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General introduction

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1.1. THE PLANT DEFENSE SYSTEM

1.1.1. General framework

At a first glance, the immobile plants seem subject to the caprices of their attackers. However, herbivores have posed selection pressures on plants that have resulted in numerous defensive adaptations. Preformed structural and chemical barriers are referred to as ‘constitutive defenses’, and offer immediate protection against many attackers. However, defenses are costly to produce and maintain, and therefore not all defenses are active at all times (Kessler and Baldwin, 2002). If the constitutive defenses prove ineffective, a second line of defense can be activated, the ‘induced defenses’ (Kessler and Baldwin, 2002). Discriminating experimentally between constitutive and induced defenses is often not easy since there can be considerable overlap. For example, the size and density of physical barriers such as spines and plant hairs (Glas et al., 2012), which are commonly considered to function as constitutive defenses, can also be increased by induction (Traw and Dawson, 2002).

One can also make a distinction between direct and indirect defense mechanisms. Direct defenses are any plant traits that by themselves affect the susceptibility to and/or the performance of attacking arthropods. These include thorns, silica, trichomes and primary and secondary metabolites (Kessler and Baldwin, 2002). Indirect defense refers to plant traits that enhance attraction or arrestment of natural enemies of the herbivore, such as predators and parasitoids (Sabelis et al., 2001). Often, this type of defense is inducible. Attacked plants can release volatile compounds, which reveal the presence of herbivores to nearby natural enemies (Dicke and Sabelis, 1987; Vinson, 1976). Furthermore, natural enemies can be arrested by providing them with food such as extrafloral nectar (Pemberton and Lee, 1996) or with shelter (domatia) such as cavities or tufts of hair (Walter, 1996).

Next to herbivores, plants are also threatened by attackers such as pathogens. Each requires different defense measures. In general, phytopathogens are less mobile than herbivores, and migration across or through their host plant is often passive and occurs over relatively short distances. Hence, the infected host can attack such relatively immobile pathogens at the site of infection, and plants will often respond by producing structural reinforcements or toxins, or by initiating programmed local cell death (apoptosis) to isolate and possibly kill the pathogen (Dangl and Jones, 2001). Programmed cell death, orchestrated by the hypersensitive

response, is highly efficient in preventing pathogens from spreading and is therefore one of the most common anti-parasite defense strategies found in nature. Herbivores, however, are not very susceptible to this ‘isolate-and-kill’ strategy because most are mobile. Therefore, anti-herbivore defenses generally come down to a ‘go-away-or-die’ strategy or a ‘slow-them-down’ strategy, and these two strategies share many physiological characteristics. In both instances, plants will mount a sequence of defense programs that serve to interfere with herbivore growth and development on the one hand and to reallocate resources on the other, in order to delay growth of the herbivores into larger individuals, stages or populations, which consume more plant tissue. Hence, the simultaneous reallocation of resources may not only serve to rescue resources so that the plant can use them later for growth and reproduction, but may also serve to deprive the herbivore of food. This may be an effective strategy, especially when the plant is attacked by small herbivores or relatively immobile stages. Consequently, herbivores that decide to stay on defended plants select plant tissues where defenses are lower (Paschold et al., 2007; Shroff et al., 2008; Stork et al., 2009) and/or increase their feeding intensity to gain sufficient biomass, thus compensating for the decreased efficiency of food conversion (Gómez et al., 2012). Plants, in turn, often initiate systemic (i.e., in the entire plant) responses (Pieterse et al., 2009) to decrease the chance that the herbivore will simply move to undefended tissues (Paschold et al., 2007) and furthermore produce secondary metabolites to constrain compensatory feeding responses (Steppuhn and Baldwin, 2007). The sequence of defense programs executed by plants under attack appears not to be fully hard-wired, suggesting a certain degree of herbivore-specific tailoring by the plant, and while the early responses seem usually aimed at rescuing the attacked tissue, they may shift towards senescence and tissue death (reminiscent of the hypersensitive response) after a couple of days (Steinbauer et al., 2014). From the herbivore’s point of view, resource depletion at the feeding site may represent a more difficult problem to deal with than toxins, because they cannot develop resistance to an absence of nutrients. However, some herbivores such as gallmakers have evolved abilities to manipulate plant resource flows and turn their feeding site into a sink for resources (Tooker et al., 2008). Whereas the role of resource allocation remains under-studied, induced plant defenses and their effects on herbivores have been analyzed in great detail.

It should be clear by now that the regulation of plant defenses is a highly complex process. Indeed, defenses are preferentially activated only at times when needed, at places where needed, and should be effective against the attacker at hand, while avoiding wasting resources for defenses which don't harm or even help the attacker.

1.1.2. The induction of plant defenses against herbivores

During herbivore feeding, plant tissue is damaged, which alerts the plant's immune system. The herbivore's feeding style has an impact on the inflicted damage and the extent to which defenses are activated. Chewing insects, such as many lepidopteran larvae, remove relatively large quantities of leaf material. The induced defense response will be different from that of piercing-sucking herbivores which feed by means of needle-shaped mouthparts called stylets. Aphids for example, suck plant sap from vascular tissue and during this process plant cells are barely damaged (Elzinga et al., 2014). However, chemical compounds act as powerful cues for defense induction (Hogehout and Bos, 2011). Furthermore, plants may sense the arrival of a herbivore even before feeding has taken place. Components in the fluids of deposited eggs (Fatouros et al., 2008), and activities like herbivore movement (Hall et al., 2004; Peiffer et al., 2009) can betray the presence of an attacker.

A four-phase model of plant immunity has been presented by Jones and Dangl (2006). This 'zigzag' model was originally used to describe plant-pathogen interactions. However, it can be applied to plant-herbivore interactions as well since a high degree of analogy between plant-pathogen and plant-herbivore interactions has become apparent (e.g., Heil, 2008; Hogehout and Bos, 2011; Rodriguez et al., 2017; Stuart, 2015; Zhao et al., 2015). However, the extent of this analogy definitely requires further research. During feeding, there is an interaction at the molecular level between plant and herbivore (FIGURE 1.2). Non-feeding related cues, such as egg components can, however, be recognized as well (Erb et al., 2012; Fatouros et al., 2008). Plants carry pattern recognition receptors (PRRs) at their cell surface that mediate detection of a herbivore attack. These transmembrane receptors recognize evolutionary conserved molecular features denominated 'herbivore-associated molecular patterns' (HAMPs). Additionally, plant-derived 'damage-associated molecular patterns' (DAMPs), such as plant cell wall fragments, can also be recognized (van Schie and Takken, 2014). In phase 1 of the zigzag model, HAMP/DAMP recognition by PRRs acti-

vates a plant defense response named ‘HAMP triggered immunity’ (HTI). Herbivores have, however, evolved means to overcome PRR responses by the secretion of effector molecules. In phase 2, these effectors can interfere with PTI, resulting in effector-triggered susceptibility (ETS). Plants in turn evolved means to overcome ETS, as in phase 3, effectors can specifically be recognized by nucleotide binding leucine rich repeat (NB-LRR) proteins, activating effector-triggered immunity (ETI), an accelerated and

