The salivary proteome of Tetranychus urticae

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6

General discussion

6.0. Abstract

In the previous chapters, the salivary proteome of spider mites was investigated, with a focus on the extremely generalist species *Tetranychus urticae*. The main aims were to identify effectors, digestive proteins and proteins involved in detoxification of secondary plant metabolites. Effectors are molecules that the mite evolved in order to manipulate the plant's cellular actions so that the plant becomes a better food source. Both a bio-informatics prediction approach and a proteomics-based approach revealed that the salivary proteome is key to *T. urticae*'s polyphagous nature. The datasets produced in this PhD study will be a starting point to further unravel the molecular interplay between spider mites and their host plants. This may eventually lead to the discovery of plant resistance (R) and susceptibility (S) genes, in turn aiding the development of crops with an enhanced resistance or a reduced susceptibility to spider mites. However, the sustainable application of these (modified) plant genes in crop protection might prove challenging. After all, *T. urticae* likely exhibits polymorphism in effector genes and is, above all, a master in detoxification as well.

6.1. Introduction

Spider mites are important crop pests with a worldwide distribution. The two-spotted spider mite, *T. urticae*, is the most notorious one as it can thrive on a wide range of different host plants and possesses the ability to quickly develop resistance against acaricides with various modes of action (Van Leeuwen et al., 2010). Furthermore, many arthropod herbivores have been shown to manipulate defense responses allowing them to thrive on their host plants. The main molecular mediators, so-called effectors, are assumed to be transferred through saliva (Mutti et al., 2008; Hogenhout and Bos, 2011; Musser et al., 2012). Recent research suggests this is also the case for spider mites (Takabayashi et al., 2000; Kant et al., 2008; Sarmento et al., 2011, Alba et al., 2015; Schimmel et al., 2017).

Although *T. urticae* is a model organism for studies on the evolution of host specialization (Fellous et al., 2014), hardly any information about spider mite saliva was available at the onset of this PhD research project. We aimed to fill this void in scientific knowledge by using a multi-disciplinary approach based on proteomics, transcriptomics and *in silico* predictions, supplemented with comparative genomics and functional analysis. In what follows, the findings from the different approaches are integrated in the plant-mite molecular interaction and linked to the broader context of spider mite biology.

6.2. Evaluation of the used approaches

In this thesis, two different approaches were used to identify spider mite salivary (effector) proteins. A first approach relied heavily on a bio-informatics pipeline to filter relevant protein sequence from genome and transcriptome datasets (**Chapter 2**), while a second approach was founded on nano-LC tandem mass spectrometry of secreted mite saliva (**Chapter 3**).

The first approach, an *in silico*-based method, allowed the identification of putative effector proteins of *T. urticae* and *T. evansi* (**Chapter 2**). Such bio-informatics approaches produce, depending on the stringency of the selection criteria, long lists of candidate effectors which require experimental confirmation to verify if these proteins are indeed effectors. A first condition is that the proteins should be secreted into the host. Confirmation of expression of the corresponding gene in the salivary glands by *in situ* hybridization (ISH) is a strong indication that the identified protein is secreted into the host plant and therefore may be an effector (Haegeman et

al., 2012). However, this ISH technique is labor-intensive, hampering its use as a screening technique. Therefore, stringent selection criteria were used in the *in silico* approach to come up with a top list of high quality effector candidates. This inevitably may involve the loss of promising effector proteins from the dataset. A complementary proteomic approach was therefore initiated, focusing on the analysis of artificial diet fed upon by *T. urticae* (**Chapter 3**). Mite lines adapted to distinct hosts were used to ascertain a broad range of salivary proteins could be identified, including those with a host-dependent production level. Identification of proteins by this proteomics approach is in itself already a good indication that the protein is secreted into the host. Implementing proterosoma-specific expression data further aided in distinguishing between salivary proteins and contaminating proteins, while also *in silico* selection criteria (e.g., the presence of a signal peptide) were used to prioritize candidates for further study. For example, on the one hand, protein tetur03g07920 was identified in artificial diet fed upon by *T. urticae* and is annotated as a glutathione S-transferase, which is known to act as a salivary effector in aphids (Kettles and Kaloshian, 2016). However, the expression of the encoding gene is downregulated in the mite body region that contains the salivary glands (proterosoma) and the protein is not predicted to be secreted. As such, this glutathione S-transferase likely does not end up in the saliva and does not act as an effector. Proteins belonging to Tu_MCL_211 and Tu_MCL_25, on the other hand, were also identified in the artificial diet, yet the encoding genes are highly expressed in the proterosoma. Additionally, they are predicted to be secreted. Whole-mount ISH supported the salivary nature of these proteins, while some family members were indeed shown to act as effectors (**Chapter 2**). Thanks to the stringent selection criteria, most of the selected genes (17/18) eventually tested with whole-mount ISH proved to be expressed in the salivary glands (**Chapter 3**). These genes should be prime candidates for further study.

Both the bio-informatics- and proteomics-based approaches have their limitations. For example, only a fraction of the peptides present in a proteomics sample will be detected using a specific LC-MS/MS procedure (Muntel et al., 2015). Also, if a protein is not produced or secreted at the time of saliva collection, for example due to host plant-specificity, it will not be detected. Hypothetically, a spider mite may sense that it is feeding on non-host material (i.e., the artificial diet), and secrete saliva with another

composition. *Tetranychus urticae* specimens fed well on the artificial diet hemispheres, as assessed by blue-staining of the gut in control mites feeding on erioglaucine-supplemented diet. In addition, the presence of proteins in the diet, of which the production site has been confirmed to be the salivary glands (dorsal and anterior podocephalic glands), confirms that the secretions of the glands are injected into the diet. Therefore, it is assumed that the artificial diet is accepted as a food source by the mite. Further, the qualitative composition of saliva that is secreted in the artificial diet within the 24 h time window of collection is assumed to be representative of saliva secreted in the original host, while the quantitative composition may differ slightly from the saliva secreted in the plant due to 'host-dependent' transcriptional plasticity. Indeed, the expression of certain salivary genes may change as soon as the mite is transferred to the artificial diet, which may be perceived by the mite as a new host plant. Therefore, gene expression data from the mite lines collected directly from their hosts should be used as an additional source of quantitative information on the salivary protein composition and its host-dependency (**Chapters 4 & 5**).

