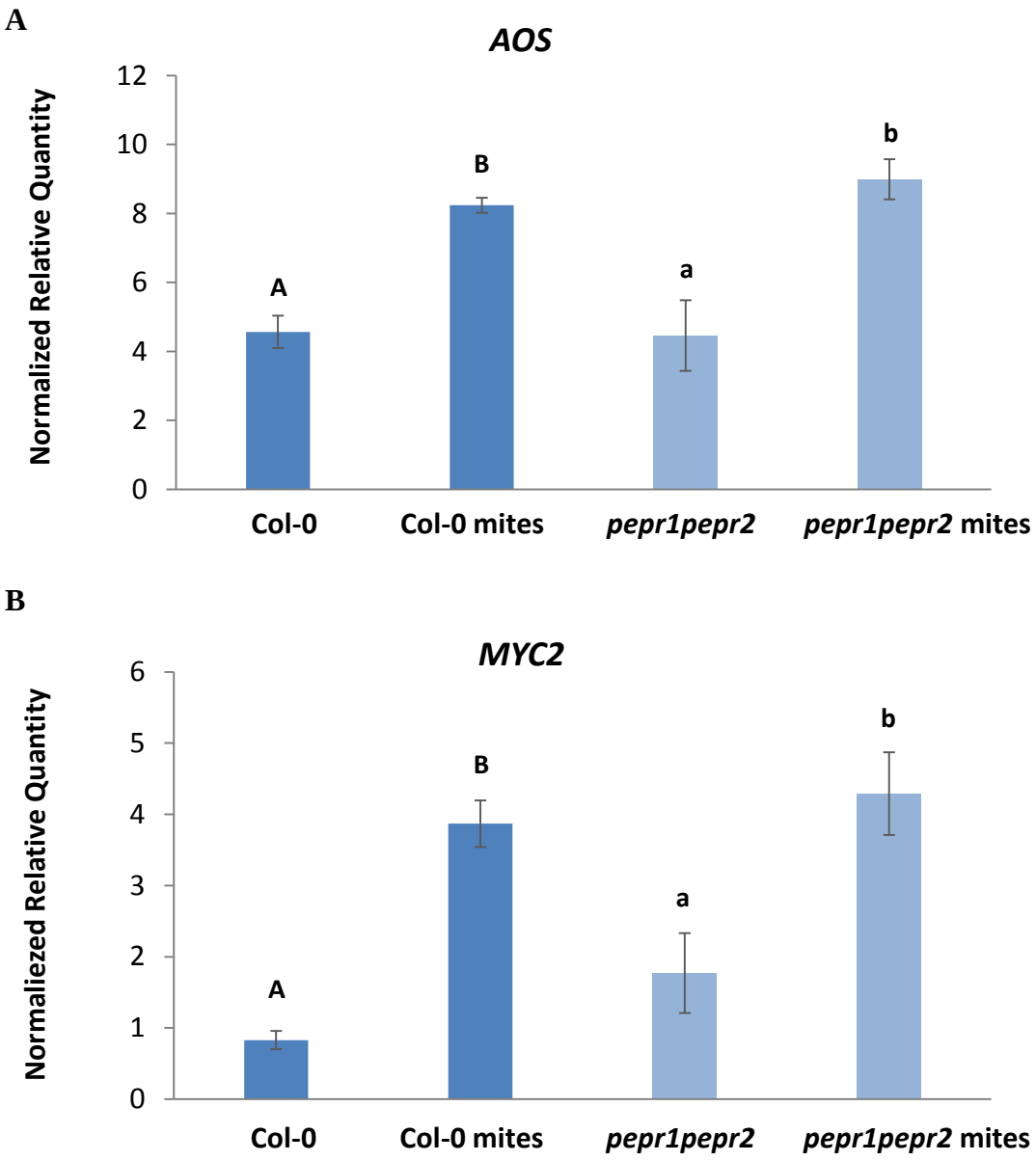

Figure 3.7 *AOS* and *MYC2* marker gene analysis in Col-0 (WT) and *pepr1pepr2* double mutant plants. *AOS* (**A**) and *MYC2* (**B**) gene transcript levels upon feeding of 10 spider mites for 1 h on Col-0 (WT) and *pepr1pepr2* double mutants plants. Mean fold changes detected by qRT-PCR (n = 3). Error bars are ± 1 SEM. Different letters indicate significant differences within genotype (uppercase – Col-0, lowercase – *pepr1pepr2*, Tukey HSD test, $p < 0.05$).

3.3 Response: Indole Glucosinolates are Effective Secondary Metabolites in the Defence Response to Spider Mite Feeding and are JA Dependent

3.3.1 Plant damage and spider mite larvae developmental and mortality assays using *A. thaliana* mutants lacking indole glucosinolates

Due to the effect of JA-dependent responses on spider mite larval mortality in mutants lacking a functional JA pathway, I hypothesized that upon feeding, JA-regulated defence compounds are synthesized. In microarray data, genes associated with tryptophan catabolic and indoleacetic acid biosynthetic processes are induced (Zhurov *et al.*, 2014). Plant damage, and mite developmental and mortality assays were performed using a mutant that lack genes encoding IG-committing enzymes CYP79B2 and CYP79B3 (Figure 1.3). An indole glucosinolate mutant with a reduced subset of IG metabolites was also used (*cyp81f2*, lacking 4-OH-I3M and 4-MO-13M, 4-methyl glucosinolates) (Pfalz *et al*., 2009) (Figure 1.3). The *cyp79b2cyp79b3* double mutant, lacking IGs, showed an increase in plant damage following spider mite feeding and displayed improved spider mite performance as observed by faster development and significantly lower mortality of mite larvae feeding on the *cyp79b2cyp79b3* double mutant (Figure 3.8A, B and C). The *cyp81f2* mutant, lacking a subset of IG metabolites showed increase in plant damage and a clear trend of enhanced spider mite performance, though it was not statistically significant (Figure 3.9A, B and C). Plant damage assays were performed by Dr. Marie Navarro, plant damage quantification and statistical analysis was performed by myself. Spider mite development/mortality assays were performed by myself. Interestingly, in contrast to IGs, spider mite herbivory did not induce the expression of genes involved in the biosynthesis of aliphatic glucosinolates and mutants lacking the regulators of aliphatic glucosinolate biosynthesis, *myb28*, *myb29*, and *myb28myb29*, showed no difference in plant damage relative to controls following spider mite herbivory and the double mutant showed no difference in mite performance assayed by developmental timing and mortality (Zhurov *et al.*, 2014).

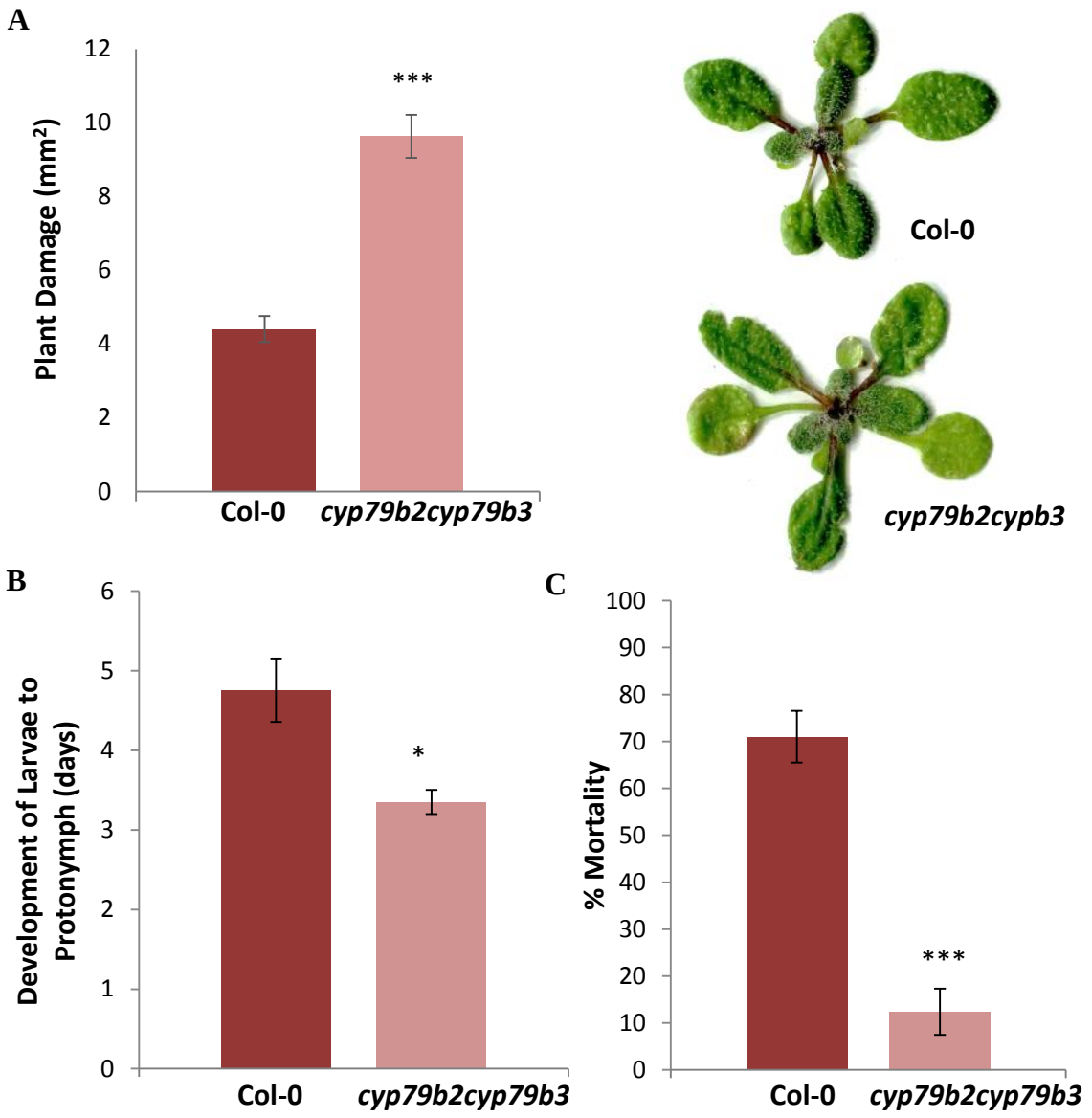

Figure 3.8 Role of indole glucosinolates in *A. thaliana* defence response to spider mites. **A** Relative plant damage of Col-0 (WT) and *cyp79b2cyp79b3* mutants as assayed by mean chlorotic spot area following feeding by 10 spider mites for 3 days (n = 4 to 7 plants per genotype). Pictures of representative plants from each genotype shown on the right. **B** Spider mite larval performance assayed by average number of days required for larvae to become protonymphs. **C** Mean percentage of larval mortality after feeding on Col-0 or *cyp79b2cyp79b3* detached leaves. Error bars are ± 1 SEM (n = 5 replicates with 30-60 larvae each; unpaired t-test, * - $p < 0.05$, *** - $p < 0.001$). Published in Zhurov *et al.* (2014).