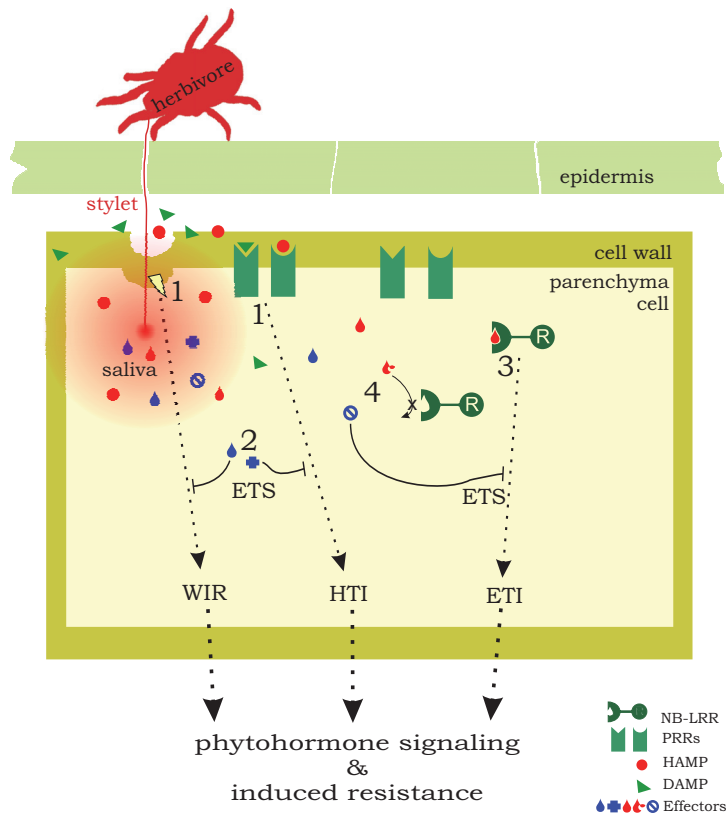


FIGURE 1.2. Molecular interaction between plants and herbivores. (1) Basal plant immunity involves the molecular recognition of herbivore- or damage-associated molecular patterns (HAMPs & DAMPs). The conserved HAMPs and DAMPs are recognized by pattern recognition receptors (PRRs), leading to HAMP/DAMP-triggered immunity (HTI). In addition, mechanical wounding in itself can lead to wound induced resistance (WIR). (2) In response, pathogens, and likely also herbivores evolved effectors to suppress HTI or WIR, resulting in effector-triggered susceptibility (ETS). These effectors are typically produced in the salivary glands and may, depending on the feeding mode, be delivered in the plant cells via the stylet. (3) Plants evolved resistance gene (R gene) products, which specifically recognize effectors and activate effector-triggered immunity (ETI). This R gene mediated immunity supplements basal immunity. (4) Selection favors the development of new or altered effectors, which are no longer recognized by the R gene product or suppress ETI. Redrawn after (Erb et al., 2012; Hogenhout and Bos, 2011; Jones and Dangl, 2006; Kant et al., 2015).

amplified PTI response. Recognition of effectors occurs either through direct interaction, or indirectly, by monitoring the integrity of host cellular targets of effector action (the so-called guard hypothesis) (Jones and Dangl, 2006). This effector, which formerly enhanced herbivore performance, then has become a liability since it acts as an elicitor of plant defenses. In phase 4, effector recognition by the plant therefore resulted in natural selection which drove herbivores to avoid ETI by either diversifying the recognized effector, or by getting rid of it altogether. Alternatively, additional effectors can be acquired that suppress the ETI (Jones and Dangl, 2006). This process of herbivore defense avoidance and plant countermeasures is repeated over and over again, and represents a good example of an evolutionary arms race (Heil, 2008).

Due to the context dependency (herbivore and plant genotype), effector proteins are no longer defined as molecules which specifically enhance herbivore (or pathogen) performance. Now, a broader inclusive definition (Hogenhout et al., 2009) is favored and effectors are defined as ‘all pathogen- or herbivore-secreted proteins and small molecules that alter host-cell structure and function’. Based on this broader definition, HAMPs can also be referred to as effectors (elicitors) (Sonah et al., 2016). However, whereas HTI confers broad herbivore recognition, ETI is more specific as effectors are highly polymorphic (van Schie and Takken, 2014).

The proteins that plants use to perceive and respond to effector activity are called resistance (R) proteins (Stuart, 2015), typically NB-LRR proteins, encoded by R genes. Several R genes conferring resistance against herbivores are currently known (e.g., Kaloshian, 2004; Zhao et al., 2015). *Mi-1* (resistance to the nematode *Meloidogyne incognita*) from tomato, is a well-known example (Rossi et al., 1998). R protein activity only results in resistance if a matching effector gene is present in the attacking herbivore. The absence of either the plant R gene or the herbivore effector gene results in successful herbivory. One can therefore speak of a gene-for-gene complementarity (Kaloshian, 2004; van Schie and Takken, 2014). Another kind of genes encountered in this context are the susceptibility (S) genes, which are all plant genes that facilitate herbivory and support compatibility. A mutation or a loss of an S gene can limit the ability of the herbivore to feed on the plant. Resistance conferred by the loss or alteration of S genes is generally recessive, whereas R genes are typically dominant (van Schie and Takken, 2014).

Between herbivore recognition and the anti-herbivore response lays a highly complex signaling network, mainly mediated by phytohormones.

1.1.3. Plant defense signaling

1.1.3.1. Defense-regulating plant hormones

Three phytohormones play a primary role in regulating defense responses (Pieterse et al., 2009): jasmonic acid (JA) (Wasternack and Hause, 2013), salicylic acid (SA) (Vlot et al., 2009) and the volatile ethylene (ET) (Adie et al., 2007). The central roles of JA and SA are substantiated by the fact that biosynthesis mutants are hypersensitive to a wide range of attackers. Several other phytohormones are known to play a secondary role in plant defense by modulating it, including abscisic acid (ABA) (Dinh et al., 2013), auxin (Kazan and Manners, 2009), cytokinin (Choi et al., 2011), gibberellic acid (GA) (Yang et al., 2012), brassinosteroids (Nakashita et al., 2003) and possibly strigolactones (Torres-Vera et al., 2014). In concert with these hormones, a small set of signalling peptides, such as systemin (Ryan, 2000) and the Peps from *Arabidopsis* (Huffaker et al., 2006), are also involved in orchestrating plant defenses. The peptide systemin of tomato, *Solanum lycopersicum* (Pearce et al., 1991), functions upstream of JA biosynthesis and may facilitate priming of the plant's JA response (Kandath et al., 2007).

1.1.3.2. Jasmonate as a regulator of plant defenses against herbivores

Jasmonic acid regulates the core defenses of dicots against herbivorous arthropods (Howe and Jander, 2008) and necrotrophic pathogens (Glazebrook, 2005). The biosynthesis of JA was elucidated by Vick and Zimmerman (1984) and seems quite conserved across species. In tomato, JA biosynthesis was shown to take place in the chloroplast and peroxisomes of the phloem companion cells (Howe, 2004). Briefly, the first step in JA biosynthesis comprises the formation of α -linolenic acid, which is released from the galactolipids of chloroplast membranes by the action of one or more phospholipases, although it is still unclear which roles the different lipase candidates play in α -linolenic acid formation during different plant-herbivore interactions (Wasternack and Hause, 2013). Subsequently, α -linolenic acid is converted via three enzymatic steps into 12-oxophytodienoic acid (OPDA), and dinorOPDA is also formed in *Arabidopsis* (Stintzi et al., 2001). OPDA is then imported into the peroxisomes, where it is converted by OPDA reductase OPR3, followed by three cycles of β -oxidation

into JA. Finally, JA diffuses into the cytosol, after which a range of JA conjugates and derivatives are formed (Yan et al., 2013), among which is jasmonoyl isoleucine (JA-Ile), which is the main bioactive form of JA (Fonseca et al., 2009). Although JA-Ile has a well-established role in regulating defense gene expression, OPDA may also function as such independently (Taki et al., 2005).