The bio-informatics based approach consisted of a filtering strategy based on two main phases: firstly genome-wide secretome prediction and secondly effector repertoire prediction (**Chapter 2**). In the approach, selecting for proteins with a signal peptide (SP) was one of the main selection steps (Bos et al., 2010; Carolan et al., 2011; Atamian et al., 2013; Thorpe et al., 2016). Even though this selection criterion is often used for secretome prediction, proteomics studies did point out that many proteins detected in herbivore saliva may not possess such SPs. Indeed, proteins might be secreted using a secretory pathway independent of the canonical endoplasmic reticulum-Golgi network (Haegeman et al., 2012). Furthermore, much depends on the quality of the used database. For example, SPs can simply be overlooked because 5'-sequences required for SP prediction can be missing from datasets such as (*de novo*) transcriptome sequences (Thorpe et al., 2016). In our proteomics-based approach (**Chapter 3**), the presence of a SP was used as a means to prioritize candidates rather than as a strict selection criterion (i.e., proteins without predicted SP were maintained in the dataset). Indeed, the detection of a protein in collected salivary secretions rendered the SP-criterion rather accessory. From our data (TABLE 3.1), however, it was clear that most identified salivary proteins possess a SP. In addition, the apparent absence of a SP for some of the detected proteins

appeared to be due to incomplete annotation. It can be concluded that 'presence of a SP' is indeed a valuable criterion to select for salivary (effector) proteins in bio-informatics approaches, and that a potential loss of some false negatives is currently acceptable.

In **Chapter 2**, five candidate effector families from the bio-informatics prediction approach were selected for further analysis, being family numbers 19, 28, 84, 90 and 128 (TABLE 2.1). Proteins belonging to Tu28 and Tu84 were indeed shown to act as effectors. Proteins from these two groups were also identified in the proteomics approach, as were proteins belonging to Tu128 (TABLE 3.1) (**Chapter 3**). Although no proteins belonging to the Tu18 and Tu90 family could be identified using the proteomics approach, some homologs of Tu90 family members were found (DATA S4.1 and S4.2) (**Chapter 4**). This illustrates there is overlap between the bio-informatics and the proteomics results, and both can be used to identify effector proteins. Unless strict selection criteria are used, the bio-informatics method may, however, deliver irrelevant proteins. The proteomics approach likely underestimates the number of salivary proteins, although a genome search for homologs may add missed candidates.

The whole-mount ISH technique was of utmost importance during this PhD study. In both the bio-informatics as the proteomics approach, ISH was used to prove that the genes encoding some of the identified proteins are expressed in the salivary glands. As such, ISH made clear that the majority of the identified proteins which fulfill the selection criteria are indeed *bona fide* salivary proteins. Additionally, ISH allowed us to partly unravel the functional complexity of spider mite salivary glands. The salivary genes were shown to be expressed in either the dorsal or the anterior podocephalic gland. Such 'division of labor' has been described for insects such as aphids (Mutti et al., 2008; Pan et al., 2015; Wang et al., 2015) and thrips (Stafford-Banks et al., 2014) (**Chapter 3**), yet the functional relevance in spider mites is still unknown.

6.3. A PROPOSED FUNCTIONAL FRAMEWORK FOR *T. URTICAE* EFFECTORS

6.3.1. The 'zigzag' model and polyphagous attackers

A four phased 'zigzag' model of the plant immune system (FIGURE 1.2) has been presented by Jones and Dangl (2006). Basically, it distinguishes two related categories of immunological mechanisms. Firstly there is basal or

innate immunity, and secondly there is R-gene-mediated immunity (gene-for-gene interactions), in which an evolutionary arms race between the host and its antagonist results in an oscillation between susceptible and resistant states of the host over time. A decade has passed since the publication of this landmark paper, and it has become clear that the use of the model is not limited to plant-pathogen interactions but applies to plant-herbivore interactions as well (Poland et al., 2009; Stuart, 2015; Rodriguez et al., 2017) (**Chapter 1**). R-gene-mediated immunity has been described for the interaction between (amongst others) the Asian rice gall midge (*Orseolia oryzae*) and rice (*Oryza sativa*), the small brown planthopper (*Nilaparvata lugens*) and rice, and the Hessian fly (*Mayetiola destructor*) and wheat (*Triticum* spp.) (reviewed in Stuart, 2015). However, it is important to keep in mind that none of these herbivores are polyphagous, and all have specialized to feed on a limited number of related host plants. This implies a close molecular interaction, much alike in most plant-pathogen interactions. The Hessian fly, for example, forces its host plants to create an enriched gall nutritive tissue that feeds the larva (Harris et al., 2006; Stuart et al., 2012). Notably, the defense response of rice plants against *O. oryzae* involves a hypersensitive response (HR) (Rawat et al., 2012; Stuart, 2015), which has been argued to be the most common plant resistance mechanism against insect herbivores that have intimate associations with their host plants, including gall formers (Fernandes and Negreiros 2001). Incompatibility in these intimate associations has severe consequences on the survival of the antagonist. As such, the plant defense response appears to result in a 'black or white' situation: if there is an interaction between an R-gene-product and the cognate effector, the host is fully resistant. If there is no interaction, the host is fully susceptible. However, resistance levels are in fact shades of gray ranging from complete resistance to incomplete resistance. These two general categories of plant disease resistance are conditioned by a single gene or by multiple genes of partial effect, respectively. Other terms have been used to refer to this dichotomy, including 'qualitative disease resistance' versus 'quantitative disease resistance', where R genes are sometimes used to refer to genes that confer complete effects explicitly (Poland et al., 2009).