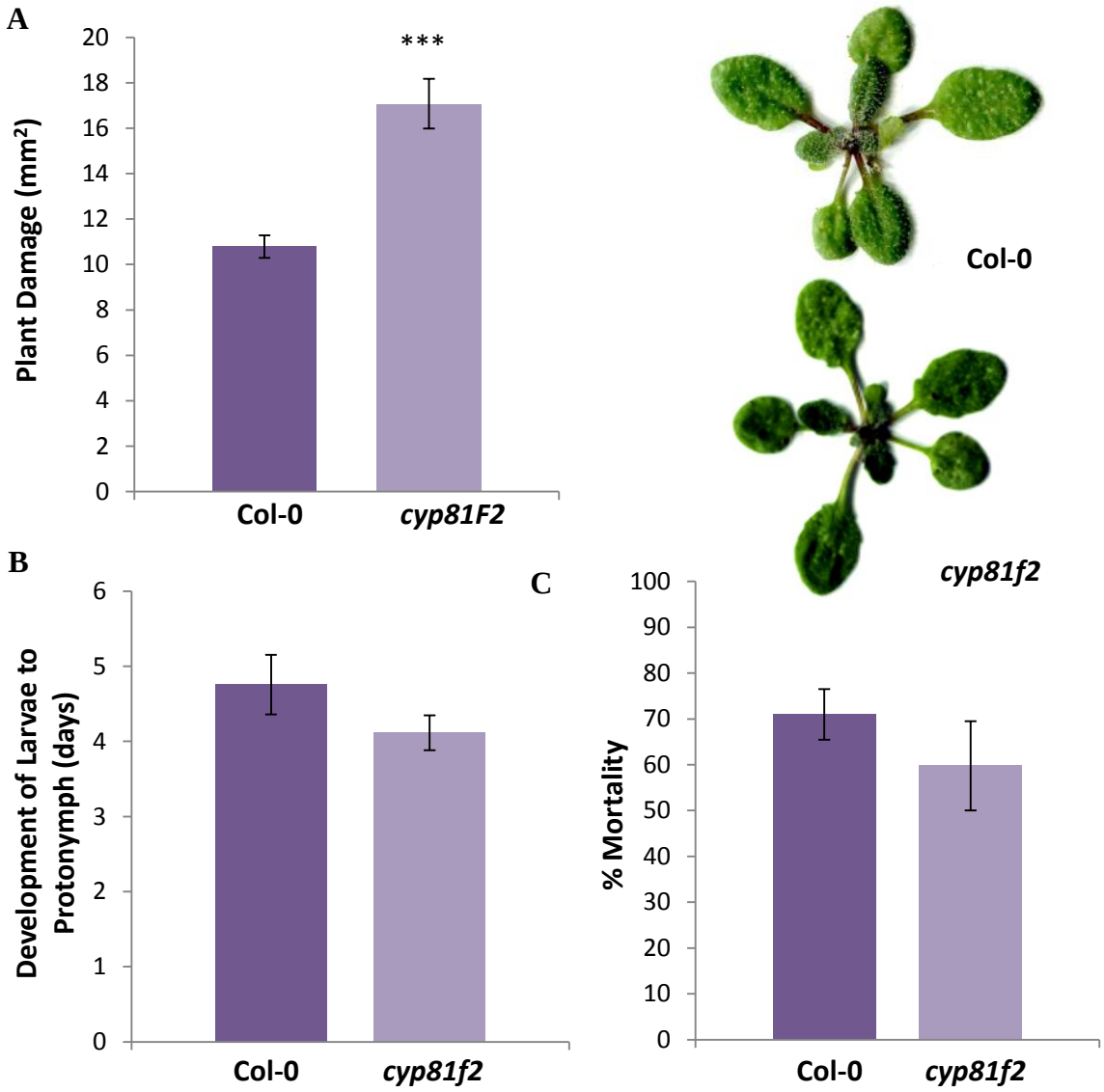

Figure 3.9 Role of a subset (4-OH-I3M and 4-MO-13M, 4-methyl glucosinolates) of indole glucosinolates in *A. thaliana* defence response to spider mites. **A** Relative plant damage of Col-0 (WT) and *cyp81f2* mutants as assayed by mean chlorotic spot area following feeding by 10 spider mites for 3 days (n = 4 to 7 plants per genotype). Pictures of representative plants from each genotype shown on the right. **B** Spider mite larval performance assayed by average number of days required for larvae to become protonymphs. **C** Mean percentage of larval mortality after feeding on Col-0 or *cyp81f2* detached leaves. Error bars are ± 1 SEM (n = 5 replicates with 30-60 larvae each; unpaired t-test, *** - $p < 0.001$). Published in Zhurov *et al.* (2014).

3.3.2 *CYP79* marker gene analysis in *pepr1pepr2* double mutant

Although JA marker gene analysis showed no dependence on PEPR mediated perception of spider mite feeding, marker gene analysis was performed using *CYP79B2* and *CYP79B3* as marker genes of IG biosynthesis in response to spider mite feeding in Col-0 (WT) and *pepr1pepr2* double mutants. qRT-PCR was used to determine the expression of *CYP79B2* and *CYP79B3* in Col-0 and *pepr1pepr2* plants without mites and plants inoculated with 10 adult female mites for 1h. There was no significant difference observed in transcript levels of *CYP79B2* or *CYP79B3* when comparing Col-0 and *pepr1pepr2* within the same treatment group (Figure 3.10A and B). Lack of significant/high levels of induction of *CYP79B2* and *CYP79B3* in Col-0 WT is probably due to the short duration of mite feeding (1 h) where induction of these genes following perception of spider mite happens around 6 h post inoculation as indicated by microarray data (Zhurov *et al*., 2014). The data revealed that there was no difference in the basal levels of expression of these genes in Col-0 and *pepr1pepr2* mutant plants. Therefore, any difference in response to spider mite herbivory in the *pepr1pepr2* mutant with respect to IG is presumably associated with perception of feeding and not differences in constitutive defence states.

3.3.3 *CYP79* marker gene analysis in *aos* mutant

To assess whether the induction of IG secondary metabolites are behaving in a JA dependant manner, marker gene analysis was performed in *aos* mutants incapable of synthesizing JA. I assayed the transcript levels of *CYP79B2* and *CYP79B3* genes encoding enzymes required for IG biosynthesis. Both *CYP79B2* and *CYP79B3* are induced upon spider mite feeding in the Col-6 WT control (Figure 3.11A and B). However, this induction was completely absent in *aos* mutants where there was no significant increase in gene expression following feeding of 10 female adult spider mite for 6 h (Figure 3.11A and B). I can conclude that the levels of transcript induction were different in Col-6 and *aos* plants. Also, lower basal levels of *CYP79B2* and *CYP79B3* transcripts are observed in the *aos* mutant (asterisks, Figure 3.11A and B).

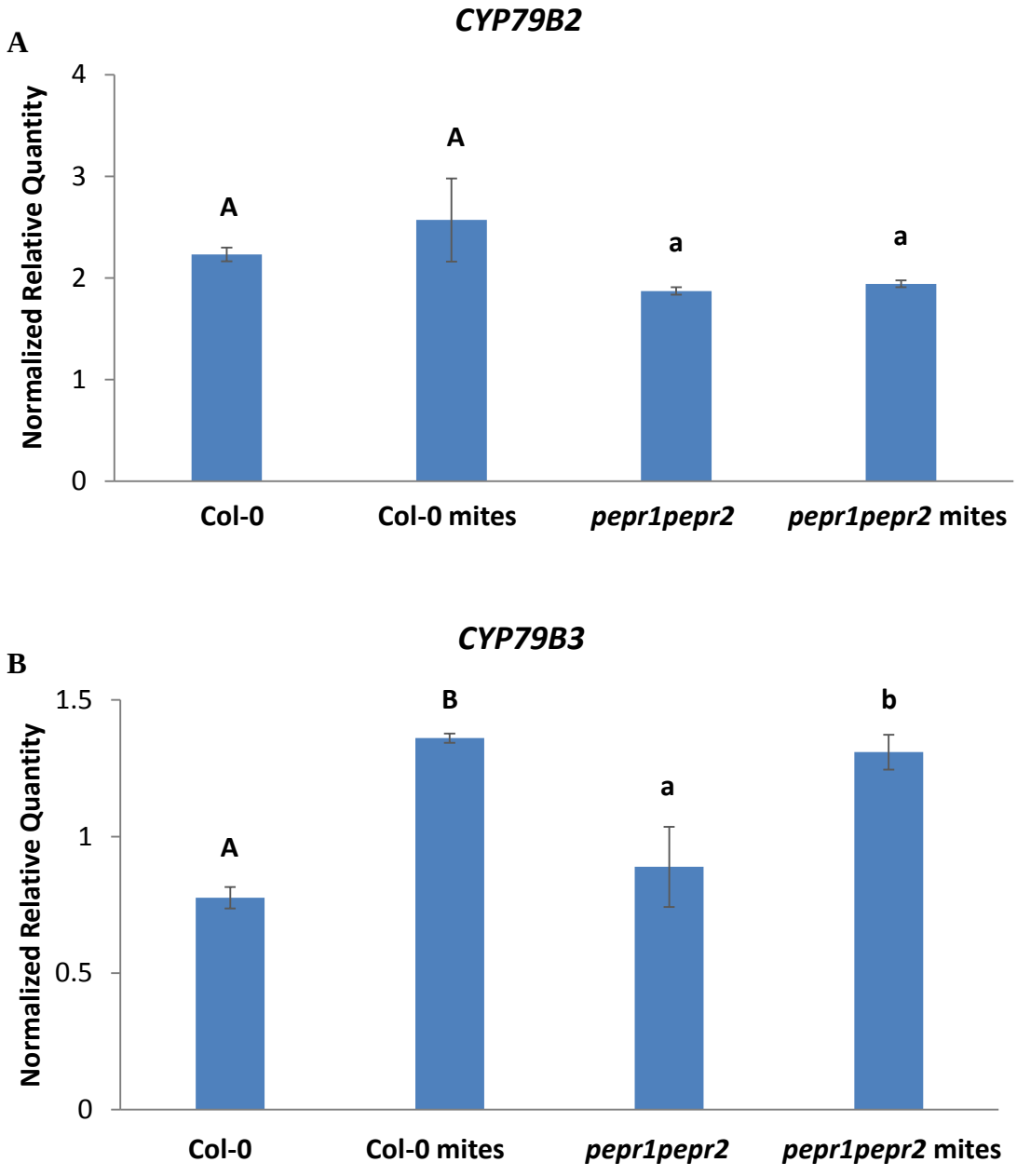

Figure 3.10 *CYP79B2* and *CYP79B3* marker gene analysis in Col-0 (WT) and *pepr1pepr2* double mutant plants. *CYP79B2* (**A**) and *CYP79B3* (**B**) gene transcript levels upon feeding of 10 spider mites for 1 h on Col-0 (WT) and *pepr1pepr2* double mutants plants. Mean fold changes detected by qRT-PCR (n = 3). Error bars are ± 1 SEM. Different letters indicate significant differences within genotype (uppercase – Col-0, lowercase – *pepr1pepr2*, Tukey HSD test, $p < 0.05$).