Before induction, JA-dependent responses are constitutively blocked due to repressor proteins (FIGURE 1.3), called jasmonate ZIM domain (JAZ) proteins, bound to transcription factors that otherwise would promote defense gene expression (Thines et al., 2007), including several MYC (Chini et al., 2007; Fernández-Calvo et al., 2011) and MYB (Qi et al., 2011) transcription factors. The JAZ proteins have two types of functional domain: ZIM domains and Jas domains. The ZIM domains establish homo- or heterodimerization among individual JAZ proteins but also interactions with additional (co-)suppressors, such as TOPLESS and NINJA (Pauwels et al., 2010). The Jas domains establish the interaction with the transcription factors, which prevents these from functioning. Transcriptional (de)repression also regulates the synergistic action of JA and ethylene since JAZ proteins repress not only the transcriptional activity of the ethylene-stabilized transcription factors EIN3 and EIL1 but also interfere with their transcription by promoting histone acetylation. However, induced JA-Ile interrupts the interaction between the JAZ proteins and EIN3/EIL1 to enhance their transcriptional activity (Zhu et al., 2011) by promoting the ubiquitination–degradation of JAZ proteins via a protein complex called the SCF^{COI1} complex. Hence, activation of JA-responsive genes largely is obtained by derepression of transcription. In *Arabidopsis*, the JA responses downstream of SCF^{COI1} are executed via two different branches: one branch that is dependent on MYC transcription factors (referred to as the MYC branch) (Dombrecht et al., 2007) and the other depending on transcription factors like ETHYLENE RESPONSE FACTOR1 (ERF-1) and OCTADECANOID-RESPONSIVE ARABIDOPSIS 59 (ORA59), which is referred to as the ERF/ORA59 branch (Pré et al., 2008; Zhu et al., 2011). These branches are known to antagonize each other: the MYC2 transcription factor suppresses expression of ERF-dependent JA-responsive genes and *vice versa* (Dombrecht et al., 2007; Lorenzo et al., 2004). The levels of JA in *Arabidopsis* leaves can start to rise within 30 s after wounding (Glauser et al., 2009). The burst is transient: levels decrease again after a few hours

(Reymond et al., 2000; Schittko et al., 2000); however, two consecutive bursts have been observed in *S. nigrum* (VanDoorn et al., 2011). In *N. attenuata*, large veins can constrain the spatial spread of JA bursts, and while a second elicitation can suppress a (second) burst, a third elicitation can induce it again (Stork et al., 2009). Subsequently, induction of JA accumulation can also occur in distal leaves (Glauser et al., 2008). Spatiotemporal

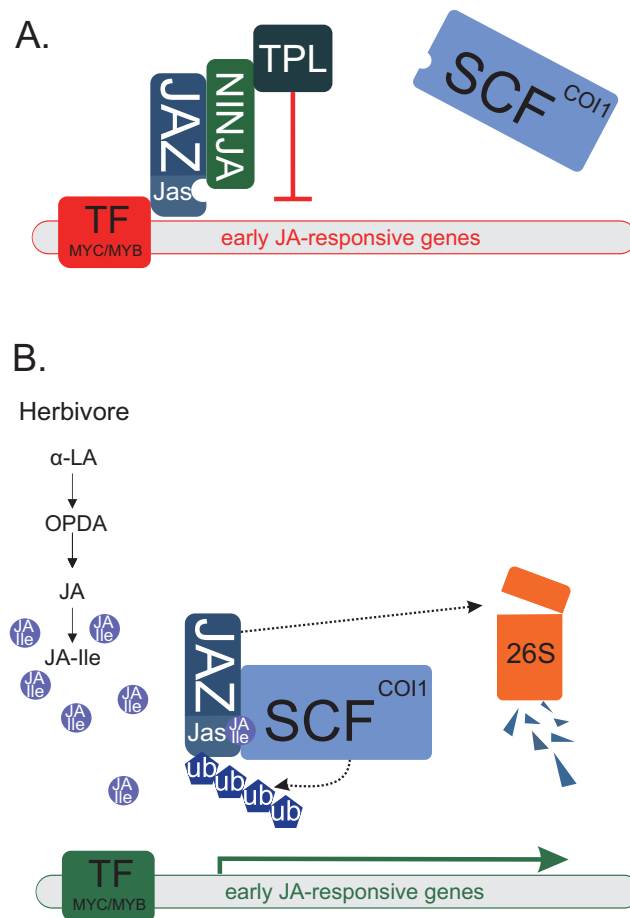


FIGURE 1.3. Activation of the JA pathway by herbivores. (A) In the absence of attackers and the associated bioactive JA, transcription of defense-related genes is blocked by jasmonate ZIM domain (JAZ) proteins, which are bound to transcription factors (TFs) such as MYC and MYB. JAZ repressor proteins control gene expression through the interaction with the NINJA adapter and TOPLESS (TPL) corepressor proteins. (B) Upon herbivore recognition, JA biosynthesis starts with the release of α -linolic acid (α -LA) from membrane lipids. α -LA is converted into OPDA which is used to generate JA. Next, the bioactive JA conjugate JA-Ile is formed. JA-Ile brings about the interaction between the Jas domain of the JAZ proteins with SCF^{COI1} ubiquitin ligase, triggering ubiquitination (ub) and targeting them for degradation by the 26S proteasome. Eventually, transcription of defense genes is derepressed. Redrawn after Ballaré (2011), Pauwels et al. (2010) and Pieterse et al. (2012).

variability in JA accumulation may be a defensive tactic by itself because it makes it difficult for herbivores to anticipate which tissues are defended poorly and which strongly (Stork et al., 2009).

1.1.3.3. Ethylene as a regulator of plant defenses against herbivores

Ethylene (ET) is a gaseous hormone and is involved in development, senescence and defense against necrotrophic pathogens (Chen et al., 2005b). Endogenous ET concentrations in plant tissues depend on the activities of two biosynthetic enzymes, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACC oxidase), which convert S-adenosyl-Met to ethylene, but also on the rates of outward diffusion and metabolization (Wang et al., 2002). Transcription factors that control ethylene-responsive genes are constitutively repressed by proteins such as JAZ (Zhu et al., 2011) and ET perception controls the ethylene response. *Arabidopsis* contains five ET transmembrane receptors, located in different organelles (Kendrick and Chang, 2008). These receptors are active in the absence of ET (Hua and Meyerowitz, 1998), and suppress the ethylene response by constitutively stimulating phosphorylation of the ET signaling hub EIN2 (ETHYLENE INSENSITIVE2), leading to its degradation (Qiao et al., 2009). Upon binding to ET, the receptors become inactive, allowing unhindered accumulation of EIN2 in the cytosol. This initiates degradation of the ET transcriptional repressors and thus the activation of ET-responsive genes in the nucleus (An et al., 2010).