Because of its mobility (relative to pathogens and gall formers such as *O. oryzae* and *M. destructor*) and its extensive detoxification mechanisms, it can be assumed that the occurrence of complete or qualitative R-gene-

mediated resistance will be rare against *T. urticae*. Quantitative or incomplete resistance is, however, likely to exist and may aid in reducing spider mite performance. In light of this, a 'moderated' version of the zigzag model has here been applied to the plant-mite interaction.

6.3.2. The plant-mite molecular battlefield: the role of salivary components

As soon as *T. urticae* arrives on its host plant, it may start gathering information about the host plant identity. Indeed, it may 'taste' or 'smell' plant components via cuticular pores in neuron-rich setae on its palps and legs (Bostanian and Morrison, 1973), which likely represent chemosensory sensilla (Ngoc et al., 2016). Clusters of chemosensory receptors are expanded in *T. urticae*. These receptors may allow the polyphagous mite to detect a huge variety of different compounds, potentially including sex pheromones (Ngoc et al., 2016), although it would not come as a surprise if most of these receptors would recognize molecules of plant origin. Chemosensory perception may affect gene expression changes and may activate detoxification pathways (Ngoc et al., 2016). Although no evidence is at hand, it is tempting to speculate on a mechanism whereby the chemosensory perceptions influence expression levels of some effectors as well (**Chapter 5**). As such, *T. urticae* may be prepared to deal with the host plant at hand, even before feeding has started. This is still hypothetical and may prove interesting for future research.

Tetranychus urticae inserts its stylet between epidermal pavement cells or through stomatal openings in order to feed on the underlying mesophyll cells (Bensoussan et al., 2016). It is known that certain enzymes of phytophagous insects allow stylet penetration through the intercellular matrix. For example, the aphid stylet path travels intercellularly. Pectic compounds, cementing adjacent cells, can be dissolved by pectinases contained within the aphid's saliva (McAllan and Adams, 1961; Celorio-Mancera et al., 2009; Raman, 2012; Stafford-Banks et al., 2014). Likely, enzymes with such function will also be among the identified *T. urticae* salivary proteins. We have shown that *T. urticae* saliva contains several proteins with a glycoside hydrolase function (Table S3.6). Two of such proteins (encoded by *tetur16g03420* and *tetur28g00360*) are annotated as β-mannosidase and are highly upregulated in the proterosoma, while one of the genes (*tetur28g00360*) was shown to be specifically expressed in the anterior podocephalic glands (Table 3.1).

Clearly, these are likely *bona fide* salivary proteins. Glycoside hydrolases are known to be involved in the degradation of plant cell wall polysaccharides (Minic and Jouanin, 2006). As such, these two proteins may facilitate stylet penetration in *T. urticae*, or they may be involved in extra-oral digestion of plant cell walls. However, a detailed study of their physiological role during spider mite feeding remains to be made.

Spider mites belong to the Chelicerata, and in predatory chelicerates such as spiders, horseshoe crabs and scorpions, pre-oral digestion is a common digestive strategy. Also predatory mites utilize secreted proteins to facilitate prey consumption (Cohen, 1995; Bensoussan et al., 2016). For example, the predator mite *Phytoseiulus persimilis* spends the first 20 minutes of its feeding on *T. urticae* to a pre-oral digestive process (Pérez-Sayas et al., 2015). Analogously, the duration of the consumption of a single mesophyll cell by *T. urticae* ranges from several minutes to more than half an hour (Bensoussan et al., 2016). This time window seems reasonable for pre-oral digestion reactions to occur. A way to check if extra-oral digestion also occurs in spider mites would be to add compounds such as proteins and carbohydrates to the artificial diet used in **Chapter 3** and to verify if break-down products can be detected in the diet hemisphere after spider mite feeding. The detection of cleaved peptides, originating from salivary proteins, in the artificial diet points towards the activity of proteases in spider mite saliva (**Chapter 5**). The top-10 list of the most abundantly identified *T. urticae* salivary proteins contains two proteins annotated as serine protease. (Table 3.1). Serine proteases are common salivary proteases attributed to digestion of dietary proteins by phytophagous insects (Stafford-Banks et al., 2014) and based on our results we concluded that they are also important in phytophagous mites.

As soon as the mite starts feeding, constitutive plant defensive compounds may be partially disarmed by the mite's extensive detoxification pathways (Wybouw et al., 2012; Dermauw et al., 2013). While it can be suggested that detoxification mainly occurs inside the mite body (e.g., ABC transporters are membrane-spanning proteins; Dermauw, 2013), the mite injects putative salivary detoxification proteins into the host cells as well (**Chapter 3**). The expression of mite genes involved in detoxification pathways can be upregulated upon initial exposure to the novel plant, increasing short-term reproductive performance (Wybouw et al., 2015). As mentioned before, chemosensory receptors may be involved in the activation of this process (Ngoc et al., 2016, Van Leeuwen and Dermauw 2016).

In the generations following the colonization of a new host plant, mites will adapt and improved detoxification genotypes will gradually be selected out of the founder population. Indeed, mites exhibit genetic polymorphism in resistance characteristics (Kant et al., 2008). The host history of the mites will determine the performance on the new host. For instance, mite adaptation to a certain host plant can lead to pre-adaptation to live on other plant species through pleiotropy (Fellous et al., 2014). Alternatively, adaptation can result in a loss of the ability to grow on an original host (Fellous et al., 2014). Mites are likely to have a higher success in colonizing plants which are closely related to the original host, as, for example, the salivary composition (**Chapter 4**) and the detoxification metabolism are already predisposed to deal with certain classes of phylogenetically specific allelochemicals.