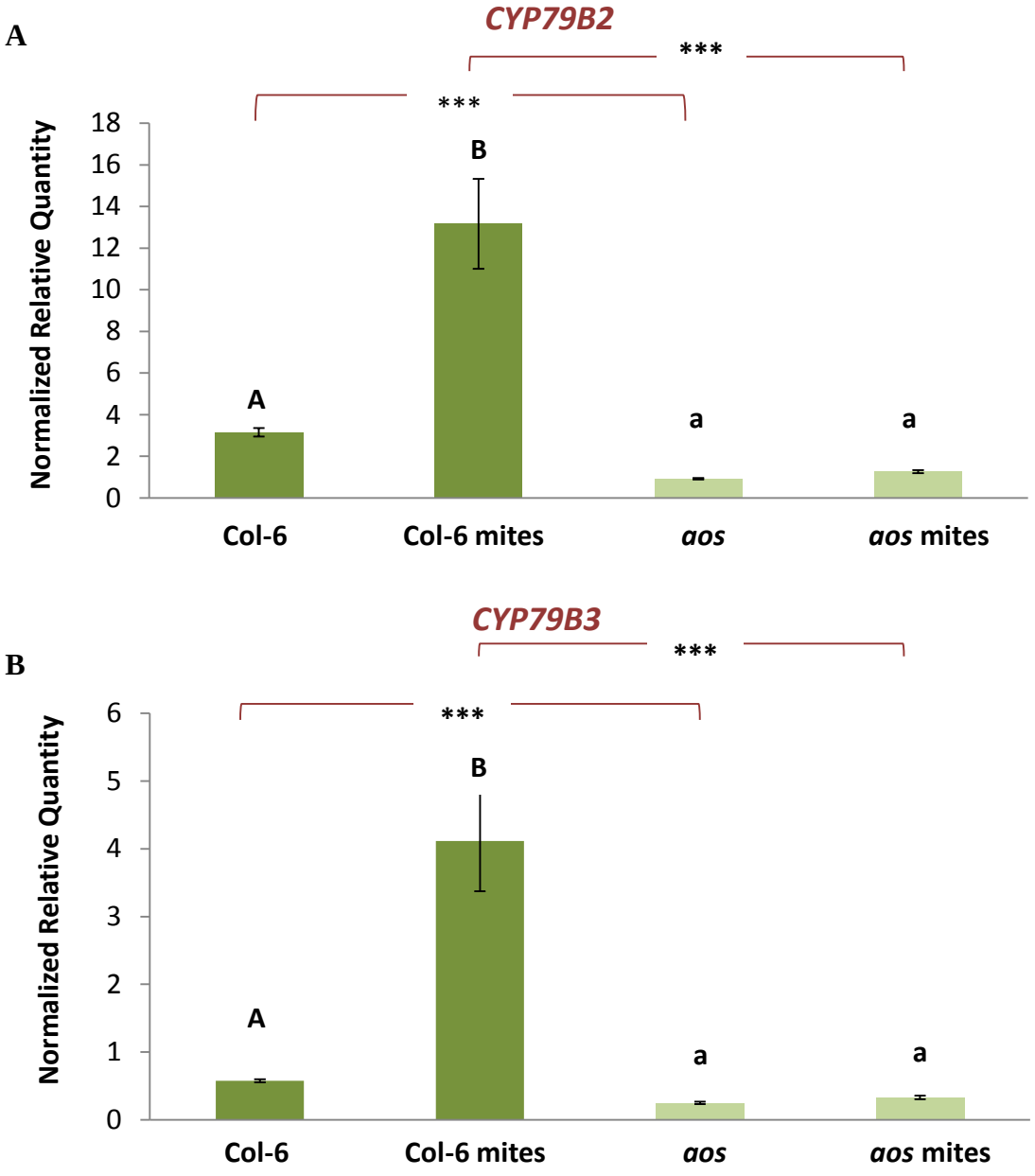

Figure 3.11 *CYP79B2* and *CYP79B3* marker gene analysis in Col-6 (WT) and *aos* mutant plants. *CYP79B2* (**A**) and *CYP79B3* (**B**) gene transcript levels upon feeding of 10 spider mites for 6 h on Col-6 (WT) and *aos* mutant plants. Mean fold changes detected by qRT-PCR (n = 4). Error bars are ± 1 SEM. Different letters indicate significant differences within genotype (uppercase – Col-6, lowercase – *aos*, Tukey HSD test, $p < 0.05$). Published in Zhurov *et al.* (2014).

Chapter Four – Discussion

With regards to the microarray data, GO terms found exclusively enriched in the feeding site sample were associated with perception signalling and transcriptional activation. GO terms specific to the 3 to 24 h response were implicated in the production of defence compound and metabolic changes through enzymatic activities involved in defence against herbivore attack (Zhurov *et al.*, 2014). Using this information, this study used physiological assays of performance on both plant and spider mite, as well as plant gene expression analysis to verify the molecular players identified in microarray data analysis.

4.1 DAMP receptors PEPR1 and PEPR2 may be two among several/many receptors involved in perception

Plant damage assays of *pepr1pepr2* double mutants revealed a moderate but significant increase in plant damage compared to Col-0 controls, where single *pepr* mutants did not display a significant increase in damage (Figure 3.1). There was no difference in spider mite larval developmental timing in any of the *pepr* mutants (Figure 3.2). However, in all 3 repetitions of the mortality assay, a trend for *pepr1pepr2* and *pepr1* with respect to larval mortality was observed (Figure 3.3). The fact that the *pepr2* single mutant always displayed the same phenotype as the wild type in all assays does not give many clues about which Pep ligand(s) is/are responsible for activity following perception of feeding, as PEPR1 recognizes all Peps (1-6) and PEPR2 only perceives Pep1 and Pep 2. Of interest, however, is the fact that PEPR2 binds to Pep1 with a higher affinity than PEPR1 (Yamaguchi *et al.*, 2010). This is interesting upon analysis of *PROPEP* transcription data, as revealed by qRT-PCR in the time course experiment. *PROPEP1* is actually repressed upon perception of spider mite herbivory during the first 6 h before raising to basal levels again (Figure 3.4C), and this is consistent with the *pepr2* phenotype similarity to Col-0 in damage assays (Figure 3.1) The kinetics of *PROPEP2* shows a trend of increased expression in the 3-6 h time frame (Figure 3.4D). *PROPEP3* expression varied considerably throughout the experiment but increased in expression towards 24 h (Fig 3.4E). *PROPEP4* shows a similar pattern of expression, increasing 4 fold over control levels by 12 h (Figure 3.4F). This may suggest possible roles for Peps 2,

3 and 4 in the amplification of a defense response, though validation of increased levels of functional Pep peptides would have to be confirmed before this hypothesis is valid. The analysis of *PEPR* transcript levels after spider mite feeding revealed no dramatic increase, which is contrary to what microarray data revealed. However, *PEPR* expression was identified as differentially expressed in the feeding site data (Zhurov *et al.*, 2014), where hundreds of mites were allowed to feed on plants for one hour as opposed to this time course experiment where only 10 spider mites were feeding and it is likely that this local response was diluted when whole plant tissue was collected for RNA isolation and subsequent qRT-PCR analysis.

There was a high degree of biological variation in physiological assays of spider mite performance, consistent with the hypothesis that PEPRs would be serving as one of several/many PRRs involved in spider mite herbivory associated HAMP and DAMP perception. Other studies thus far have focused on plant responses to *PROPEP* overexpression, where overexpression of *PROPEP1* and *PROPEP2* enhanced resistance to the root pathogen *P. irregulare* and causes constitutive expression of the defence gene defensin, *PDF1.2* (marker of the ERF branch of the JA pathway; Huffaker *et al.*, 2006). *PROPEP* genes are differentially expressed following spraying intact plants with methyl jasmonate and methyl salicylate and when excised leaves are supplied with peptides derived from the C terminus of each of the PROPEP proteins (mimicking functional Peps) through cut petiols (Huffaker and Ryan, 2007). In another species, *Zea mays* (corn), a homolog of *PROPEPs* in *A. thaliana*, *ZmPROPEP3* has been demonstrated to be effective in regulating the defence responses against the herbivore *Spodoptera exigua* (beet armyworm). *ZmPROPEP3* was rapidly induced upon application of *S. exigua* OS to scratched leaves. Microarray analysis of excised leaves treated for 12 h either water or ZmPep3 indicated that ZmPep3 stimulated the production of JA and ET, and increased expression of genes encoding proteinase inhibitors and biosynthetic enzymes for production of volatile terpenes and benoxazinoids. Exogenous application of ZmPep3 stimulated the production of JA and ET. Also, it was shown that direct and indirect defences induced by ZmPep3 contribute to reduction of larval growth of *S. exigua*, with larvae gaining considerably less biomass on ZmPep3 pre-treated leaves compared to undamaged water controls (Huffaker *et al.*, 2013). Although all of the studies mentioned

above consisted of well performed experiments, none of them were performed in such a way as to be considered reflective of a real biological interaction between plant and herbivore. Many of them involved exogenous application of a synthetic peptide/hormone or OS. Other experiments have investigated the overexpression of *PROPEP* genes.

To my knowledge, this is the first study to look at the kinetics of *PROPEP* and *PEPRs* gene transcription during the 'natural' interaction of *A. thaliana* and spider mites. Also, the plant damage and spider mite developmental and mortality assays were performed using knockout mutants of PEPRs, without any other known physiological consequences. Indeed, the fact that marker gene analysis in *pepr1pepr2* mutant revealed no difference in basal levels of *AOS, MYC2, CYP79B2*, and *CYP79B3* compared to Col-0 (WT) (Figures 3.7 and 3.10) suggests that the results of increased *pepr1pepr2* plant damage (Figure 3.1) and the trend of decreased mortality on *pepr1pepr2* (Figure 3.3) are a result of induced responses (or lack thereof) that would have been present following proper perception of Pep peptides following plant damage by spider mites.

Unfortunately, not much is known about how Peps are processed and how they end up in the apoplast to be perceived by PEPRs. The enzyme required to cleave the PROPEP precursor proteins into functional Peps remains unknown and represents another level of regulation that is not taken into account in this study. This study only examined responses at the transcriptional level and can therefore not be considered completely applicable to the functional protein level. In the context of herbivory, the proposed model for PEPR involvement in spider mite resistance would occur as a result of spillage of cytoplasmic content into the apoplast following cell damage during feeding, thus negating the need for PEPs to be actively transported intro the apoplast. In this context, upon spider mite feeding, it is possible that the cell damage occurring as a result of cell puncture from the spider-mite stylet allows processed Peps to spill into the apoplast to be perceived by PEPRs on the cell surface of adjacent, intact cells, and aid in the induction of defence genes as well as PEP precursors, thus triggering defence responses in adjacent, intact living cells (Figure 5.1).

From literature it appears that Pep ligand activity is largely specific to native plant families; however, their function as amplifiers of pathogen and herbivorous pest responses appears to be a conserved motif across diverse plant species (Huffaker *et al.*, 2013). Although results from this study are not conclusive in implicating PEPRs or any specific Peps in the resistance response to spider mites, their small but reproducible effect may be biologically relevant as it is very likely that other receptors are involved in perceiving spider mite attack through spider mite derived HAMPs and DAMPs (Figure 5.1). Indeed, given the fact that it is extremely likely that other receptors, including receptors of cell wall fragments and possibly other unidentified cytoplasmic peptides, are involved in perceiving spider mite herbivory, the results here from the loss of just two homologous receptors is noteworthy. Further research into the role of PEPRs and Peps should be explored. Although other studies using *PROPEP* overexpressing plants and direct application of synthetic Peps may not yield results that are consistent with results gathered from direct plant-herbivore interactions, they may give more clear results as to their implication in spider mite feeding responses.

4.2 Defence signalling involves MYC2 branch of JA pathway

Mutants compromised in one or more elements of the JA pathway are more susceptible to wide range of arthropod herbivores including: caterpillars (Lepidoptera), beetles (Coleoptera), thrips (Thysanoptera), leafhoppers (Homoptera), spider mites (Acari), fungal gnats (Diptera) and mired bugs (Heteroptera) (Bostock, 2005; Howe, 2004; Kessler and Baldwin, 2002). Therefore, it is of no surprise that results from this study identify JA as the key phytohormone involved in resistance signalling in response to spider mite feeding on *A. thaliana* as indicated by a severe susceptible phenotype in *aos* mutant plants (Figure 3.5). Clearly, with almost a 5-fold increase in plant damage of *aos* plants compared to Col-6 controls, when JA biosynthesis is compromised, the plant lacks a significant portion of its effective defences (Figure 3.5A). This is also evident in spider mite larvae developmental assays where larvae feeding on Col-6 WT leaves take twice as long to develop into protonymphs as they do on *aos* leaves (5.6 days compared

to 2.8 days, Figure 3.5B). Also, there is almost no larvae mortality when feeding on *aos* plants (3.6 % compared to 90% on Col-6, Figure 3.5C), further supporting the hypothesis that most of the effective defenses that *A. thaliana* employs against spider mites are JA dependant.

It is also clear from the results of this study that the MYC2 branch of the JA pathway is important in the defence signalling associated with spider mite herbivory. Similar to results of *aos* mutants, there is a ~5-fold increase in plant damage of the *myc2myc3myc4* triple mutant compared to the Col-0 WT control (Figure 3.6A), indicating that the plant is severely compromised in its ability to defend itself against spider mites. Consistent with plant damage assays, and again similar to results using *aos* mutants, mite larvae develop into protonymphs faster on *myc2myc3myc4* leaves compared to Col-0 leaves (3 days compared to 5.3 days, Figure 3.6B). A very low mortality (4.6 %) on *myc2myc3myc4* mutant (compared to 89 % on Col-0, Figure 3.6C) suggests that most effective defences against spider mites require the MYC2 branch of the JA pathway. The prioritization of the MYC2 branch over the EFR branch during spider mite herbivory is not surprising, as it has been associated with anti-herbivore defence in numerous other studies (De Vos *et al.*, 2005; Verhage *et al.*, 2011). This may also suggest that ethylene may not be present or induced at high levels following spider mite perception, as its presence would have an antagonistic effect on the MYC2 branch (Pieterse *et al.*, 2009).