1.1.3.4. Salicylate as a regulator of plant defenses against herbivores

Salicylate mediates defenses against biotrophic pathogens (Glazebrook, 2005) and phloem-feeding herbivores (Kaloshian and Walling, 2005). During pathogen infections, defense responses can spread systemically, so are also expressed in uninfected tissues, and this is referred to as systemic acquired resistance. Several candidate signals have been reported to play a role in systemic acquired resistance, including the SA-derivative methyl salicylate. However, SA is the central local regulator because plants that are unable to accumulate SA are often highly susceptible to pathogen infections (Klessig, 2012). In rice (a monocot), the JA and SA pathways are thought to regulate a common set of defense genes that are effective against both biotrophic and necrotrophic pathogens (De Vleeschauwer et al., 2013). Salicylate is derived from chorismate, the end-product of the shikimate

pathway. From there it can be synthesized in plants via at least two distinct biosynthetic routes. The first route delivers SA in two steps and depends on the enzymes isochorismate synthase, which is induced upon pathogen infection (Wildermuth et al., 2001), and isochorismate pyruvate lyase. The second route depends on the phenylpropanoid pathway. This is a pathway responsible for a variety of products, such as flavonoids and lignins, but also for SA, and there may be parallel sub-branches within the branch leading to SA (Boatwright and Pajerowska-Mukhtar, 2013). Which of these pathways or branches determines induced SA levels most strongly may also differ across plant species. Once formed, SA may be modified further by glucosylation, methylation or amino acid conjugation. Most of these derivatives are inactive and may serve to fine-tune local and systemic SA accumulation and function or may provide safe storage. Methyl salicylate is inactive but easier to transport to distal tissues, either actively via the phloem or passively via the air (Dempsey et al., 2011). A central role is played by the NONEX-PRESSOR OF PR GENES (NPR) protein family. It has recently been discovered that NPR3 and NPR4 are SA receptors, whereas NPR1 acts a master regulator of SA-mediated responses (Yan and Dong, 2014). NPR1 proteins are constitutively present in the cytosol of the cell as oligomers (Tada et al., 2008) and their concentration increases upon induction (Spoel et al., 2009). Accumulation of SA causes an increase in the levels of reduced glutathione (the antioxidant form of glutathione), thereby changing the redox status of the cell, i.e., the balance between oxidants and antioxidants (Spoel and Loake, 2011), and this generates NPR1 monomers by the thioredoxin-catalysed reduction of monomeric disulphate bridges. Subsequently, NPR1 monomers migrate into the nucleus (Mou et al., 2003; Tada et al., 2008). Without NPR1, the expression of the SA-responsive genes is repressed by TGA transcription factors. After NRP1 has arrived in the nucleus, a portion of it is phosphorylated. Phosphorylated NPR1 binds to the TGA transcription factors and this complex allows the expression of target genes such PR-1. Unphosphorylated NPR1 may assemble together with different transcription factors and give rise to TGA-independent expression of other target genes. After a round of transcription initiation, the NPR1 protein complexes are degraded via the proteasome and new monomeric NPR1 proteins need to enter the nucleus from the cytosol to keep the response going (Mukhtar et al., 2009).

1.1.3.4. Hormonal crosstalk in plant defenses against herbivores

The distinct defense signaling pathways that are regulated by phytohormones interact directly and indirectly, forming complex networks, and these interactions can be additive, antagonistic or synergistic (Koornneef and Pieterse, 2008). Of all the interactions that occur between hormonal defense signaling pathways, crosstalk between the JA and SA pathways has received most attention, after it was discovered that SA can inhibit the plant's wound response (Doherty et al., 1988) and indications were found for the opposite (Sano et al., 1994). Under most conditions, crosstalk between SA and JA is antagonistic (Thaler et al., 2012), but when applied to plants in specific ratios synergistic interactions were also observed (Mur et al., 2006), and other plant hormones may modulate this crosstalk (Robert-Seilaniantz et al., 2011). Suppression of the JA response by SA occurs downstream of JA-Ile perception, depending on *ORA59* (Van der Does et al., 2013). Other regulators include *NPR1* and some of its interacting partners, including the *TGA* and *WRKY* transcription factors (Pieterse et al., 2012). Nuclear localization of *NPR1* is not required for the suppression of JA signalling, and it has therefore been suggested that the role of *NPR1* in mediating JA/SA crosstalk depends on a function executed in the cytosol (Spoel et al., 2003). The adaptive value of the JA/SA (antagonistic) crosstalk is not clear. It has frequently been suggested that it allows plants to fine-tune the balance between different defensive strategies, depending on the type of attacker, or in case the plant is attacked by multiple attackers, depending on the timing and sequence of infestation (Pieterse and Dicke, 2007). However, whether JA/SA antagonism is adaptive remains an open question (Thaler et al., 2012).

1.1.4. Molecules used by plants to resist herbivores

The collective hormonal responses and their interactions induced by herbivores determine which defenses are established in which host plant tissues and to what extent. Induced plant defenses upon herbivory are seldom lethal, the fact that herbivores can move away from defended tissues will usually prevent them from ingesting a fatal dose. Hence, plant defenses induced by herbivores will cause them to depart or, alternatively, slow down their development and population growth. Many of these herbivore-induced plant defenses rely on the direct antagonistic action of enzymes that interfere with feeding activities, digestive processes and gut integrity

(Carlini and Grossi-de-Sá, 2002). One can distinguish defense proteins and defense metabolites.

1.1.4.1. Defense proteins

Several types of defense proteins exist, including (1) protease inhibitors (PIs) which are believed to inhibit the action of protein-digesting enzymes (proteases) in the herbivore's gut (Jongsma and Beekwilder, 2011). Other defensive proteins are (2) peptidases/proteases, which attack the herbivore's peritrophic membrane or inactivate the herbivore's digestive enzymes by proteolysis (Fescemyer et al., 2013; Fowler et al., 2009; Lomate et al., 2013; Pechan et al., 2002). Additionally, plants produce (3) amino acid degrading proteins, which degrade the free amino acids, released from the proteins through herbivore digestion, before they can be taken up (Chen et al., 2005a; Gonzales-Vigil et al., 2011). (4) Oxidases, including polyphenol oxidases (PPOs) which generate quinones, highly reactive molecules that can either spontaneously polymerize or damage proteins, amino acids and nucleic acids via an alkylation reaction (Constabel and Barbehenn, 2008). Other oxidases, such as peroxidase and lipoxygenase, may play a functional role in plant defenses by creating potent electrophiles or interfering with the accumulation of essential nutrients (Zhu-Salzman et al., 2008). Plant defensive (5) lectins are associated with the disruption of several processes involved in the digestion of food and nutrient uptake in herbivores (Michiels et al., 2010). Lectins comprise a diverse family of proteins that bind specifically with mono- and oligosaccharides (Komath et al., 2006). Due to their high affinity with oligosaccharides, it is assumed that lectins can interact with glycoproteins in the digestive tract of the herbivore and bind to the insect's intestinal epithelial cells or its peritrophic membrane and disrupt these tissues (Macedo et al., 2004). (6) Pathogenesis-related (PR) proteins include a wide variety of proteins with diverse functions, predominantly associated with resistance to pathogens. These proteins are often used as defense marker genes, though not all of them are functionally understood. Most of them are classified as a glucanase, chitinase, thaumatin, PI or peroxidase. They are defined as pathogen-induced proteins and for the majority of them evidence is largely lacking that they play a significant role in anti-herbivore defenses (Chen et al., 2007; Sels et al., 2008). Another type of defense proteins are the (7) small cysteine-rich defense proteins, which are generally assumed to disrupt membranes or to inhibit gut enzymes (Stotz et al., 2009).