Tetranychus urticae appears to feed in a way that limits host plant mechanical damage (Bensoussan et al., 2016). Nevertheless, plant defenses are induced, in many cases leading to visible chlorotic spots. Plants carry pattern recognition receptors (PRRs) at their cell surface that mediate the recognition of slowly evolving pathogen-derived molecules, referred to as PAMPs (pathogen-associated molecular patterns) (Jones and Dangl, 2006; Schie and Takken, 2014). In analogy, it can be assumed that PRRs also recognize 'herbivore-associated molecular patterns' (Mithöfer and Boland, 2008; Hogenhout and Bos, 2011). These HAMPs are conserved molecules, and avoidance of recognition through alterations (caused by mutations) in these molecules are assumed to be rare since most would have severe consequences for mite survival. A proposed example of such spider mite HAMP is chitin, the main component of the exoskeleton and gut lining (Merzendorfer and Zimoch, 2003; Díaz-Riquelme et al., 2016). Interestingly, a putative chitin-degrading protein (chitinase, encoded by *tetur01g11910*) has been identified in *T. urticae* saliva (**Chapter 3**). The relevance of this protein in the molecular battlefield between *T. urticae* and its hosts remains to be determined. In plant-parasitic nematodes chitinase has been attributed effector-like properties (Gao et al., 2002, 2003). Yet, plants also produce chitinases in response to spider mite attack (Ozawa et al., 2011). Clearly, further research is needed.

Next to HAMPs, plant-derived DAMPs (damage-associated molecular patterns), including cell wall fragments, are also recognized by PRRs. Whether the putative digestive enzymes in *T. urticae*'s saliva generate such DAMPs (e.g., by the hypothetical extra-oral digestion of plant cell walls) is unknown. HAMP or DAMP recognition by PRRs activates plant defense

responses designated 'HAMP-triggered immunity' (HTI). Since HTI is activated by evolutionary conserved molecules, it offers broad recognition. During mite attack, it may for example recognize a shared component of all Acari or even all Arthropoda. HTI involves a whole set of actions aiming to counter the mite attack, and may include the production of proteinase inhibitors, polyphenol oxidases and indole glucosinolates. These defense compounds are host specific and reflect differences in secondary metabolism between these plants (Díaz-Riquelme et al., 2016). In response to this diversity in potentially encountered compounds, the polyphagous *T. urticae* likely evolved a plethora of salivary protein variants (expansions) (**Chapters 2 & 3**). Due to their limited host range, mono- or oligophagous spider mites such as *T. lintearius* and *T. evansi* are exposed to a lower diversity of plant allelochemicals. This could be at the basis of their less varied salivary protein repertoire (**Chapter 4**).

The ubiquitous presence of different (serine) proteases in *T. urticae* saliva may be a means to compensate for the inactivation of gut proteases by plant proteinase inhibitors. The use of such 'decoys' has been described in plant pathogens (Ma et al., 2017). Furthermore, salivary 'trypsin inhibitor-like' proteins may inhibit digestion-inhibiting plant serine proteases. In addition, spider mites likely evolved a plethora of salivary effector molecules which alter host cell structure and function (**Chapters 2, 3, 4 & 5**), and whose activity may lead to effector-triggered susceptibility (ETS) (Jones and Dangl, 2006; van Schie and Takken, 2014). This ETS is unlikely to involve a complete shutdown of the plant immune system, but each effector may turn the plant into a more susceptible host for *T. urticae*. Findings advocating such 'incomplete susceptibility' are presented throughout this thesis and in literature about plant-mite interactions (e.g., Kant et al., 2008). As shown in previous chapters, the number of putative effector proteins contained within the saliva is plentiful. Each likely has a partial effect on the eventual defense state of a particular host. Some of the effectors will act as elicitors (e.g., potentially SHOT1 & 2 in non-fabacean hosts; **Chapter 5**), activating certain defense responses. Others will act as virulence factors, manipulating a specific reaction or interaction somewhere in the complex defense pathway. Since the effector-mediated defense suppression appears to occur downstream of the main phytohormone hub (Alba et al., 2015), it is conceivable that only one of the many downstream defense branches is targeted by a single effector, and the realized mite fit-

ness on a particular host is the result of positive and negative actions of several effectors. Also, recombinant expression of individual effectors in leaves only had a small effect on spider mite performance, which may support this hypothesis (**Chapters 2 & 5**). Alternatively, however, it is suggested that – given the broad host range of *T. urticae* – defense manipulation must occur at early and conserved elements in the pathway. Otherwise, specific mechanism should have evolved for each of the host plant species (Van Leeuwen and Dermauw, 2016). Additional research is clearly needed to help resolve this contradiction.

While effectors are most often associated with defense-suppression, the broad inclusive definition (Hogenhout et al., 2009) allows several other potential functions. Salivary proteins involved in pre-digestion can hardly be seen as effectors, but mite proteins redirecting the plant nutrient flow towards the feeding site or the aboveground tissues fully pertain to the effector definition. Indeed, they are secreted by the attacker (i.e., pathogen or herbivore) and alter host-cell structure or function (Hogenhout et al., 2009). Such effectors, turning the feeding site in a better food source are described in other herbivores, including the Hessian fly (Stuart et al., 2012) and several nematode species (Haegeman et al., 2012). It is possible that such molecules are also included in spider mite saliva, although they may not necessarily be proteins. Phytohormone-mimics have been suggested to occur in spider mite saliva (Storms, 1971), yet this still needs to be confirmed.