Marker gene analysis in the *pepr1pepr2* double mutant suggests that the induction of signalling via the JA pathway is not dependent on perception of spider mite herbivory by DAMP receptors PEPR1 and PEPR2. Induction of both *AOS* (involved in JA biosynthesis) and *MYCs* (JA responsive TF), is the same in both Col-0 WT plants and in *pepr1pepr2* double mutant plants (Figure 3.7A and B), suggesting that JA signalling induction following spider mite feeding is not dependent of the perception of Peps by PEPRs. It is also important to note that basal levels of *AOS* and *MYC2* were not significantly different in the *pepr1pepr2* mutant, indicating that the increase in plant damage seen in the *pepr1pepr2* mutant is not due to differing constitutive defense states (Figure 3.7A and B). These results are not surprising given that multiple other

HAMP/DAMP receptors are likely involved in spider mite feeding perception and they would act redundantly in activating a defence response through JA signalling (a conserved response to perception of HAMPs and DAMPs associated with numerous herbivores, Figure 5.1).

Overall, results from this study identify JA as necessary for signalling an effective defence against spider mites and, furthermore, it is the MYC2 branch of this signalling network that is prioritized and responsible for this defence response. It has been shown previously that AP2C1 (a PP2C-Type Phosphatase) negatively regulates JA-induced herbivore defences in *A. thaliana,* where spider mites feeding on an *ap2c1* mutant showed reduced fecundity (Schweighofer *et al.*, 2007), suggesting JAs importance in the resistance to spider mites; however, to my knowledge, this is the first study to directly look at both plant and spider mite performance with respect to the JA signalling pathway and specifically the importance of the MYC2 branch. These novel findings in conjunction with literature suggest a conserved response through JA signalling by many HAMPs and DAMPs associated with herbivory from a wide range of attackers. This is favorable to the plant because HAMPs and DAMPs consist of evolutionarily conserved patterns associated with many arthropod herbivores, and presumably the plant cannot distinguish between herbivore species based on these cues alone. Therefore, having a common signalling response to many herbivores is efficient and having more than one PRR perceiving many different elicitors present during attack is prudent so as not to put all stock into the perception of just one or few of them.

4.3 JA dependent indole glucosinolates are effective secondary metabolites in the defence response to spider mite feeding in *A. thaliana*

Glucosinolates are a relatively small but diverse group of secondary metabolites, largely contained to *Brassicaceae* species. They are hydrophilic, stable metabolites that are normally sequestered in plant vacuoles. It is the loss of cell wall integrity that causes glucosinolates to come into contact with and be hydrolysed by myrosinase, which are localized in idioblasts (myrosin cells; Grubb and Abel, 2006). The biosynthesis of

primary glucosinolates (indole, aliphatic and aromatic) begins with the oxidation of precursor amino acids to aldoximes by side chain-specific cytochrome P450 monooxygenases (cytochrome P450) of the CYP79 family (Grubb and Abel, 2006). *A. thaliana* has seven CYP79s, five of which have known enzymatic functions. CYP79B2 and CYP79B3 enzymes are responsible for the production of IGs. Results from this study indicate that IGs are effective secondary metabolites against spider mites, affecting both larval development and mortality. Plant damage analysis on *cyp79b2cyp79b3* double mutant plants revealed a ~2-fold increase in plant damage relative to Col-0 controls, which suggests that IGs play a role in spider mite defence (Figure 3.8A). It should be noted that while the *cyp79b2cyp79b3* double mutant has a relatively severe plant damage phenotype, it is not as great as the damage increase seen in *aos* or *myc2myc3myc4* mutants, suggesting that while IGs might be the main contributing factor in spider mite defence, there are probably other, as yet unidentified, secondary metabolites acting against spider mites. This is corroborated in spider mite larvae development and mortality assays, where larvae feeding on *cyp79b2cyp79b3* double mutant leaves developed faster than those on Col-0 controls (3.4 days compared to 4.8 days, Figure 3.8B), which is meaningful but not as dramatic a difference as seen in JA mutants (Figures 3.5B and 3.6B). Also, larvae mortality is reduced when feeding on *cyp79b2cyp79b3* leaves (12% compared to 71%, Figure 3.8C), but is still higher than seen on JA mutants (Figures 3.5C and 3.6C). The effect of IGs on adult mite mortality has also been demonstrated, where the effect of increase mortality on adult female mites correlated with an increase in IG content within the plant (Zhurov *et al.*, 2014). Therefore, IGs are effective secondary metabolites in affecting spider mite performance; however, other secondary metabolites are probably involved as well, which makes sense from an evolutionary perspective, as relying on one metabolite for defence would quickly select for herbivores capable of overcoming its effect. This is especially true for herbivores such as spider mites, which are known for their detoxifying ability.

CYP81F2 is an enzyme required for the production of a subset of IGs (4-OH-I3M and 4-MO-13M, 4-methyl glucosinolates) that have been shown to contribute to defence against the green peach aphid (*Myzus persicae*), but not to larvae of 4 lepidopteran species (Pfalz *et al*., 2009). A mutant lacking the CYP81F2 enzyme was tested for its role

in spider mite defence. Results indicate that the IGs derived from CYP81F2 do play a role in the defence against spider mites as revealed in the plant damage assay using the *cyp81f2* mutant, which displayed a 1.5-fold increase in damage caused by mite feeing (Figure 3.9A). Mite larvae development and mortality assays show no differences in larvae feeding on *cyp81f2* leaves compared to Col-0 control, though there was a trend towards enhanced larval performance on *cyp81f2* leaves (Figure 3.9B and C). These results indicate that the subset of IGs derived from CYP81F2 activity are probably involved in the defence against spider mites, but there are other IGs involved as the data from the *cyp81f2* mutants and those from the *cyp79b2 cyp79b3* mutants were not the same. MYB28 and MYB29 TFs are regulators of aliphatic glucosinolate biosynthesis. In another experiment, the *myb28myb29* double mutant showed no difference in plant damage or mite performance assays compared to the control (Zhurov *et al*., 2014) indicating aliphatic glucosinolates have little or no effect on spider mites.

Marker gene analysis in the *pepr1pepr2* double mutant revealed no difference in *CYP79B2* or *CYP79B3* expression compared to Col-0 controls in both control plants and plants treated with 10 adult female spider mites feeding for 1 h (Figure 3.10). The lack of a substantial induction of *CYP79B2* and *CYP79B3* in Col-0 and *pepr1pepr2* is probably due to the short duration of feeding (1 h), as IG biosynthesis occurs closer to 6 h. What is important to take away from these results is that there was no difference in basal expression of *CYP79B2* or *CYP79B3* between Col-0 and *pepr1pepr2* plants. Consequently, the increase in *pepr1pepr2* plant damage cannot be attributed to differences in basal defensive states, and result from compromised perception of attack and subsequent defence program induction. Results from *CYP79* marker gene analysis in the *pepr1pepr2* mutant do not give conclusive evidence that IG induced biosynthesis is independent of Pep perception by PEPRs (lack of induction in Col-0 means we cannot compare with induction in *pepr1pepr2*). However, it is unlikely that PEPRs are required for *CYP79B2* and *CYP79B3* induction as *AOS* and *MYC2* marker gene analysis in *pepr1pepr2* mutants showed no dependence on PEPRs (Figure 3.7A and B) and *CYP79B2* and *CYP79B3* induction is dependant of a functional JA pathway (described below).

Marker gene analysis using the *aos* mutant (lacking JAs) reveal that *CYP79B2* and *CYP79B3* induction following spider mite feeding is dependent on a functional JA signalling pathway. Both *CYP79B2* and *CYP79B3* are highly induced upon spider mite feeding in Col-6 WT plants following 6 h of feeding by 10 female adult spider mites, but this induction is completely absent in *aos* plants (Figure 3.11). Also, basal levels of *CYP79B2* and *CYP79B3* expression are significantly decreased in *aos* mutants (Figure 3.11), suggesting JA is not only required for induction of expression of these genes, but their constitutive levels as well. Therefore, the severe susceptible phenotype in JA mutants may be attributed to a loss of basal levels of IGs as well as loss of IG accumulation upon feeding. Marker gene analysis of *MYB28* and *MYB29* TFs revealed a very small (perhaps biologically irrelevant) increase in induction of *MYB28* in *aos* treated plants compared to Col-0 treated plants, but no other differences were observed, corroborating results that aliphatic glucosinolates have little effect on plant resistance to spider mites (Appendix 1).

Chapter Five – Conclusion

This study shows that it is possible to use high throughput microarray data to unveil underlying global responses to a biotic stress. I successfully used microarray data gathered from susceptible and resistant *A. thaliana* accessions to investigate the entire window of herbivore-plant interaction in the aspect of induced plant resistance from perception through signalling and culminating in response. Specifically, this study identified: 1) potential DAMP receptors involved in perception of wounded self during spider mite feeding, 2) the main signalling pathway involved in initiating defence, and 3) effective secondary metabolites whose biosynthesis is induced upon spider mite feeding.

Defences regulated by JA have been described to be affective against spider mites in several plant species (Li *et al.*, 2002; Ament *et al.*, 2004; Li *et al.*, 2004; Schweighofer *et al.*, 2007; Zheng *et al.*, 2007; Zhang *et al.*, 2009), suggesting JA-dependent regulatory mechanisms responsible for spider mite induced defence programs are widely conserved across plant species (Zhurov *et al.*, 2014). However, the conservation of downstream regulated pathways that mediate plant resistance is unclear. For example, in tomato, JA-

inducible serine proteinase inhibitors can be used as reliable markers of JA-induced tomato defenses to spider mite herbivory (Ament *et al*., 2004), whereas only a handful out of the 50 annotated proteinase inhibitors in *A. thaliana* were weakly induced in response to spider mite feeding (Zhurov *et al.*, 2014). Spider mite herbivory induces the biosynthesis of glucosinolates, metabolites known to accumulate as a result of herbivory in *A. thaliana* (Zhurov *et al.*, 2014). Specifically, spider mite feeding induces the expression of IG biosynthetic genes (Figure 3.11), and accumulation of IGs (Zhurov *et al.*, 2014). However, feeding of *Spodoptera exigua* (another generalist herbivore) induces the transcription of aliphatic glucosinolate biosynthetic genes and increases accumulation of aliphatic glucosinolates (Mewis *et al*, 2005: 2006), despite common JA signalling initiation of defenses (Zhurov *et al.*, 2014; Chung *et al.*, 2008). Therefore, although conservation of JA-regulated defenses against herbivores are seen in many plant species against many different herbivores, there is also evidence for plant species and herbivore specific responses (Zhurov *et al.*, 2014).

Results from this study indicate that IGs have toxic effects on mites (Figure 3.8), whereas other studies have identified deterrent and anti-feedant properties of glucosinolates, causing reduced weight gain and fecundity of the herbivore (Kim and Jander, 2007; Kim *et al.*, 2008: Muller *et al.*, 2010). This is further supported when you look at spider mite performance on beans (host mites are reared on) compared to Col-0 (Appendix 2), where fecundity is lower for mites feeding on Col-0 plants for 7 days. The results of the spider mite performance assay in Appendix 2 is not comparable to the other mite performance assays described in this thesis as a different methodology was used. The activity of IGs against spider mites may be myrosinase dependant, and this can easily be tested using plant damage and spider mite development and mortality assays using the mutant *tgg1tgg2*, which lacks the two known myrosinase enzymes in *A. thaliana*. Comparable plant and mite performance observed in the *A.* thaliana - spider mite interaction using the *tgg1tgg2* mutant compared to *cyp79b2cyp79b3* would indicate the proportion of total activity of IGs is dependent on hydrolysis by myrosinases.