1.1.4.2. Defense metabolites

While defensive plant proteins play a significant role in direct interactions between herbivores and plants, the role of non-protein secondary metabolites is just as big. The term ‘secondary’ is used to contrast them with metabolites that are directly involved in growth, development or reproduction, although it is not always possible to determine the precise physiological role of each metabolite. Across the plant kingdom, there is a staggering diversity of secondary metabolites, and they can be distinct for small phylogenetic groups. Despite the rich diversity of secondary metabolites, their biosynthetic origins allow them to be classified into three basal groups: the phenolics; the isoprenoids; and the nitrogen-containing compounds. Defense metabolites have a variety of functions, including attraction of natural enemies, toxicity, and protection against UV radiation. While defense metabolites can be constitutively present in plant cells, their production and transport may also be increased upon herbivore attack (Kant et al., 2015; War et al., 2012).

1.2. HOW HERBIVORES COPE WITH DEFENSES

1.2.1. Defense and non-defense related adaptation to plant feeding

Plants and herbivores have coevolved for over 350 million years (War et al., 2012). While specific anti-herbivore compounds and structures are indeed challenging for herbivores, some more general issues related to plant feeding have to be overcome as well. Phloem, for example, is a troublesome food source since its sugar content is high, yet it lacks essential components such as amino acids (Douglas, 2006). Herbivores, however, established relationships with microbial symbionts (Engel and Moran, 2013), solving some of the issues. In addition, some herbivores acquired microbial genes through horizontal gene transfer, enabling them to degrade troublesome plant components (Pauchet and Heckel, 2013). As such, a herbivore’s host plant range is closely linked to its digestive physiology (Pearse et al., 2013). In addition to these general plant-feeding issues, of which a thorough description is outside the scope of this introduction, herbivores have been under pressure to evade defenses specifically produced to discourage feeding (reviewed in Alba et al., 2011). Hence, behavioral adaptations have evolved that allow herbivores to avoid defended plant tissues as much as possible (Paschold et al., 2007; Perkins et al., 2013; Shroff et al., 2008) or to dismantle defensive

structures such as trichomes (Cardoso, 2008) and latex channels (Rodrigues et al., 2010). However, herbivores have also evolved a variety of mechanisms to cope with deterrent substances produced by their host plants.

Two general mechanisms allow herbivores to cope with the xenobiotics from their environment: mechanisms that decrease exposure (pharmacokinetic responses) and mechanisms that decrease sensitivity (pharmacodynamic responses). Pharmacokinetic responses comprise a variety of adaptations that reduce uptake, increase catabolism and allow sequestration, whereas the pharmacodynamic response types comprise adaptations at the level of interactions between allelochemicals and their target-site(s) (Kennedy and Tierney, 2013; Taylor and Feyereisen, 1996; Van Leeuwen and Dermauw, 2016). Together, these mechanisms determine the level of tolerance of herbivores to xenobiotics.

Although the influence of these mechanism on herbivore success cannot be over-estimated, they are beyond the scope of this general introduction and I instead refer to Kant et al. (2015) for a thorough overview. Rather, I will focus on molecular compounds (more specifically effectors) used by herbivores to reduce plant defense induction in the first place, i.e., the herbivores interpretation of the saying ‘prevention rather than cure’.

1.2.2. Effectors involved in plant defense suppression by herbivores

There are indications that plant-defense-suppressing herbivorous arthropods secrete effectors via their saliva into their host, similar to pathogens and nematodes. The first of such salivary components that was discovered was the enzyme glucose oxidase (GOX), which is the most abundant molecule in the oral secretions of *Helicoverpa zea* caterpillars (Musser et al., 2002). This enzyme catalyzes the oxidation of glucose to D-gluconic acid and thereby generates hydrogen peroxide. The amount of GOX applied to *Nicotiana tabacum* plants correlates with an increase in SA and a decrease in the accumulation of nicotine. Possibly, GOX suppresses or attenuates JA and ET responses by crosstalk with SA (Diezel et al., 2009; Eichenseer et al., 2010). GOX has been found in many more caterpillar species (Eichenseer et al., 2010) and other herbivorous insects, such as aphids and non-herbivores such as honeybees (Harmel et al., 2008; Iida et al., 2007). A comprehensive study by Eichenseer et al. (2010) showed large variation in GOX activity within families and subfamilies of 88 caterpillar species, but

these activities depended on the host plant species as well. Moreover, a recent report showed that *H. zea* GOX elicits the JA pathway in tomato (Tian et al., 2012). Taken together, these studies suggest that some plants, such as tomato, may have evolved a recognition mechanism for GOX, resembling R-gene-mediated recognition of effector proteins in plant–pathogen interactions.

Advances in genomics and proteomics have greatly facilitated the discovery of more effector proteins in insects. After the *Acyrtosiphon pisum* (peach aphid) salivary glands were sequenced, the first aphid effector was discovered. This protein is a 22-kDa salivary-secreted protein of unknown function called C002 (Mutti et al., 2008). RNAi-mediated knockdown of C002 expression affected *A. pisum* foraging and feeding behavior and reduced aphid fitness. Bos et al. (2010) used *Nicotiana benthamiana* to ectopically express C002 from *Myzus persicae* (green peach aphid) and showed that aphid fecundity increased on these plants. Transient overexpression of a second aphid protein, Mp10, sufficed to suppress the flagellin-triggered oxidative burst in *N. benthamiana*, but aphid reproduction was lower on these plants. A subsequent study characterized two additional aphid proteins, Mp1 (PIntO1) and Mp2 (PIntO2), which correlate positively with aphid fecundity on *Arabidopsis*. Interestingly, the performance of *M. persicae* did not improve on *Arabidopsis* plants expressing the *A. pisum* orthologues of both effectors (Pitino and Hogenhout, 2013). Finally, two putative effectors of *Macrosiphum euphorbiae* were found, Me10 and Me23, both of which increased aphid fecundity on *N. benthamiana*, whereas only Me10 increased their fecundity on tomato (Atamian et al., 2013).

Research on gall midges has provided independent evidence for a role of effector proteins in plant-herbivore interactions. Early larval stages of the Hessian fly, *Mayetiola destructor*, are plant parasites. When they colonize wheat (*Triticum* spp.), the larvae induce feeding cells in their host, which provide them with food until they develop into adults (Harris et al., 2003). More than 30 Hessian fly resistance genes have been found in wheat, some of which are predicted to encode typical R proteins (Liu et al., 2005). On resistant wheat, Hessian fly larvae are unable to induce feeding cells, but instead induce a hypersensitive-like response that prevents them from eating (Harris et al., 2010). One *M. destructor* gene, *vH13*, functions as an avirulence factor (elicitor) on wheat carrying the *H13* resistance gene. In contrast, larvae from populations that are virulent on *H13* wheat did not

express *vH13*, while RNAi-mediated knockdown of *vH13* in avirulent larvae made some of them virulent (Aggarwal et al., 2014). These data suggest that *vH13* may function as an effector in non-resistant wheat varieties.

Thus, there are indications that herbivores may make use of effectors, just as pathogens do. This notion is strengthened by the existence of anti-herbivore R genes such as *Mi-1*, *Vat* and *Bph14* (Dogimont et al., 2008; Du et al., 2009; Rossi et al., 1998). The high diversity found among pathogen effectors discourages the use of protein homology as a strategy to identify herbivore effectors (Rep, 2005). Nevertheless, most effector proteins share structural features that can be easily recognized, such as an amino-terminal signal peptide, the absence of transmembrane domains and a small protein size. Furthermore, effectors that operate in the plant apoplastic space are usually rich in cysteine residues (Rooney et al., 2005). Several studies have exploited these common properties to find novel effector-encoding genes from sequenced pathogen genomes or transcriptomes. Comprehensive datasets on herbivore transcriptomes and proteomes (DeLay et al., 2012; Grbić et al., 2011; Su et al., 2012) will probably give rise to the discovery of new effectors in the near future.