To counter the activity of effectors, plants evolved the ability to recognize either the effector itself or its modification inflicted on a host protein using resistance (R) genes, in most cases encoding intracellular nucleotide-binding leucine-rich-repeat (NB-LRR) proteins. R-gene-mediated immunity is referred to as effector-triggered immunity (ETI). ETI is more specific than HTI as effectors are highly polymorphic, and ETI can be considered as an amplified version of HTI (Jones and Dangl, 2006; van Schie and Takken, 2014). ETI would favor mites which have lost the elicitor, or which have an effector variant which does no longer trigger ETI. Indeed, effectors which trigger ETI are under strong negative selection pressure and the encoding genes often evolve rapidly. The resulting arms race forces the mite to continuously evolve new strategies to evade or suppress HTI and ETI (Schie and Takken, 2014). This arms race is traceable in genomes of herbivores (Stuart, 2015). This is also the case for *T. urticae* since it possesses an arsenal of spider mite-specific expanded effector-encoding gene fam-

ilies (**Chapters 2, 3 & 5**). Furthermore, R-gene-mediated recognition might hypothetically favor mites which limit the expression of the associated gene when the mite is feeding on particular plant species (**Chapter 4**).

After colonization of a new host plant, gene expression changes may occur during the first generation. Over several generations, the frequency of mites with 'optimal' effector genotypes and detoxification genotypes increases. An important factor in this process may be the size of the founder population. Colonization of a new host plant by a group of spider mites (e.g., in a 'collective silk ball'; Clotuche et al., 2011), will likely have higher chances of being successful than colonization by a single mite, since a more diverse mite genepool will be present for selection to work on. After some generations, a well-adapted mite strain may become established. Formerly abundant alleles may persist in the background and re-emerge if the gene product offers the mite a fitness advantage when the conditions change, e.g., after a host plant shift or when a new host plant genotype appears due to co-evolution. Indeed, in an environment where frequent host shifts occur, balancing selection may sustain genotypic and phenotypic diversification of resistance and defense manipulation characteristics. While data on the evolution of (putative) effectors and their effect on mite fitness are not provided by this PhD study, the absence of *SHOT3* genes in the *Ulex* specialist *T. lintearius*, for example, may illustrate that a useless effector can be lost if a mite strain is maintained on a particular host for a long period of time (**Chapter 5**). Additionally, new genotypes might arise through mutations. Again, an example may be found in **Chapter 5**: it can be hypothesized that *SHOT1 & 2* genes evolved from *SHOT3* genes, and that this event co-occurred with a host transfer from the common ancestor of *T. urticae* and *T. lintearius* to a fabacean host.

During the evolution of effectors, different mite genotypes may have an influence on each other. Indeed, defense-suppressing mite genotypes may enhance the fitness of non-defense-suppressing genotypes residing on the same plant (Kant et al., 2008; Alba et al., 2015). As such, it can be hypothesized that they temporary prevent the extinction of these genotypes, and the loss of potentially 'valuable' alleles from the population.

The mesophyll cell on which the spider mite is feeding is unlikely to mount a defense response by itself. Indeed, stylet puncturing will disturb intracellular ion balances, and mite-derived digestive proteins may likely disable the cell's signaling processes. Furthermore, the content will be withdrawn from the plant cell within several minutes (Bensoussan et al.,

2016). It is therefore conceivable that the defenses are initiated in the neighboring plant cells. Upon puncturing the plant cell and injecting it with saliva, molecules derived from the mite and the injured plant cell [e.g., phosphatidic acid (Kant et al., 2004), ROS, Ca2+, extracellular ATP, effectors (Guiguet et al., 2016)] spread throughout the apoplast and the plasmodesmata and will interact with extra- and intracellular receptors of the neighboring cells. Electrical signals may be involved as well. In these cells, the defenses are then activated, and signals are sent to distant plant parts to prepare them for future mite attack (systemic acquired resistance; SAR).

6.4. R and S genes: mite-resistant crops

Among the more than thousand different *T. urticae* host plants, 150 are of great economic value (Santamaría et al., 2012). Some important crops were used in this study to establish mite lines on: bean, cotton, maize, soybean and tomato (**Chapter 3 & 4**). Other well-known agriculturally important plants which can be affected include grape vine, corn, apple, strawberry, pea, pepper and cucumber (Migeon and Dorkeld, 2006-2017). To limit the associated crop loss, farmers commonly rely on acaricides. However, *T. urticae* is known to quickly evolve resistance to these compounds (Van Leeuwen et al., 2015; Van Leeuwen and Dermauw, 2016), an asset that appears to be linked to the mite's evolutionary history. Indeed, the adaptation to an extreme diversity of host plant species has equipped *T. urticae* with metabolic tools allowing it to deal with a variety of plant toxins, tools which come in handy when a synthetic chemical is encountered as well (Grbić et al., 2011; Dermauw et al., 2013; Van Leeuwen et al., 2015; Van Leeuwen and Dermauw, 2016). Nowadays, *T. urticae* has developed resistance to almost all chemical classes used for its control. Furthermore, the application of pesticides may negatively affect the environment and human health, and as such, alternative control measures are desirable (Van Leeuwen et al., 2015). One of the proposed alternatives are biotech crops, engineered for mite resistance.