Our current understanding of *A. thaliana* responses to spider mite feeding is shown in Figure 5.1. Although the presence of DAMP receptors, such as PEPR1 and

PEPR2, suggest that *A. thaliana* is capable of perceiving the effect of herbivory (tissue damage), there are likely other receptors capable of recognising plant damage and herbivore derived elicitors. For example, cell wall fragments, like oligogalacturonides, have been shown to act as DAMPs and one would expect that they would form upon disruption of the feeding cell during spider mite herbivory. In addition, one would expect the presence of mite derived HAMPs (and their receptors), as numerous elicitors from other herbivores have been identified; for example, β-glucosidase from OS of *Pieris brassicae* (white cabbage butterfly) and inceptin from *Spodoptera frugiperda* (fall armyworm) have been shown to act as HAMPs in cabbage and cowpea plants respectively.

The receptors of various elicitors associated with mite feeding are shown as asterisks in Figure 5.1. The prediction is that cells surrounding the feeding cell will perceive these elicitors to trigger defense responses. Following the paradigm of plant-pathogen interaction, HAMPs are evolutionarily conserved molecular patterns among herbivores, implicating that the plant would not be able to identify a specific herbivore engaged in feeding. Thus, the expectation is that upon perception of various HAMPs/DAMPs, there is initiation of a conserved response. Indeed, we have identified induction of a JA-mediated defense response that is conserved across responses to many herbivores of different feeding guilds (Howe and Jander, 2007). Despite expected conservation of defense responses, there are subtle differences. This may reflect HAMP presence/absence and perhaps differences in feeding mode that can provide the plant with additional cues to modify the final defense output to be as effective as possible. In this study, the induction of IG biosynthesis upon spider mite herbivory was observed as opposed to biosynthesis of other glucosinolates that are induced by other herbivores and were shown to have little to no effect on spider mites.

Finally, robust results from this study support the use of *A. thaliana* and *T. urticae* as viable and informative model organisms for the study of plant-herbivore interaction. Future work on the interaction between *A. thaliana* and *T. urticae* should focus on identification of mite specific triggers and responses. Although the Col-0 and Col-6 wild type genotypes did behave similarly overall, there was considerable variation between

plant performance (as assayed by plant damage) when you compare genotypes from different replications of the same experiments. Mite development and mortality also varied when feeding on the same genotype in different experiments. From this I conclude that analyzing performance of two different interacting organisms will undoubtedly generate a considerable amount of biological variation; however, the same conclusions can be drawn from replicated experiments of the same comparison between genotypes.

It should be remembered that although this research focused on the response of one plant species to one arthropod herbivore, in reality, most interactions in the field are composed of simultaneous attack by other herbivores and/or pathogens as well as abiotic stressors, each of which may induce a semi-specific response (Kessler and Baldwin, 2002). Future work on herbivore-plant interaction in general should include assaying plant responses to multiple attackers at various times in the plants' life history. This will be difficult due to the large amount of crosstalk between pathways and responses, allowing plants to modulate their response depending on biotic and abiotic factors, but this is what will be required for a complete understanding of plant response to herbivores.

The identification of IGs as effective secondary metabolites against spider mites provides an opportunity to utilize them against spider mites and other herbivores in the context of agriculture. This study revealed that IGs as a family have toxic effects on spider mites; however, before IGs can be considered a viable pesticide, the individual IG(s) responsible for the toxic effect on spider mites must first be identified. This can be done using spider mite artificial diets complimented with one or a known combination of individual IGs and analysing their effect on spider mite development and mortality. Using IGs as a pesticide against spider mites (and potentially other herbivorous pests that are susceptible to IGs) is promising; however, it must be noted that spider mites are well known for their ability to develop resistance to pesticides through detoxification mechanisms. Therefore, for IG-derived pesticides to be effective long-term, an integrated pest management (IPM) approach must be used. IPM combines pest management practices including host plant resistance, biological control (use of natural pest predators), cultural control, and other methods (Smith and Clement, 2012). Importantly, IG-derived pesticides should not be used in isolation or for many growing seasons continuously

without use of other pest control practices due to the high probability of spider mites developing resistance to the IG-derived pesticides as they would provide a strong selective pressure against spider mites using a mode of action spider mites are known to overcome given time.

This study provides support for the use of non-synthetic, IG-derived pesticides as a control against spider mites in agriculture; however, further research and development of this potential, through promising, pesticide must be performed before it can be considered commercially and environmentally viable.

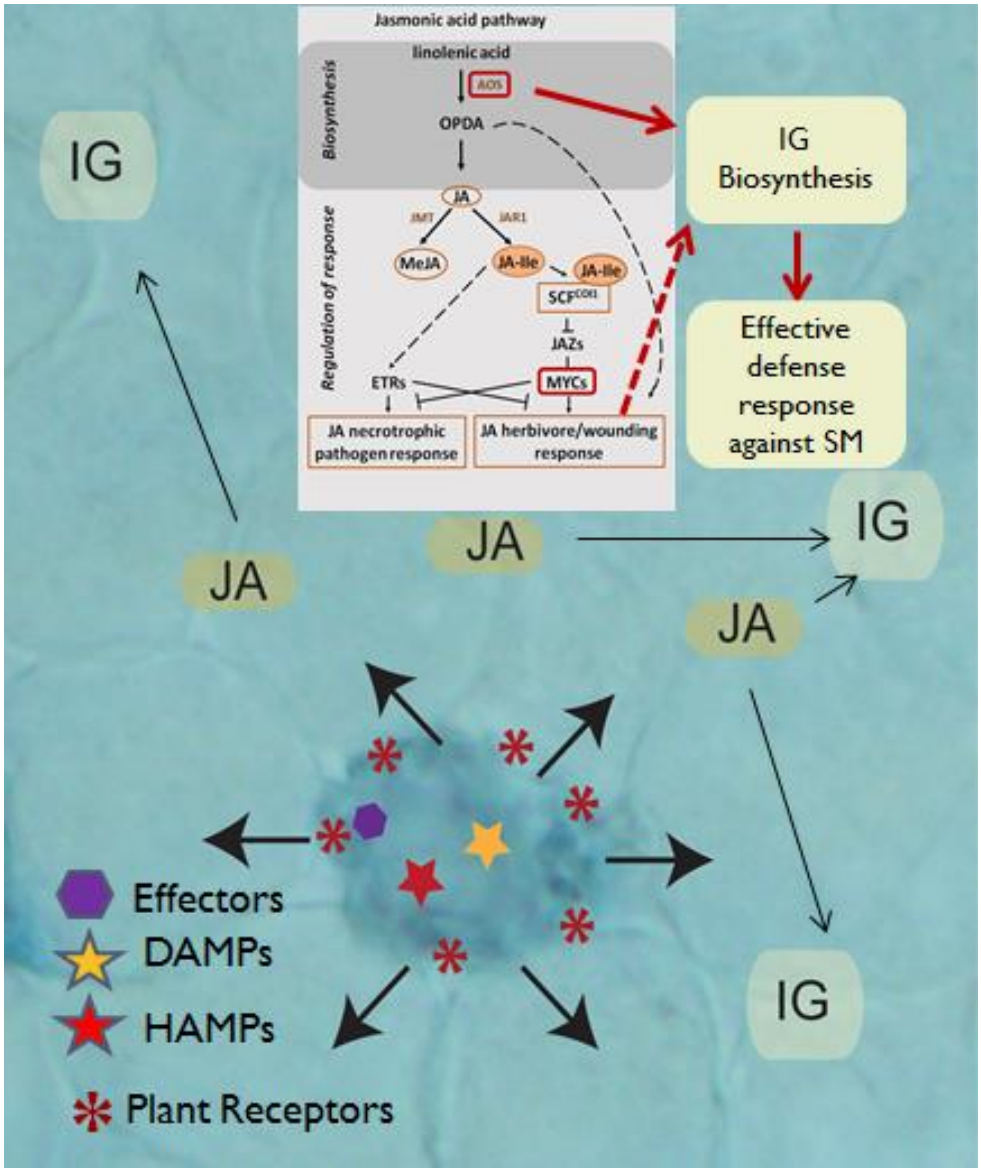

Figure 5.1 Predicted model of *A. thaliana* response to spider mite herbivory. Perception occurs via recognition of HAMPs (★) likely present in spider mite OS and DAMPs such s Pep peptides (☆) as well as other unknown endogenous and exogenous elicitors by PRRs on adjacent intact cells (*). It is also probable that the spider mite can inject effectors (⬣) that could be effective in suppressing plant defences of some species. This leads to the initiation a signalling response through the MYC2 branch of the JA pathway and production of IGs following local perception of feeding.

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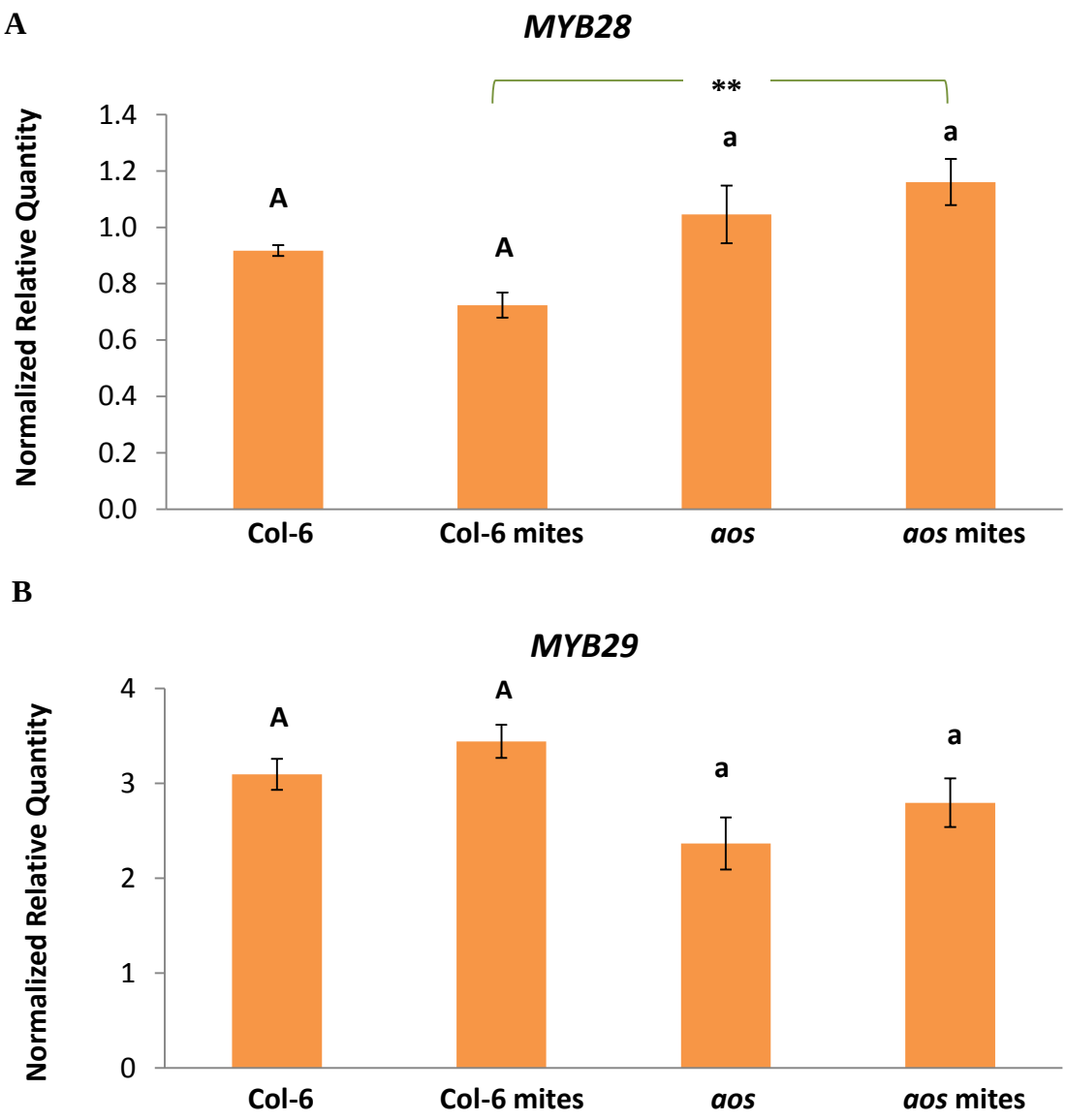
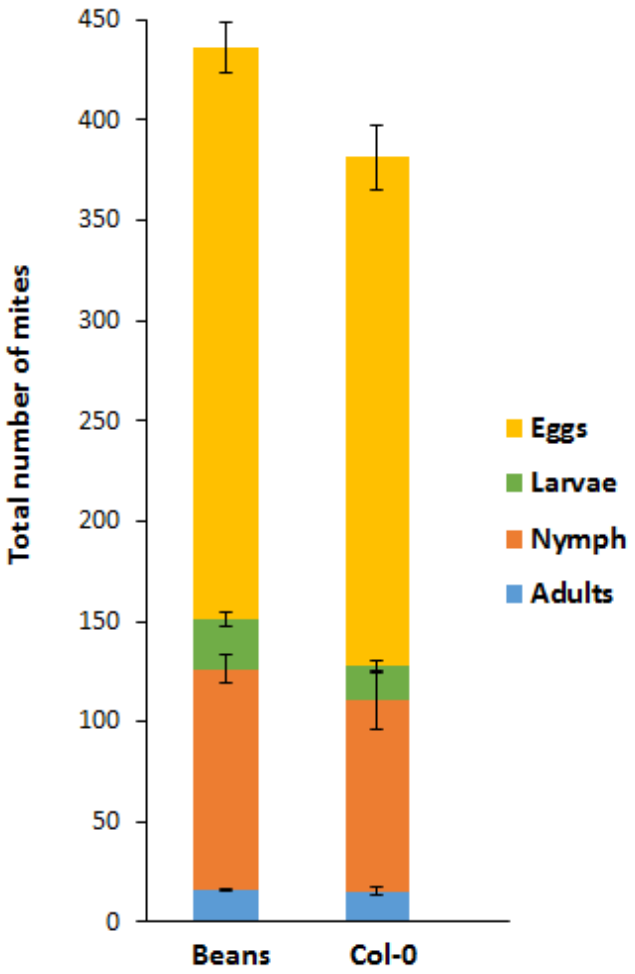
Appendices