1.3. *TETRANYCHUS URTICAE* AS A HERBIVORE

1.3.1. General biology of *Tetranychus urticae*

Spider mites belong to the subphylum Chelicerata (e.g., spiders and scorpions), which together with the Myriapoda (e.g., centipedes and millipedes), Crustacea (e.g., shrimps, crabs and water fleas) and Hexapoda (e.g., insects) constitute the phylum Arthropoda. Mites and insects diverged over 400 million years ago (Weygoldt, 1999), and as such do not share a recent history. Therefore, traits that allow herbivorous insect and mite species to deal with plant defenses are likely to have evolved independently (Kant et al., 2015).

Tetranychus urticae Koch 1836 (Acari: Tetranychidae) is commonly known as the two-spotted spider mite. The ‘spider’ component in its name is derived from its ability to produce a silk-like webbing, while ‘two-spotted’ refers to the bilateral symmetrical dark spots on its opisthosoma (Grbić et al., 2011; Helle and Sabelis, 1985). Another common name is ‘red spider mite’. However, next to a red body color, green forms exist as well. Due to the existence of these two colors, the red form was previously known as ‘*T. cinnabarinus*’. Today, however, both forms are considered the same species,

and *T. cinnabarinus* is synonymous to *T. urticae* (Auger et al., 2013; Hino-moto et al., 2001).

Spider mites have a haplodiploid sex-determination system and fertilized eggs give rise to diploid females, while unfertilized haploid eggs develop into males (i.e., arrhenotoky) (Grbic et al., 2007; Li and Margolies, 1993b). Mated females produce both fertilized and unfertilized eggs (Helle, 1967), and the sex ratio is usually 3:1 female to male (Krainer and Carey, 1990). *Tetranychus urticae* has five life stages: egg, larva, protonymph (first nymphal stage), deutonymph (second nymphal stage) and adult. Before each of the last three stages, a quiescent phase occurs, respectively named protochrysalis, deutochrysalis and teleochrysalis. Larva, protonymph, deutonymph and adult all actively feed on plant tissue (Helle and Sabelis, 1985). A mated female can produce over 50 female offspring. Together with a very short life cycle of 8 to 12 days (Jeppson et al., 1975), this high fecundity results in exponential population growth which subsequently leads to host plant overexploitation (Alba et al., 2015; Clotuche et al., 2011; Sarmiento et al., 2011). *Tetranychus urticae* may disperse by active movement (i.e., walking) and through passive transport by another organism (phoresy) (Clotuche et al., 2011). Spider mites also disperse aerially, either individually or collectively, on air currents to surrounding areas and can establish new colonies rapidly (Clotuche et al., 2011; Li and Margolies, 1993b; Smitley and Kennedy, 1985). Furthermore, adult females can go into diapause to survive unfavorable seasonal conditions (Bryon et al., 2013).

Tetranychus urticae has a worldwide distribution and has been recorded on a staggering amount of different host plants. More specifically, it has been documented to feed on over 1100 species distributed over more than 140 families (Migeon and Dorkeld, 2006-2016) and it appears to be adapted to using a series of temporary hosts (Li and Margolies, 1993a). Potential hosts include important crops and ornamental plants, which makes of *T. urticae* a major agricultural pest (Van Leeuwen et al., 2010). Although *T. urticae*, as a species, is extremely generalist, it does not necessarily consist of generalist individuals (Fox and Morrow, 1981; Kant et al., 2008). Indeed, *T. urticae* has high intraspecific genetic variability (Magalhaes et al., 2007) and exhibits genetic polymorphism (Kant et al., 2008). Local populations form host races that do not perform equally well on all potential host plants species (Agrawal et al., 2002; Díaz-Riquelme et al., 2016; Gotoh et al., 1993; Navajas, 1998). An illustration of this can be found in Zhurov et al. (2014),

where the preference and performance of bean-adapted *T. urticae* was even highly dependent on the ecotype of one and the same plant species, *A. thaliana* (Zhurov et al., 2014). Adaptation to particular hosts might limit the colonization success on alternative plants (Agrawal, 2000; Agrawal et al., 2002; Gould, 1979), and may depend on the characteristics of the new plant (e.g., secondary metabolites), and the detoxifying and digestive toolkit of the mite. This genetically determined toolkit is likely to be partly optimized for the original host, yet may prove less suited for a newly colonized plant (Agrawal, 2000; Agrawal et al., 2002; Dermauw et al., 2013a). If a host plant is favorable, *T. urticae* has the tendency to remain within the infestation area. On unfavorable hosts, however, it tends to disperse or, in extreme cases, to leave the plant (Díaz-Riquelme et al., 2016; Hussey and Parr, 1963; Margolies and Kennedy, 1985).

The genome of *T. urticae* (90 Mb) was recently sequenced and annotated (Grbić et al., 2011) and represents a rich source of information to study important features of *T. urticae*, including its ability to rapidly develop resistance against various pesticides (Van Leeuwen et al., 2013) and its extremely polyphagous nature (Migeon and Dorkeld, 2006–2016). Both of these features appear to be correlated. Indeed, synthetic chemicals, including pesticides, can be dealt with in a similar way as plant metabolites, and mechanisms to overcome plant defensive compounds predisposed the development of pesticide resistance (Dermauw et al., 2013b).

1.3.2. Spider mite feeding

Two interlocking cheliceral digits form a tube with a single canal of approximately 2 μm in diameter (André and Remacle, 1984; Bensoussan et al., 2016). With this so-called ‘stylet’ of about 150 μm in length, *T. urticae* punctures leaf mesophyll cells and empties them by lacerate-and-flush feeding (Alba et al., 2015; Jeppson et al., 1975). The details of this feeding mechanism are still a matter of dispute. A recent study by Bensoussan et al. (2016) offered some valuable insights. When feeding on plant tissue, *T. urticae* inserts its stylet in between epidermal pavement cells or through the stomatal opening, rather than penetrating epidermal cells. This way, damage to the epidermis is avoided. During a feeding event, a process that is initiated by the settlement at a particular leaf spot and is terminated when the mite raises its head away from the leaf surface, spider mites usually seem to consume individual mesophyll cells. This punctured cell is generally located immedi-

ately below the epidermal layer the mite stands on. The stylet is assumed to deliver saliva into the plant tissue, as stated by Hislop and Jeppson (1976), yet rejected by André and Remacle (1984). How plant nutritive fluid is eventually transported to the mite is also still controversial. In the latest research paper on this subject, the scenario in which the cell content is sucked up through the stylet is favored (Bensoussan et al., 2016). How the plant fluid then ends up in the oesophagus is unclear since no direct connection between the oral orifice and the stylet tube has ever been found (Beard et al., 2012). Alternatively, one has claimed that the buccal cavity is used to ingest cell fluid that is extruded to the leaf surface after the cell has been punctured by the stylet (Alberti and Crooker, 1985; Baker and Connell, 1963; Beard et al., 2012). Clearly, future research is needed to settle this disagreement.