Resistance against herbivorous arthropods in transgenic plants is commonly obtained through the insertion of toxin genes, e.g., Bt (*Bacillus thuringiensis*) toxin genes. These Bt genes encode Cry proteins, which are solubilized and activated in the insect midgut, after which they form pores in the gut epithelial membrane, leading to cell lysis and eventually death (Vachon, 2012; Lombardo, 2016). Next to Bt toxin genes, one relies on transgenic plants overexpressing inhibitors of proteinases, lectins, α-amylase inhibitors,

chitinases and biotin-binding proteins. These compounds combat phytophagous pests by altering their digestive system or by reducing the availability of nutrients (Lombardo et al., 2016). Instead of developing plants which produce such anti-herbivore compounds, an alternative strategy may be to modify plants in a way that they gain the ability to recognize the attacker (via R genes), or do not longer possess certain genes that facilitate infection and support compatibility (S genes). Contrary to R genes, which are typically dominant, resistance conferred by loss or alteration of S genes is generally recessive. Hence, S gene mediated resistance is assumed to be more durable then R gene mediated resistance. Indeed, for an attacker to overcome R gene-based resistance, a simple point mutation in a protein or effector recognized by an NB-LRR or PRR may be sufficient to evade recognition. However, many effectors are recognized indirectly by monitoring the host target. As such, an effector would need to alter its activity on the host target, or it would need to disappear altogether. Since effectors are often redundant, the fitness penalty of losing the effector may be small making the recognition of conserved effectors more durable (Schie and Takken, 2014). For the attacker to overcome S gene-based resistance, it must overcome its dependency on a host factor. This involves the acquisition of a new function, which is more difficult to accomplish than a loss-of-function (Schie and Takken, 2014).

The list of spider mite salivary proteins presented in this thesis, including several putative effector families, can be a starting point for the identification of plant R and S genes. These genes can then be used by breeders to develop mite-resistant plants. Research and development costs for genetically engineered crops are high, and their widespread use will therefore depend on their effective lifetime. The main threat to this lifetime is the evolutionary change in the targeted pest species (Rausher, 2001). The overall rate of evolution depends on the turnover rate of individuals in populations (generation time) and the genomic variation among individuals (Gillooly et al., 2005). *Tetranychus urticae* has a very short life cycle (8-12 days) and a high fecundity, which leads to exponential population growth (Jeppson et al., 1975). As a result, a relatively high number of random mutations may occur during a given period of time. In addition, *T. urticae* has a worldwide distribution and has been recorded on an extremely high number of host plants (Migeon and Dorkeld, 2006-2017). Even within populations, there are different plant-defense-related genotypes (Kant et al., 2008; Alba et al., 2015). As such, the genomic variation among individuals can considered to be high.

Despite their huge potential, the development of sustainable mite-resistant crops mediated by R and S genes will not be straightforward, particularly not for *T. urticae*. Although we showed that salivary effectors are playing a role in the host plant colonization success of *T. urticae*, the mite's capability to feed on thousands of different hosts is presumably mainly accomplished by its extensive detoxification mechanisms. The relative importance of defense manipulation on the one hand, and detoxification of induced and constitutive defensive compounds on the other hand (FIGURE 6.1), remains to be determined. Furthermore, variation in this relative importance between mite strains is to be expected, while also the host plant may play a role (Kant et al., 2008; Díaz-Riquelme et al., 2016).

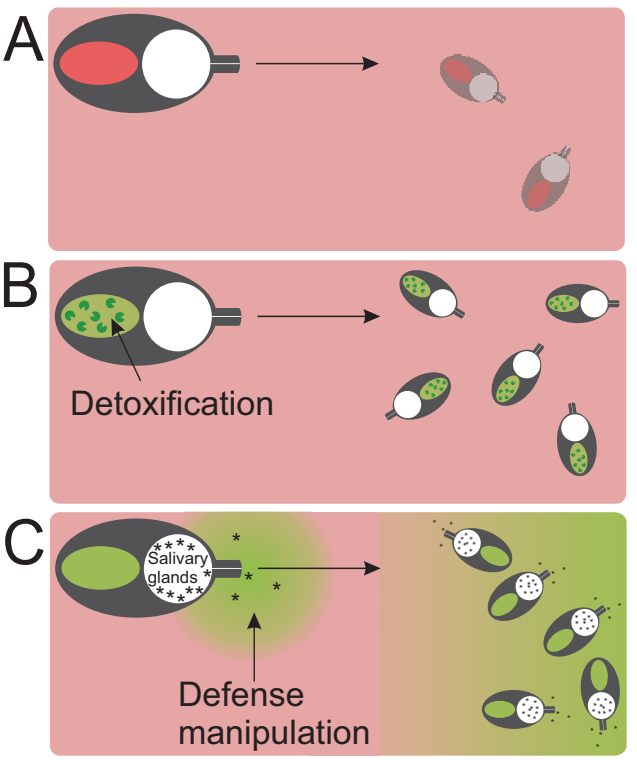

FIGURE 6.1. Detoxification and defense manipulation. **(A)** If plant defenses are present (pink leaf), and the mites do not possess the appropriate detoxification mechanisms, mites are unable to colonize the plant. **(B)** If the mites possess the appropriate detoxification mechanisms, a population can be established. Detoxification occurs inside the mite body, although detoxification proteins were also found in the saliva. **(C)** If the plant possesses strong defense mechanisms which mites cannot detoxify, a population can be established if salivary effectors (*) are employed that interfere with host defenses (green). The ability of spider mites to colonize a host thus depends on detoxification and/or defense manipulation. The relative importance of both is likely to be host plant and mite strain dependent.