Appendix 1 *MYB28* and *MYB29* marker gene analysis in Col-6 (WT) and *aos* mutant plants. *MYB28* (**A**) and *MYB29* (**B**) gene transcript levels upon feeding of 10 spider mites for 6 h on Col-6 (WT) and *aos* mutant plants. Mean fold changes detected by qRT-PCR (n = 4). Error bars are ± 1 SEM. Different letters indicate significant differences within genotype (uppercase – Col-6, lowercase – *aos*, Tukey HSD test, $p < 0.01$). Published in Zhurov *et al.* (2014).



Appendix 2 Spider mite performance on beans (host used to rear spider mite colony) and on Col-0, WT genotype used in many experiments in this study. One experimental replication shown. Total number of spider mites at all developmental stages were counted after 7 days following transfer of 20 adult female mites (synchronized in terms of development and age) onto beans or Col-0 plants. Experiment was performed by Huzefa Ratlamwala.

T-tests

t-test: Two-Sample Unequal Varianace Assumed	Fiugre 1.1B		Figure 1.1B	
Non-equal sample sizes	**Developmental Assay**		**Mortality Assay**	
	Bla-2	**Kon**	**Bla-2**	**Kon**
Mean	4.827532	3.753516	58.68839	14.48177
Variance	0.031517	0.0597871	70.72799	35.73651
n	4	5	4	5
Hypothesied Mean Difference	0		0	
df	6.972		5.27	
t Stat	7.6256		8.8717	
p -value	0.0001262		0.0002305	

t-test: Two-Sample Unequal Varianace Assumed	Figure 3.5A		Figure 3.5B		Figure 3.5C	
Non-equal sample sizes	**Plant Damage Assay**		**Developmental Assay**		**Mortality Assay**	
	Col-6	***aos***	**Col-6**	***aos***	**Col-6**	***aos***
Mean	7.472222	38.472222	5.58	2.813159	89.698003	3.564815
Variance	1.529405	82.286241	0.557	0.03072092	29.95596	11.93844
n	10	9	5	5	5	5
Hypothesied Mean Difference	0		0		0	
df	8.297		4.44		6.751	
t Stat	10.1583		-8.0702		-29.7562	
p -value	0.0000058		0.0008145		0.00000002	

t-test: Two-Sample Unequal Varianace Assumed	Figure 3.6A		Figure 3.6B		Figure 3.6C	
Non-equal sample sizes	**Plant Damage Assay**		**Developmental Assay**		**Mortality Assay**	
	Col-0	***myc2/3/4***	**Col-0**	***myc2/3/4***	**Col-0**	***myc2/3/4***
Mean	7.765625	41.625	5.28	2.960258	88.670001	4.565957
Variance	1.735884	79.948864	2.567	0.06478067	11.07915	55.18762
n	12	12	5	5	5	5
Hypothesied Mean Difference	0	0	0		0	
df	11.477		4.202		5.544	
t Stat	-12.9777		3.1974		23.1022	
p -value	0.0000000		0.0307600		0.00000100	

t-test: Two-Sample Unequal Varianace Assumed	Figure 3.8A		Figure 3.8B		Figure 3.8C	
Non-equal sample sizes	**Plant Damage Assay**		**Developmental Assay**		**Mortality Assay**	
	Col-0	***cyp79b2/b3***	**Col-0**	***cyp79b2/b3***	**Col-0**	***cyp79b2/b3***
Mean	4.40625	9.633929	4.75619	3.352155	71	12.4
Variance	0.4934896	2.4237351	0.7876916	0.1141681	153.5	120.3
n	4	7	5	5	5	5
Hypothesied Mean Difference	0	0	0		0	
df	8.802		5.136		7.884	
t Stat	-7.6284		3.3059		7.9189	
p -value	0.00003652		0.02050000		0.00005097	

t-test: Two-Sample Unequal Varianace Assumed	Figure 3.9A		Figure 3.9B		Figure 3.9C	
Non-equal sample sizes	**Plant Damage Assay**		**Developmental Assay**		**Mortality Assay**	
	Col-0	***cyp81f2***	**Col-0**	***cyp81f2***	**Col-0**	***cyp81f2***
Mean	10.7885	17.08088	4.75619	4.114524	71	59.73506
Variance	3.15366	20.2367	0.7876916	0.2659059	153.5	474.4354
n	13	17	5	5	5	5
Hypothesied Mean Difference	0	0	0		0	
df	21.969		6.424		6.343	
t Stat	-5.2565		1.3978		1.0052	
p -value	0.00002850		0.20850000		0.35160000	

Appendix 3 Summary tables of *t*-tests for plant damage, and spider mite larval development and mortality assays.

ANOVA and Tukey's HSD tests

one-way ANOVA Plant damage assay: Col-0 vs *peprs* (Figure 3.1)			
	Df	F	*P*
Genotype	3	6.915	0.0006130

Tukey HSD test Plant damage assay Relevant contrasts: Col-0 vs peprs (Figure 3.1)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	3.9186150	0.0704925	7.7667380	0.0445075
Col-0 - *pepr1*	-2.4274840	-6.3549580	1.4999900	0.3628829
Col-0 - *pepr2*	-1.0212340	-4.9487080	2.9062400	0.8992130
pepr1pepr2 - *pepr1*	6.3460990	2.4186254	10.2735730	0.0004850
pepr1pepr2 - *pepr2*	-4.9398490	-8.8673233	-1.0123750	0.0084509
pepr1 - *pepr2*	1.4062500	-2.5990033	5.4115030	0.7857947

one-way ANOVA Plant damage assay: Col-0 vs *peprs* (Appendix 1A)			
	Df	F	*P*
Genotype	3	2.766	0.0521000

Tukey HSD test Plant damage assay Relevant contrasts: Col-0 vs peprs (Appendix 1A)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	3.5576923	-0.2636247	7.3790093	0.0763467
Col-0 - *pepr1*	0.2736378	-3.6264775	4.1737531	0.9976520
Col-0 - *pepr2*	0.2788462	-3.5424708	4.1001631	0.9973614
pepr1pepr2 - *pepr1*	3.2840545	-0.6160608	7.1841698	0.1267252
pepr1pepr2 - *pepr2*	-3.2788462	-7.1001631	0.5424708	0.1160834
pepr1 - *pepr2*	0.0052083	-3.8949070	3.9053236	1.0000000

one-way ANOVA Plant damage assay: Col-0 vs *peprs* (Appendix 1B)			
	Df	F	*P*
Genotype	3	4.362	0.0098000

Tukey HSD test Plant damage assay Relevant contrasts: Col-0 vs *peprs* (Appendix 1B)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	1.8821678	0.1151575	3.6491781	0.0330509
Col-0 - *pepr1*	1.2086538	-0.4923998	2.9097075	0.2414829
Col-0 - *pepr2*	-0.2144231	-2.1327253	1.7038792	0.9904374
pepr1pepr2 - *pepr1*	0.6735140	-0.9832604	2.3302884	0.6964286
pepr1pepr2 - *pepr2*	-2.0965909	-3.9757400	-0.2174418	0.0236772
pepr1 - *pepr2*	-1.4230769	-3.2403440	0.3941901	0.1702930

Appendix 4 Summary tables for ANOVA and relevant Tukey HSD comparisons for *pepr* mutant plant damage assays.

ANOVA and Tukey's HSD tests

one-way ANOVA Larvae developmental assay: Col-0 vs peprs (Figure 3.2)			
	Df	F	*P*
Genotype	3	0.473	0.7060000

Tukey HSD test Larvae developmental assay Relevant contrasts: Col-0 vs peprs (Figure 3.2)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	-0.1563127	-0.8957350	0.5831096	0.9257784
Col-0 - *pepr1*	-0.1471210	-0.7874795	0.4932375	0.9074623
Col-0 - *pepr2*	-0.2610657	-0.9014242	0.3792928	0.6456380
pepr1pepr2 - pepr1	-0.0091917	-0.7486140	0.7302306	0.9999822
pepr1pepr2 - pepr2	-0.1047531	-0.8441754	0.6346693	0.9755427
pepr1 - pepr2	-0.1139447	-0.7543032	0.5264138	0.9535802

one-way ANOVA Larvae developmental assay: Col-0 vs *peprs* (Appedix 2A)			
	Df	F	*P*
Genotype	3	0.631	0.6070000

Tukey HSD test Larvae developmental assay Relevant contrasts: Col-0 vs pepr (Appedix 2A)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	-0.0429238	-1.9226300	1.8367820	0.9998897
Col-0 - *pepr1*	-0.5709524	-2.4506580	1.3087540	0.8136851
Col-0 - *pepr2*	-0.6338095	-2.5135160	1.2458960	0.7628105
pepr1pepr2 - pepr1	0.5280286	-1.0998450	2.1559020	0.7828035
pepr1pepr2 - pepr2	-0.5908857	-2.2187590	1.0369870	0.7210810
pepr1 - pepr2	-0.0628571	-1.6907300	1.5650160	0.9994687

one-way ANOVA Larvae developmental assay: Col-0 vs *peprs* (Appendix 2B)			
	Df	F	*P*
Genotype	3	0.473	0.7060000

Tukey HSD test Larvae developmental assay Relevant contrasts: Col-0 vs *peprs* (Appendix 2B)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	0.4666303	-0.0962788	1.0295394	0.1232211
Col-0 - *pepr1*	-0.0654197	-0.6283288	0.4974894	0.9868470
Col-0 - *pepr2*	0.2433333	-0.3195758	0.8062424	0.6136758
pepr1pepr2 - pepr1	0.5320499	-0.0308592	1.0949590	0.0673207
pepr1pepr2 - pepr2	-0.2232969	-0.7862060	0.3396122	0.6741453
pepr1 - pepr2	0.3087530	-0.2541561	0.8716621	0.4224889

Appendix 5 Summary tables for ANOVA and relevant Tukey HSD comparisons for spider mite larvae developmental assays on *pepr* mutants.