According to Bensoussan et al. (2016), mite feeding in itself does not cause the formation of visible chlorotic spots, contrary to what has been proposed in earlier reports. Indeed, a study by Liesering (1960), based on long term mite feeding, led to the conclusion that single spider mites damage about 20 plant cells per minute, directly leading to the formation of visible spots. Now it is stated that the consumption rate of plant cells is much lower. During feeding, the content is removed from a single or a limited number of mesophyll cells that are on the stylet path. Surrounding cells remain intact with unperturbed internal organization. As such, the visible chlorotic spots are no immediate consequence of spider mite-induced damage, but are rather caused by the plant response to this limited damage (Bensoussan et al., 2016). In addition, not all host plants develop macroscopic chlorotic spots. *Vitis vinifera*, for example, accumulates red/brown spots (leaf bronzing) instead (Díaz-Riquelme et al., 2016). Furthermore, the Kanzawa spider mite *T. kanzawai*, induces different scar colors in lima bean, depending on the mite strain (Matsushima et al., 2006). Clearly, these observations are unlikely to be caused by differences in inflicted mechanical damage. Which plant- or mite-derived signals lead to these variations in local plant responses is currently unknown. Likely, effectors contained within the saliva are involved.

The number of plant cells that are emptied during a feeding event and the way plant fluids are transported to the mite are likely to have implications on the nature of the saliva. Indeed, plant chloroplasts can be removed by spider mite feeding, and the transport of these organelles – whose size exceeds the stylet diameter – would be facilitated by pre-oral digestion. This digestion could be brought about by mite salivary enzymes, yet also

by enzymes originating from the plant (e.g., vacuolar hydrolytic enzymes) (Bensoussan et al., 2016). Hemolysis experiments executed decades ago already suggested that saliva is injected into leaves by *T. urticae*, and that this saliva contains one or more proteolytic enzymes (Hoof, 1958; Storms, 1971). Such enzymatic reactions require time, and the duration of a feeding event (about 13 minutes on average; Bensoussan et al., 2016) seems rather adequate for these reactions to take place.

1.3.3. Spider mite molecular adaptations to polyphagy

1.3.3.1. Detoxification

When feeding on its many hosts, *T. urticae* is challenged by a diversity of plant defensive allelochemicals. During the course of evolution, *T. urticae* acquired systems conferring resistance to these harmful compounds. The extent of those systems reflects the xenobiotic adaptation to a wide range of potential hosts. To begin with, lineage-specific expansions and radiations have been found in the ‘classical’ detoxification gene families, including cytochrome P450 monooxygenases (P450s), carboxyl/cholinesterases (CCEs), glutathione-S-transferases (GSTs) and ATP-binding cassette (ABC) transporters (Van Leeuwen and Dermauw, 2016). P450s and CCEs generally operate during phase I of detoxification, in which the toxin is functionalized with nucleophilic groups to make it more reactive and water soluble. GSTs typically operate during phase II, which involves conjugation with endogenous metabolites to further increase toxin polarity (Kant et al., 2015; Van Leeuwen and Dermauw, 2016). ABC transporters have been reported to transport toxicants out of the cells, either directly, or after conjugation with glutathione (Dermauw et al., 2013a). As such, these are active in phase III, where the metabolites are secreted (Kennedy and Tierney, 2013; Van Leeuwen and Dermauw, 2016).

In addition to the classical ones, ‘novel’ gene families involved in detoxification have also been identified in *T. urticae*. These include the major facilitator superfamily (MFS) and the lipocalins (Dermauw et al., 2013b; Van Leeuwen and Dermauw, 2016). The MFS, also known as the uniporter-symporter-antiporter family, might effectuate the efflux of toxic allelochemicals or their metabolites out of the mite cells (Dermauw et al., 2013b). Lipocalins are capable of binding hydrophobic molecules and may as such bind allelochemicals, resulting in sequestration of these toxic compounds (Dermauw et al., 2013b).

Horizontal gene transfer (HGT), the movement of genetic material across species boundaries, has been recognized as one of the driving forces behind the xenobiotic adaptation of *T. urticae* and has been reviewed in Van Leeuwen and Dermauw (2016). The best supported example is the acquisition of β -cyanoalanine synthase from bacteria, which allows mites to detoxify cyanide released by cyanogenic plants (Wybouw et al., 2014). Another HGT that may have expanded the metabolic capacity of *T. urticae* involves a class of intradiol ring-cleavage dioxygenases (ID-RCDs). These enzymes might split aromatic ring structures in complex plant molecules (Dermauw et al., 2013b). Other examples of HGTs to *T. urticae* include UDP-glycosyl transferases (UGTs), two clusters of carotenoid synthase/cyclases and desaturases (Bryon et al., 2013), a cobalamine-independent methionine synthase, two duplicated β -fructofuranosidases (Grbić et al., 2011) and a cyanase (Wybouw et al., 2012).

In addition to these molecular adaptations, the rapid excretion of phagocytes in the mite gut might enhance the sequestration of allelochemicals (Mullin and Croft, 1983; Van Leeuwen and Dermauw, 2016). The aforementioned mite adaptations can be classified as resistance mechanisms of ‘decreased exposure’ (pharmacokinetic). Mechanisms of ‘decreased sensitivity’ (pharmacodynamic), such as target-site-insensitivity, have been described with regard to acaricides (e.g., Van Leeuwen et al., 2012). Likewise, pharmacodynamic mechanisms could confer resistance to xenobiotics of plant origin, although examples supporting this hypothesis remain to be uncovered (Van Leeuwen and Dermauw, 2016).

Furthermore, a large number of gustatory receptors has been identified in the genome of *T. urticae*. This expansion might potentially allow this spider mite to taste a greater diversity of chemical cues from its hosts and respond to it appropriately, yet further research is needed to verify the relevance of this expansion with regard to polyphagy (Van Leeuwen and Dermauw, 2016). Lastly, certain transcription factor families are expanded in *T. urticae*, potentially facilitating the coordination of xenobiotic transcriptional responses (Van Leeuwen and Dermauw, 2016). This brings us to another aspect of the mite’s plant colonization success. Most of *T. urticae*’s molecular adaptations to polyphagy are not static, and the expression levels of the aforementioned genes are often highly responsive to host plant switches (Dermauw et al., 2013b; Van Leeuwen and Dermauw, 2016; Wybouw et al., 2012, 2014). Indeed, about one fifth of all *T. urticae* genes

are differentially expressed upon host transfer, with genes in detoxification and peptidase families exhibiting the most profound changes (Grbić et al., 2011). Many differentially expressed genes lack homology to genes of known function, and genes with the most severe fold-changes are encoding putative secreted proteins or lipid-binding proteins (Grbić et al., 2011). While adaptation to challenging hosts most often results in the upregulation of genes involved in resistance, downregulation of certain genes has also been observed. This is, for example, the case for low-density lipoprotein receptor protein (LDLRs) genes. This downregulation may lead to lower receptor-mediated endocytosis of allelochemicals, and as such, a lower uptake into the mite cells (Dermauw et al., 2013b).

As such, adaptation of *T. urticae* to a new host plant alters gene expression in two ways, i.e., by changing the mite's constitutive transcript levels (stable gene expression changes over generations) and by transcriptional plasticity (within generations) (Van Leeuwen and Dermauw, 2016; Wybouw et al., 2015). Transcriptional plasticity might increase *T. urticae*'s short-term reproductive performance, yet its great adaptive potential is believed to be mainly due to the population's standing genetic variation in constitutive expression (Wybouw et al., 2015).

1.3.3.2. Defense manipulation

Although *T. urticae* can count on a variety of detoxifying systems, plant defenses nevertheless reduce spider mite reproductive performance (Alba et al., 2015; Ament et al., 2004; Ataide et al., 2016; Kandoth et al., 2007; Kant et al., 2008). In *Arabidopsis*, for example, *T. urticae* herbivory activates indole glucosinolate production. This is perceived by the spider mite, which reacts by changing the expression level of genes implicated in the detoxification. Yet, this proves insufficient to fully detoxify the secondary metabolites (Zhurov et al., 2014). Defense manipulation may therefore prove beneficial, and has indeed been shown in spider mites. For example, the Solanaceae specialist *T. evansi* performed better on tomato plants previously attacked by conspecifics than on non-attacked plants. This is quite remarkable since, in general, herbivore attack induces defenses which have a negative effect on subsequent attackers. However, not only did *T. evansi* prevent defense induction, it reduced housekeeping levels of defense-related constituents below the levels of non-attacked plants (Sarmiento et al., 2011).