Generalist species can be considered as conglomerates of specialized genotypes (Barrett and Heil, 2012). Therefore, it seems highly likely that different mite populations are characterized by different sets of salivary effectors. This can be assumed because some salivary genes show strong differential expression after just a few generations of adaptation to a new host **(Chapter 4)**. If the mite lines would be maintained on the same host for many generations, it can be assumed that useless effectors, characterized by extremely low expression levels, accumulate mutations without a fitness loss. Another cue for the existence of populations with a distinct effector set is that *T. urticae* shows distinct variation in plant-defense-associated traits (resistance/susceptibility, induction/suppression) (Kant et al., 2008; Alba et al., 2015). For some (putative) effector genes, strong differential expression is noticeable within less than 24 h after host plant transfer (**Chapter 5**), pointing towards strong transcriptional plasticity. These observation may underline the importance of effector action in some strains: the outcome of producing inappropriate effectors types, or producing them in inappropriate amounts may be severe enough that a regulatory mechanism may have evolved, allowing high or low production levels, depending on the host plant species (FIGURE 6.2.A) **(Chapter 4)**. Due to natural selection, specific effector-related mite genotypes (differences in the coding sequence of the effector itself, or in regulatory elements that lead to altered expression of the effector) giving the mite a fitness advantage in a specific interaction will increase in successive generations. Eventually an effector(-related) genotype can become ubiquitous if the population is maintained on the same host plant genotype during multiple generations (FIGURE 6.2.B). In environments where host plant shifts occur frequently, there may be a continuous alternation between different effector-related genotypes, and unfavorable effector genotypes may temporary persist in the background, ready to re-emerge after certain host plant jumps. If there is a continuous shift between groups of host plants (e.g., legumes and non-legumes) which require different effectors, fast transcriptional plasticity might offer an additional advantage (**Chapter 5**). With the presented list of salivary proteins of the London strain of *T. urticae* (originally collected from apple trees and maintained on *Phaseolus vulgaris* since 10 years (Díaz-Riquelme et al., 2016) in **Chapter 3**, we probably identified a core set of effector proteins, yet only a fraction of host plant specific effectors. As such, 'mite-resistant' plant varieties harboring specific R genes or modified S

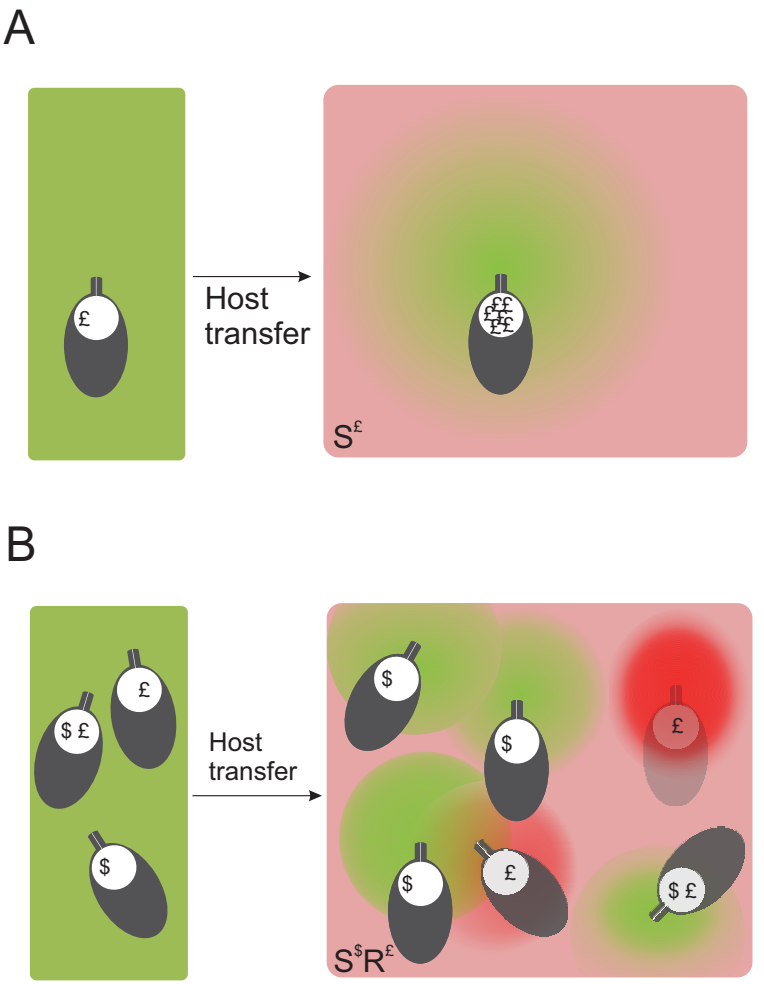

FIGURE 6.2. Host plant change and spider mite gene expression. Mites are transferred from a host on which they perform well, without effective defenses (green), to a new challenging host (pink). **(A)** The new host plant may induce the expression of effector £ in the spider mite salivary glands. Effector secretion locally interferes with defense mechanisms (green). The expression change occurs within one generation and is due to transcriptional plasticity. In an alternative situation **(B)**, spider mite effectors $ and £ may be expressed constitutively when feeding on the original host, where they have a neutral or positive effect on spider mite fitness. In this example, the founder population consist of a variety of effector genotypes ($, £, $£). The new host may possess strong defense mechanisms against spider mites (HTI, pink). Mites expressing the effector $ locally inhibit the defense responses (green) in the new host, while effector £ is recognized by the host, activating even stronger defense responses (ETI, red). Due to natural selection, the mite genotypes expressing elicitor £ gradually decrease in frequency, although they may persist for a while due to local defense inhibition by mites with a $ genotype. Also mites with $£ genotypes may decrease in frequency, although the possession of $ may attenuate the selection pressure on £. As such, £ may be maintained in the genetic background until a host is colonized where £ offers a fitness advantage again. Whether or not the effector genes are dominant or recessive further complicates the fate of the effectors.

genes offering a higher resistance against particular *T. urticae* strains might still fall prey to *T. urticae* strains with another effector set. To avoid this resistance breakdown (Jones et al., 2014), the effector repertoire of additional *T. urticae* strains or Tetranychidae species should be investigated, allowing identification of conserved or critical effectors and the associated durable plant R and S genes. Considering the speed with which mites can adapt to new host plants, it seems likely that some spider mite strains will eventually overcome the additional defenses of the engineered plant, requiring a continuous development of new mite-resistant varieties. A solution to this problem may be to combine multiple R genes with several mutations in S genes (Stuart, 2015). As such, each gene can be expected to reduce the selection pressure against the other genes in the construct (Zhu et al., 2012; Jones et al., 2014). To create such varieties within acceptable time-scales, plant breeding should be based on genetically modified (GM) methods instead of crossing with resistant wild species (Jones et al., 2014). However, the use of GM plants is restricted in Europe although newly developed technologies for resistant plants may perhaps result in a broader acceptance of biotech crops (Lombardo et al., 2016).