ANOVA and Tukey's HSD tests

one-way ANOVA Larvae mortality Assay: Col-0 vs peprs (Figure 3.3)			
	Df	F	*P*
Genotype	3	1.654	0.2340000

Tukey HSD test Larvae mortality Assay Relevant contrasts: Col-0 vs peprs (Figure 3.3)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	-13.3309446	-35.9606900	9.2987980	0.3354115
Col-0 - *pepr1*	-6.1893964	-26.4300500	14.0512610	0.7948090
Col-0 - *pepr2*	-13.6835965	-34.8517800	7.4845900	0.2651983
pepr1pepr2 - pepr1	-7.1415481	-27.3822100	13.0991090	0.7183772
pepr1pepr2 - pepr2	-0.3526519	-21.5208400	20.8155340	0.9999518
pepr1 - pepr2	-7.4942001	-26.0864300	11.0980310	0.6317390

one-way ANOVA Larvae mortality Assay: Col-0 vs *peprs* (Appendix 3A)			
	Df	F	*P*
Genotype	3	0.82	0.5060000

Tukey HSD test Larvae mortality Assay Relevant contrasts: Col-0 vs pepr (Appendix 3A)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	-12.4117440	-38.8378300	14.0143500	0.5332535
Col-0 - *pepr1*	-2.4804930	-30.1175600	25.1565700	0.9932807
Col-0 - *pepr2*	-4.2491720	-30.6752600	22.1769200	0.9639412
pepr1pepr2 - pepr1	-9.9312510	-34.2051600	14.3426600	0.6370025
pepr1pepr2 - pepr2	8.1625720	-14.7230900	31.0482400	0.7260102
pepr1 - pepr2	-1.7686790	-26.0425900	22.5052300	0.9963658

one-way ANOVA Larvae mortality Assay: Col-0 vs peprs Appendix 3B)			
	Df	F	*P*
Genotype	3	5.522	0.0103000

Tukey HSD test Larvae mortality Assay Relevant contrasts: Col-0 vs peprs (Appendix 3B)				
	diff	CI, lwr	CI, upr	adj, P
Col-0 - *pepr1pepr2*	-8.3193740	-28.1604000	11.5216519	0.6257169
Col-0 - *pepr1*	-20.7319240	-40.5729500	-0.8908985	0.0393078
Col-0 - *pepr2*	4.9151540	-15.9991230	25.8294319	0.9018149
pepr1pepr2 - pepr1	12.4125500	-6.2937480	31.1188490	0.2606569
pepr1pepr2 - pepr2	13.2345280	-6.6064980	33.0755541	0.2567763
pepr1 - pepr2	25.6470790	5.8060530	45.4881045	0.0101100

Appendix 6 Summary tables for ANOVA and relevant Tukey HSD comparisons for spider mite larvae mortality assays on *pepr* mutants.

ANOVA and Tukey's HSD tests

one-way ANOVA PEPR1 Kinetics: Col-0 (Figure 3.4A)			
	Df	F	*P*
Time point	5	2.676	0.0754000

Tukey HSD test PEPR1 Kinetics: Col-0 (Figure 3.4A) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	-0.2833333	-1.1140833	0.5474166	0.8532425
24h Control - 3h	-0.1766667	-1.0074166	0.6540833	0.9764324
24h Control - 6h	-0.4700000	-1.3007500	0.3607500	0.4461286
24h Control - 12h	-0.2166667	-1.0474166	0.6140833	0.9453354
24h Control - 24h	-0.1733333	-1.0040833	0.6574166	0.9782795
1h - 3h	-0.4600000	-1.2907500	0.3707500	0.4675577
1h - 6h	-0.7533333	-1.5840833	0.0774166	0.0840133
1h - 12h	0.0666667	-0.7640833	0.8974166	0.9997503
1h = 24h	-0.1100000	-0.9407500	0.7207500	0.9972257
3h - 6h	-0.2933333	-1.1240833	0.5374166	0.8352806
3h - 12h	-0.3933333	-1.2240833	0.4374166	0.6189946
3h - 24h	-0.3500000	-1.1807500	0.4807500	0.7184015
6h - 12h	-0.6866667	-1.5174166	0.1440833	0.1296891
6h - 24h	-0.6433333	-1.4740833	0.1874166	0.1703301
12h - 24h	-0.0433333	-0.8740833	0.7874166	0.9999701

one-way ANOVA PEPR2 Kinetics: Col-0 (Figure 3.4B)			
	Df	F	*P*
Time point	5	4.604	0.0141000

Tukey HSD test PEPR2 Kinetics: Col-0 (Figure 3.4B) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	-0.6766667	-1.8284459	0.4751126	0.4086997
24h Control - 3h	0.8733333	-0.2784459	2.0251126	0.1849739
24h Control - 6h	0.7033333	-0.4484459	1.8551126	0.3708561
24h Control - 12h	-1.2133333	-2.3651125	-0.0615541	0.0369779
24h Control - 24h	-1.5200000	-2.6717792	-0.3682208	0.0082044
1h - 3h	0.1966667	-0.9551125	1.3484459	0.9910298
1h - 6h	0.0266667	-1.1251125	1.1784459	0.9999995
1h - 12h	-0.5366667	-1.6884459	0.6151126	0.6335203
1h = 24h	0.8433333	-0.3084459	1.9951126	0.2108152
3h - 6h	-0.1700000	-1.3217792	0.9817792	0.9953948
3h - 12h	-0.3400000	-1.4917792	0.8117792	0.9120770
3h - 24h	-0.6466667	-1.7984459	0.5051126	0.4537357
6h - 12h	-0.5100000	-1.6617792	0.6417792	0.6779639
6h - 24h	-0.8166667	-1.9684459	0.3351126	0.2362042
12h - 24h	0.3066667	-0.8451125	1.4584459	0.9407017

one-way ANOVA PROPEP1 Kinetics: Col-0 (Figure 3.4C)			
	Df	F	*P*
Time point	5	146.6	0.0000000

Tukey HSD test PROPEP1 Kinetics: Col-0 (Figure 3.4C) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	3.2800000	2.6046487	3.9553513	0.0000000
24h Control - 3h	-3.4266667	-4.1020180	-2.7513153	0.0000000
24h Control - 6h	-3.5200000	-4.1953513	-2.8446487	0.0000000
24h Control - 12h	0.3933333	-0.2820180	1.0686847	0.4172948
24h Control - 24h	0.4533333	-0.2220180	1.1286847	0.2827570
1h - 3h	-0.1466667	-0.8220180	0.5286847	0.9742284
1h - 6h	-0.2400000	-0.9153513	0.4353513	0.8317778
1h - 12h	-2.8866667	-3.5620180	-2.2113153	0.0000001
1h = 24h	2.8266667	2.1513153	3.5020180	0.0000001
3h - 6h	-0.0933333	-0.7686847	0.5820180	0.9966094
3h - 12h	-3.0333333	-3.7086847	-2.3579820	0.0000000
3h - 24h	-2.9733333	-3.6486847	-2.2979820	0.0000001
6h - 12h	-3.1266667	-3.8020180	-2.4513153	0.0000000
6h - 24h	-3.0666667	-3.7420180	-2.3913153	0.0000000
12h - 24h	-0.0600000	-0.7353513	0.6153513	0.9995900

one-way ANOVA PROPEP2 Kinetics: Col-0 (Figure 3.4D)			
	Df	F	*P*
Time point	5	2.148	0.1290000

Tukey HSD test PROPEP2 Kinetics: Col-0 (Figure 3.4D) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	-0.9300000	-4.8287873	2.9687870	0.9618615
24h Control - 3h	2.8633333	-1.0354539	6.7621210	0.2085065
24h Control - 6h	2.9900000	-0.9087873	6.8887870	0.1769972
24h Control - 12h	-0.7566667	-4.6554539	3.1421210	0.9841370
24h Control - 24h	-1.3266667	-5.2254539	2.5721210	0.8543734
1h - 3h	1.9333333	-1.9654539	5.8321210	0.5757627
1h - 6h	2.0600000	-1.8387873	5.9587870	0.5140822
1h - 12h	0.1733333	-3.7254539	4.0721210	0.9999864
1h = 24h	0.3966667	-3.5021206	4.2954540	0.9992096
3h - 6h	0.1266667	-3.7721206	4.0254540	0.9999972
3h - 12h	2.1066667	-1.7921206	6.0054540	0.4918958
3h - 24h	1.5366667	-2.3621206	5.4354540	0.7675357
6h - 12h	2.2333333	-1.6654539	6.1321210	0.4337633
6h - 24h	1.6633333	-2.2354539	5.5621210	0.7084889
12h - 24h	0.5700000	-3.3287873	4.4687870	0.9955936

one-way ANOVA PROPEP3 Kinetics: Col-0 (Figure 3.4E)			
	Df	F	*P*
Time point	5	7.029	0.0027600

Tukey HSD test PROPEP3 Kinetics: Col-0 (Figure 3.4E) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	0.4433333	-1.0418826	1.9285493	0.9083828
24h Control - 3h	1.2966667	-0.1885493	2.7818826	0.1010345
24h Control - 6h	0.5266667	-0.9585493	2.0118826	0.8329580
24h Control - 12h	-0.6266667	-2.1118826	0.8585493	0.7172317
24h Control - 24h	-1.8266667	-3.3118826	-0.3414507	0.0135941
1h - 3h	1.7400000	0.2547840	3.2252160	0.0189181
1h - 6h	0.9700000	-0.5152160	2.4552160	0.3074158
1h - 12h	-1.0700000	-2.5552160	0.4152160	0.2234273
1h = 24h	2.2700000	0.7847840	3.7552160	0.0026095
3h - 6h	-0.7700000	-2.2552160	0.7152160	0.5327321
3h - 12h	0.6700000	-0.8152160	2.1552160	0.6620642
3h - 24h	-0.5300000	-2.0152160	0.9552160	0.8294827
6h - 12h	-0.1000000	-1.5852160	1.3852160	0.9998944
6h - 24h	-1.3000000	-2.7852160	0.1852160	0.0998105
12h - 24h	1.2000000	-0.2852160	2.6852160	0.1430731

one-way ANOVA PROPEP4 Kinetics: Col-0 (Figure 3.4F)			
	Df	F	*P*
Time point	5	22.39	0.0000106

Tukey HSD test PROPEP4 Kinetics: Col-0 (Figure 3.4F) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	0.8733333	-0.1984847	1.9451514	0.1380060
24h Control - 3h	0.5500000	-0.5218180	1.6218180	0.5427626
24h Control - 6h	0.7066667	-0.3651514	1.7784847	0.2988083
24h Control - 12h	-1.9433333	-3.0151514	-0.8715153	0.0005957
24h Control - 24h	-1.7800000	-2.8518180	-0.7081820	0.0012960
1h - 3h	1.4233333	0.3515153	2.4951514	0.0078341
1h - 6h	1.5800000	0.5081820	2.6518180	0.0034990
1h - 12h	-2.8166667	-3.8884847	-1.7448486	0.0000158
1h = 24h	2.6533333	1.5815153	3.7251514	0.0000292
3h - 6h	0.1566667	-0.9151514	1.2284847	0.9955978
3h - 12h	-1.3933333	-2.4651514	-0.3215153	0.0091624
3h - 24h	-1.2300000	-2.3018180	-0.1581820	0.0216743
6h - 12h	-1.2366667	-2.3084847	-0.1648486	0.0209223
6h - 24h	-1.0733333	-2.1451514	-0.0015153	0.0496039
12h - 24h	-0.1633333	-1.2351514	0.9084847	0.9946624

one-way ANOVA PROPEP5 Kinetics: Col-0 (Figure 3.4G)			
	Df	F	*P*
Time point	5	0.585	0.7110000