Next to distinct variation in traits that lead to resistance and susceptibility to plant defenses, variation also exists in traits responsible for induction or repression of these defenses (Kant et al., 2008). During adaptation, mites can acquire the ability to manipulate host physiology (Wybouw et al., 2015). Spider mites which inhibit or prevent the defense induction may have a fitness advantage, although competing herbivores can profit from these suppressed plant defenses as well (Alba et al., 2015). Distinct mite strains can trigger different defense responses in the same host plant, while also the effect of these defense responses on the mites can differ (Alba et al., 2015; Takabayashi et al., 2000). Particular spider mite lines can induce defenses and be affected by them, while other lines also induce these defenses yet are resistant to them. Again other lines are susceptible to the defenses yet repress their induction (Alba et al., 2015; Kant et al., 2008). This intraspecific variation is proposed to be common among herbivores living in environments with a diversity of plants that impose diverse selection pressure (Kant et al., 2008).

A method to identify defense suppressing mites has been described by Kant et al. (2008). First, the fecundity of JA-defense-suppressor strains should be equally high on wild type (WT) plants as on JA-biosynthesis mutants (e.g., *def-1*) since suppression will only be favored by natural selection when improving the reproductive performance. However, this relatively high reproductive performance can also be the result of direct resistance to induced JA defenses. Therefore, secondly, genuine suppressors should be able to boost the reproductive performance of defense-susceptible mites when both reside on the same leaf or leaflet (Alba et al., 2015; Kant et al., 2008).

Suppression of plant defenses by *T. urticae* and *T. evansi* occurs downstream of JA and SA accumulation and is independent of the JA-SA antagonism (Alba et al., 2015; Godinho et al., 2015; Kant et al., 2008; Sarmiento et al., 2011). *Tetranychus kanzawai*, however, is suggested to manipulate the JA-SA crosstalk to suppress JA defenses (Ozawa et al., 2011).

The relative importance of defense manipulation versus detoxification in the plant-mite interaction remains to be determined. This PhD thesis provides valuable insights to address this issue more comprehensively (**Chapter 6**).

1.3.4. Host plant responses to spider mite attack

Tetranychus urticae is a relevant organism to study plant-herbivore interactions. Due to its ability to feed on several model plants, the genome-wide

response against the two-spotted spider mite could be investigated in *Arabidopsis* (Zhurov et al., 2014), tomato (*S. lycopersicum*) (Kant et al., 2004; Martel et al., 2015) and grapevine (*V. vinifera*) (Díaz-Riquelme et al., 2016).

In attacked plants, *T. urticae* induces a set of genes associated with the biosynthesis of JA and its signaling. This set of induced genes appears to be conserved between different plant species. On the other hand, the downstream JA-regulated genes seem to be highly divergent between different hosts. This reflects differences in secondary metabolism amongst plant species (Díaz-Riquelme et al., 2016). For example, *Arabidopsis* relies on indole glucosinolates, while tomato defenses are mostly based on anti-digestive proteins such as PIs, leucine amino peptidase, threonine deaminase and PPOs. Grapevine, however, counts on both anti-digestive proteins and defensive metabolites (e.g., stilbenes) (Díaz-Riquelme et al., 2016). Next to the induction of JA, non-suppressing strains of *T. urticae* also evoke SA responses. Such cocktail of JA and SA plant defense responses is common for stylet-feeding herbivores (Alba et al., 2015; Kaloshian and Walling, 2005).

Although host plants, like grapevine, show a strong defense response against *T. urticae*, adapted mites may nevertheless flourish on these hosts. A *T. urticae* strain adapted to grapevine, for example, does not seem to rely on defense suppression but is likely to have evolved mechanisms to detoxify grapevine defensive compounds (Díaz-Riquelme et al., 2016). Furthermore, a recent study suggested that mite bacterial symbionts affect plant responses and mite performance, although clear causal links remain to be discovered (Staudacher et al., 2017). However, this suggest symbionts may further complicate the study of the plant-mite interaction.

It may be clear by now that the molecular interaction between spider mites and plants is of significant importance, yet also highly complex. During this thesis, the role of spider mite saliva in this molecular interaction is investigated, specifically in the light of the highly polyphagous nature of *T. urticae*.

1.4. GENERAL OUTLINE OF THIS THESIS

The herbivorous spider mite *Tetranychus urticae* (Acari: Tetranychidae) is notorious for having an extremely large host plant range. Being this polyphagous is exceptional since plants possess various defensive mechanisms. Herbivores need to be able to deal with these plant defenses in order to thrive on these hosts. This process requires adaptation, often leading to

specialization. Nevertheless, *T. urticae*, as a species, is able to feed on plants belonging to more than 140 families, and must therefore have evolved a variety of mechanisms to deal with a diverse arsenal of defenses. In arthropod herbivores, the saliva has been proposed as an important mediator of digestion, detoxification and defense suppression. The aim of this PhD thesis was to investigate the composition and role of spider mite saliva during host plant colonization, particularly from the perspective of the broad host plant range of *T. urticae*.

Chapter 2 describes a first strategy to determine the proteomic composition of *T. urticae* and *T. evansi* saliva. In this approach, a pipeline heavily relying on *in silico* prediction was used. Furthermore, a selection of salivary proteins was studied in more detail in an attempt to unravel their function during the plant-mite interaction.

The *in silico*-based approach proved to be a suitable method to identify spider mite effectors. Yet, this approach has its limitations. Therefore, in **Chapter 3**, the proteomic composition of secreted *T. urticae* saliva was determined using nano-LC-MS/MS analysis of spider mite-probed artificial diet. Since we suspected the salivary composition to be host plant dependent, mites from lines adapted to various host plants were included in the study, ascertaining the discovery of a broader range of salivary proteins in the diet. To filter out contaminating non-salivary proteins, the proteomics data were verified using RNAseq data of the anterior body region of *T. urticae*, which harbors the salivary glands. Finally, the expression localization of some putative salivary proteins was investigated using whole-mount *in situ* hybridization, confirming their salivary origin. As suspected, host plant-dependency of the saliva composition was evident from the proteomics data. This variability was studied in closer detail in **Chapter 4**, complementing the proteomics data with transcriptomics evidence. Furthermore, the occurrence of homologs of the identified *T. urticae* salivary proteins in the related species *T. lintearius* and *T. evansi* was investigated by *in silico* genome comparison. One salivary protein family (SHOT family) was of particular interest and was studied in more detail in **Chapter 5**. Additionally, the peptide content of secreted *T. urticae* saliva was investigated.

Finally, in **Chapter 6**, the findings are integrated and the implications of the spider mite salivary protein repertoire on the host plant range are discussed.

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General introduction

Zhurov, V., Navarro, M., Bruinsma, K.A., Arbona, V., Santamaria, M.E., Cazaux, M., Wybouw, N., Osborne, E.J., Ens, C., and Rioja, C. (2014). Reciprocal responses in the interaction between *Arabidopsis* and the cell-content-feeding chelicerate herbivore spider mite. *Plant Physiol* *164*, 384-399.