The plant-mite interaction requires a lot more research to determine the relative importance of effector action versus detoxification pathways. Indeed, even if plants can be developed which recognize specific mite effectors, it remains to be seen whether *T. urticae*, as a species, will really suffer from the associated defense responses (e.g., Kant et al., 2008). Grapevine, for example, activates strong defense responses against the adapted Murcia spider mite line, while no evidence for defense-suppression was found. This mite strain, however, has intrinsic ability to reduce or eliminate restrictions imposed by grapevine and is able to flourish on this host (Díaz-Riquelme et al., 2016). Therefore, even if a defense response (natural or transgenically introduced) reduces *T. urticae* fitness, this may not be sufficient to limit crop losses to an acceptable level. A combination with other strategies, such as biological control, may be opted to further reduce spider mite damage. However, a recent study showed that an increase in plant toxicity for resistance-breeding purposes may lead to a decreased predation of spider mite eggs by predators used for biological control (Ataide et al., 2016). Further research is needed to address this issue. In addition, one should keep in mind that plant S genes have a function other than being just a compatibility factor for antagonists. Therefore mutations in these genes, although ren-

dering the plant more resistant to an attacker, might cause pleiotropic side effects (Schie and Takken, 2014).

Despite the difficulties associated with the application of S and R genes to control *T. urticae*, additional studies on the plant-*T. urticae* interaction are of utmost importance. The fundamental lessons that will be learnt may be helpful to limit the damage inflicted by specialists such as *T. evansi*. This species has been shown to strongly inhibit tomato defenses (Sarmento et al., 2011; Godinho et al., 2015), and as such it may suffer more severely from the inhibition of this defense manipulation.

6.5. Future research and perspectives

The complex list of proteins found in spider mite salivary secretions raises more questions than answers. How is the expression of salivary protein genes regulated in function of the host plant? Why are some salivary proteins produced in the anterior podocephalic gland, and others in the dorsal podocephalic gland? Would spider mites still be able to colonize thousands of different plant species without some of these salivary proteins? Which additional salivary proteins could be identified in other populations of the cosmopolitan *T. urticae*?

The most relevant question, however, may be: what is the function of all those salivary proteins? The genetic and functional characterization of the salivary (effector) proteins is, however, an arduous task (Guiguet et al., 2016). A first important step would therefore be to narrow the field of effector candidates. One suggested approach can be to look for homologues of previously characterized effectors from other species, which should not be restricted to herbivores. Information of the interaction between blood-feeding parasites and their mammalian hosts, for example, can be useful as well. Due to their importance for human health, these interactions have been studied for a longer period of time than plant-feeding parasites, and convergent mechanisms of effectors are likely to exist (Guiguet et al., 2016). However, spider mite saliva appears to contain several expanded protein families which do not share homology to other known proteins. Furthermore, homology to functionally characterized proteins can give a clue about the means of action, but the actual interaction partner or the substrate molecule can be variable, as well as the outcome of the interaction or the resulting reaction product. As such, deriving the function of the most interesting spider mite salivary proteins based on homology to other

characterized proteins will be difficult. Alternatively, the function of proteins can be inferred by studying the nature of their interacting proteins. A promising method to study these interactions, next to yeast two-hybrid, is tandem affinity purification coupled to mass spectrometry (TAP-MS). This technique allows the highly specific isolation of protein complexes, in this case an effector and its target (or its cognate R gene product), under near-physiological conditions (Van Leene et al., 2015). The stability of the interactions, however, determines the success of this technique. Another approach may be to silence effector genes in spider mites via RNA interference (RNAi), and study the effect on the plant or on mite performance. RNAi has indeed been used to study effector action in aphids, and analogous techniques may be applicable to spider mites as well (Khila and Grbić 2007). This may, however, be complicated by the expansion of many putative effector families that lead to effector redundancy.

While a plethora of proteins has been identified in spider mite saliva, non-protein components may be present as well. Phytohormone-mimics, for example, were suggested by Storms (1971). In the future, this can also be studied using LC/MS analysis of mite secretions. Another use of the artificial diet may be to add host extracts, and to evaluate if this has an effect on expression of specific salivary protein genes (e.g., by qPCR).

The proteomics approach of mite secretions (**Chapter 3**) proved to be efficient for the identification of salivary proteins. With the genomes of *T. evansi* and *T. lintearius* at hand, a logical next step would be to allow these mite species to feed on the artificial diet and to analyze their secretions with nano LC-MS/MS. Comparing these proteomes with the salivary proteome of *T. urticae* could immediately lead to the identification of new interesting effector candidates, without the need for further 'cleanup' procedures. Indeed, the contaminating and irrelevant proteins (e.g., from cuticle, silk and eggs) in the *T. evansi* and *T. lintearius* datasets are likely to be the same as those in the *T. urticae* proteome. Therefore, proterosoma transcriptomes for the two former species can considered to be facultative. Next to the identification of new effector candidates, a comparative study would also allow the identification of core/conserved spider mite effectors.

The study of effector action is complicated by the spider mite's extensive detoxification pathways, potential interactions between effectors, effector redundancies and the effect of the host plant species or genotype. Furthermore, functional validation methods such as transient expression of

effectors are associated with several confounders such as defenses induced by the infiltration of *Agrobacteria* (Pruss et al., 2008).

6.6. Conclusions

The characterization of the salivary proteome of *T. urticae* has provided the scientific community with a foundation for a more detailed understanding of the interaction between spider mites and their host plants. While the function of the identified salivary proteins is still unknown, everything goes to show that some of these proteins are involved in the host plant specialization process. As such, we can state that the salivary protein complement of *T. urticae* is key to this mite's polyphagous nature.

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