Tukey HSD test PROPEP5 Kinetics: Col-0 (Figure 3.4G) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	0.1733333	-1.4379120	1.7845780	0.9989663
24h Control - 3h	0.4266667	-1.1845780	2.0379120	0.9419578
24h Control - 6h	-0.1566667	-1.7679120	1.4545780	0.9993649
24h Control - 12h	0.3033333	-1.3079120	1.9145780	0.9861256
24h Control - 24h	-0.1000000	-1.7112450	1.5112450	0.9999293
1h - 3h	0.6000000	-1.0112450	2.2112450	0.8045953
1h - 6h	0.0166667	-1.5945780	1.6279120	1.0000000
1h - 12h	0.1300000	-1.4812450	1.7412450	0.9997437
1h = 24h	0.2733333	-1.3379120	1.8845780	0.9912901
3h - 6h	-0.5833333	-2.1945780	1.0279120	0.8213287
3h - 12h	0.7300000	-0.8812450	2.3412450	0.6583181
3h - 24h	0.3266667	-1.2845780	1.9379120	0.9808104
6h - 12h	0.1466667	-1.4645780	1.7579120	0.9995387
6h - 24h	-0.2566667	-1.8679120	1.3545780	0.9934574
12h - 24h	0.4033333	-1.2079120	2.0145780	0.9535941

one-way ANOVA PROPEP6 Kinetics: Col-0 (Figure 3.4H)			
	Df	F	*P*
Time point	5	0.822	0.5570000

Tukey HSD test PROPEP6 Kinetics: Col-0 (Figure 3.4H) Relevant contrasts				
	diff	CI, lwr	CI, upr	adj, P
24h Control - 1h	0.4133333	-1.0766158	1.9032825	0.9304639
24h Control - 3h	0.3166667	-1.1732825	1.8066158	0.9764911
24h Control - 6h	-0.2233333	-1.7132825	1.2666158	0.9950540
24h Control - 12h	0.4100000	-1.0799492	1.8999492	0.9325967
24h Control - 24h	-0.0166667	-1.5066158	1.4732825	1.0000000
1h - 3h	0.7300000	-0.7599492	2.2199492	0.5871768
1h - 6h	0.1900000	-1.2999492	1.6799492	0.9976763
1h - 12h	-0.0033333	-1.4932825	1.4866158	1.0000000
1h = 24h	0.4300000	-1.0599492	1.9199492	0.9191938
3h - 6h	-0.5400000	-2.0299492	0.9499492	0.8207098
3h - 12h	0.7266667	-0.7632825	2.2166158	0.5914892
3h - 24h	0.3000000	-1.1899492	1.7899492	0.9813756
6h - 12h	0.1866667	-1.3032825	1.6766158	0.9978628
6h - 24h	-0.2400000	-1.7299492	1.2499492	0.9931161
12h - 24h	0.4266667	-1.0632825	1.9166158	0.9215286

Appendix 7 Summary tables for ANOVA and relevant Tukey HSD comparisons for *PEPR* and *PROPEP* gene expression kinetics analysis in Col-0 at 1 h, 3 h, 6 h, 12 h, 24 h samples treated with 10 female adult spider mites.

ANOVA and Tukey's HSD tests

two-way ANOVA Marker gene analysis in Col-0 & *pepr1/pepr2* : AOS (Figure 3.7A)			
	Df	F	*P*
Genotype	1	0.008	0.9314220
Treatment	1	26.895	0.0008370
Genotype x Treatment	1	0.323	0.5854040

Tukey HSD test: Marker gene analysis in Col-0 & pepr1/pepr2: AOS (Figure 3.7A) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-0:Control - Col-0:Treated	0.8666667	0.0166850	1.7166483	0.0457718
pepr1pepr2 :Control - *pepr1pepr2:* Treated	1.0800000	0.2300183	1.9299817	0.0151867
Col-0:Control - *pepr1pepr2* :Control	-0.0900000	-0.9399817	0.7599817	0.9855985
Col-0:Treated - *pepr1pepr2* :Treated	0.1233333	-0.7266484	0.9733150	0.9647523

two-way ANOVA Marker gene analysis in Col-0 & *pepr1/pepr2:* MYC2 (Figure 3.7B)			
	Df	F	*P*
Genotype	1	4.557	0.0653000
Treatment	1	47.03	0.0001300
Genotype x Treatment	1	2.701	0.1388800

Tukey HSD test: Marker gene analysis in Col-0 & pepr1/pepr2: MYC2 (Figure 3.7B) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-0:Control - Col-0:Treated	2.2500000	1.0514093	3.4485910	0.0014361
pepr1pepr2 :Control - *pepr1pepr2:* Treated	1.3800000	0.1814093	2.5785910	0.0254676
Col-0:Control - *pepr1pepr2* :Control	1.0000000	-0.1985907	2.1985910	0.1056981
Col-0:Treated - *pepr1pepr2* :Treated	0.1300000	-1.0685907	1.3285910	0.9845656

Appendix 8 Summary tables for ANOVA and relevant Tukey HSD comparisons for *AOS* and *MYC2* marker gene analysis in Col-0 and *pepr1pepr2* following 1 h of feeding by 10 female adult mites.

ANOVA and Tukey's HSD tests

two-way ANOVA Marker gene analysis in Col-0 & *pepr1/pepr2:* CYP79B2 (Figure 3.10A)			
	Df	F	*P*
Genotype	1	6.97	0.0297000
Treatment	1	0.895	0.3719000
Genotype x Treatment	1	0.244	0.6345000

Tukey HSD test: Marker gene analysis in Col-0 & pepr1/pepr2: CYP79B2 (Figure 3.10A) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-0:Control - Col-0:Treated	0.1700000	-0.3646319	0.7046319	0.7440728
pepr1pepr2 :Control - *pepr1pepr2:* Treated	0.0533333	-0.4812986	0.5879652	0.9878783
Col-0:Control - *pepr1pepr2* :Control	-0.2533333	-0.7879652	0.2812986	0.4711075
Col-0:Treated - *pepr1pepr2* :Treated	-0.3700000	-0.9046319	0.1646319	0.1983975

two-way ANOVA Marker gene analysis in Col-0 & *pepr1/pepr2:* CYP79B3 (Figure 3.10B)			
	Df	F	*P*
Genotype	1	0.181	0.6813620
Treatment	1	32.707	0.0004450
Genotype x Treatment	1	0.817	0.3925160

Tukey HSD test: Marker gene analysis in Col-0 & pepr1/pepr2: CYP79B3 (Figure 3.10B) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-0:Control - Col-0:Treated	0.8134162	0.2571809	1.3696514	0.0068494
pepr1pepr2 :Control - *pepr1pepr2:* Treated	0.5914104	0.0351752	1.1476456	0.0376435
Col-0:Control - *pepr1pepr2* :Control	0.1633206	-0.3929146	0.7195558	0.7850668
Col-0:Treated - *pepr1pepr2* :Treated	-0.0586852	-0.6149204	0.4975500	0.9857472

Appendix 9 Summary tables for ANOVA and relevant Tukey HSD comparisons for *CYP79B2* and *CYP79B3* marker gene analysis in Col-0 and *pepr1pepr2* following 1 h of feeding by 10 female adult mites.

ANOVA and Tukey's HSD tests

two-way ANOVA Marker gene analysis in Col-6 & *aos:* CYP79B2 (Figure 3.11A)			
	Df	F	*P*
Genotype	1	336.74	0.0000000
Treatment	1	78.44	0.0000013
Genotype x Treatment	1	31.05	0.0001210

Tukey HSD test: Marker gene analysis in Col-6 & aos: CYP79B2 (Figure 3.11A) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-6:Control - Col-6*:* Treated	2.0100000	1.4251237	2.5948763	0.0000015
aos:Control - aos*:* Treated	0.4575000	-0.1273763	1.0423763	0.1472303
Col-6:Control - *aos* :Control	1.7800000	1.1951237	2.3648763	0.0000055
Col-6:Treated - *aos* :Treated	3.3325000	2.7476237	3.9173763	0.0000000

two-way ANOVA Marker gene analysis in Col-6 & aos: CYP79B3 (Figure 3.11B)			
	Df	F	*P*
Genotype	1	246.18	0.0000000
Treatment	1	107.29	0.0000002
Genotype x Treatment	1	60.12	0.0000052

Tukey HSD test: Marker gene analysis in Col-6 & aos: CYP79B3 (Figure 3.11B) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-6:Control - Col-6*:* Treated	2.7683320	2.1265700	3.4100940	0.0000001
aos:Control - aos*:* Treated	0.3980608	-0.2437012	1.0398227	0.3021659
Col-6:Control - *aos* :Control	1.2130955	0.5713336	1.8548575	0.0005698
Col-6:Treated - *aos* :Treated	3.5833668	2.9416048	4.2251287	0.0000000

Appendix 10 Summary tables for ANOVA and relevant Tukey HSD comparisons for *CYP79B2* and *CYP79B3* marker gene analysis in Col-6 and *aos* following 6 h of feeding by 10 female adult mites.

ANOVA and Tukey's HSD tests

two-way ANOVA Marker gene analysis in Col-6 & aos: MYB28 (Appendix 4A)			
	Df	F	*P*
Genotype	1	17.8339	0.0011830
Treatment	1	0.8548	0.3734100
Genotype x Treatment	1	6.4964	0.0255220

Tukey HSD test: Marker gene analysis in Col-6 and *aos* : MYB28 (Appendix 4A) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-6:Control - Col-6*:* Treated	-0.3475000	-0.7675628	0.0725628	0.1186879
aos:Control - aos*:* Treated	0.1625000	-0.2575628	0.5825628	0.6682867
Col-6:Control - *aos* :Control	-0.1675000	-0.5875628	0.2525628	0.6477530
Col-6:Treated - *aos* :Treated	-0.6775000	-1.0975628	-0.2574372	0.0021559

two-way ANOVA Marker gene analysis in Col-6 & *aos* : MYB29 (Appendix 4B)			
	Df	F	*P*
Genotype	1	9.3266	0.0100100
Treatment	1	2.935	0.1123700
Genotype x Treatment	1	0.1722	0.6854700

Tukey HSD test: Marker gene analysis: MYB29 (Appendix 4B) Relevant contrasts of Genotype x Treatment interaction				
	diff	CI, lwr	CI, upr	adj, P
Col-6:Control - Col-6*:* Treated	0.1525000	-0.3407216	0.6457216	0.7959246
aos:Control - aos*:* Treated	0.2500000	-0.2432216	0.7432216	0.4645414
Col-6:Control - *aos* :Control	0.4075000	-0.0857216	0.9007216	0.1192966
Col-6:Treated - *aos* :Treated	0.3100000	-0.1832216	0.8032216	0.2920559

Appendix 11 Summary tables for ANOVA and relevant Tukey HSD comparisons for *MYB28* and *MYB29* marker gene analysis in Col-6 and *aos* following 6 h of feeding by 10 female adult mites.

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Best wishes,

Jon Munn

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Zhurov, V., Navarro, M., Bruinsma, K.A., Arbona, V., Santamaria, M.E., Cazaux, M., Wybouw, N., Osborne, E.J., Ens, C., Rioja, C., Vermeirssen, V., Rubio-Somoza, I., Krishna, P., Diaz, I., Schmid, M., Gomez-Cadenas, A., Van der Peer, Y., Grbic, M., Clark, R.M., Van Leeuwen, T., Grbic, V. (2014). Reciprocal responses in the interaction between *Arabidopsis* and the cell-content feeding chelicerate herbivore *Tetranychus urticae*. Plant Physiology *164*, 384-399.

Cazaux, M., Navarro, M., Bruinsma, K.A., Zhurov, V., Negrave, T., Van Leeuwen, T., Grbic, V., and Grbic, M. (2014). Application of two-spotted spider mite *Tetranychus urticae* for plant-pest interaction studies. Journal of Visual Experiments.