From induction to suppression: How to manipulate plant defenses
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1.1 Feeding the world
By the year 2050, earth is estimated to be inhabited by 9.7 billion people (UN, 2015). It was estimated that, to feed all of them, we will roughly have to double our current crop production (Godfray et al., 2010; Tilman et al., 2011). Yet, at this moment, over 10% of the world population does not have access to sufficient amounts of food (FAO et al., 2015), emphasizing that we already fail to feed the present-day world. On top of that, the land area suitable for crop production is expected to decrease as a result of climate change (Nelson et al., 2010; Challinor et al., 2014). Ironically, agriculture itself is a major negative force behind climate change and other environmental threats such as biodiversity loss and eutrophication, while it also ‘consumes’ enormous amounts of land, freshwater and energy (Tilman et al., 2001; Foley et al., 2005). Hence, if we are to secure future food production, while simultaneously reducing the environmental impact of agriculture, revolutionary changes in the way we produce, process, distribute and consume food are required (Foley et al., 2011; Tilman et al., 2011; Bajželj et al., 2014; West et al., 2014).

Although the efficiency of our food supply system can be significantly enhanced by improving market infrastructure and consumer awareness to reduce the post-harvest loss of high quality food (FAO, 2011), future food security critically depends on further increasing crop yields (Ray et al., 2013), which requires sustainable agricultural intensification (Garnett et al., 2013). This can in the first place be attained by closing existing ‘yield gaps’ (the gap between the actual yield and what the yield could be under ideal conditions) by optimizing the global distribution of resources (such as water, nutrients, seeds, technology) to utilize the full potential of all agricultural land (Foley et al., 2011; Mueller et al., 2012). In the second place this can be attained via conventional breeding or genetic modification strategies to generate higher-yielding crop varieties (Rossi et al., 2015), plus to reduce yield losses to abiotic and biotic stresses (Boyd et al., 2013; Cominelli et al., 2013). The latter is an important goal considering that annual pre-harvest crop losses due to phytophagous pests (e.g., animals, bacteria, fungi, weeds) on average range from 25 to 40% (Oerke, 2005; Pimentel & Peshin, 2014). Unfortunately that is not all, because pests additionally claim about 20-25% of the harvest during storage. Insects and mites are responsible for most of these losses. Shockingly, without the intensive use of pesticides (a staggering 3 million tons per year), and other non-chemical control methods, average pre-harvest crop losses were predicted to be as high as 70% (Pimentel & Peshin, 2014), leaving many more people hungry.
The above suggests that plants are merely helpless victims that are at the mercy of their attackers and rely on our pesticides to survive. Obviously, this cannot be true, as the terrestrial world around us appears green and plants withstood the struggle for life long before we humans appeared. Indeed, compared to agriculture, damage inflicted by herbivores and pathogens to plants in natural ecosystems is generally low (estimated at a maximum of 15%) and inconspicuous, accordingly pest outbreaks that lead to complete defoliation or death of a plant are rare (Schowalter et al., 1986; Gilbert, 2002). Why then, do our crops suffer so much from pests?

There are two main reasons why cultivated crops are damaged more severely by pests than plants in natural ecosystems: (i) loss of resistance during breeding, and (ii) the lack of (diverse) natural enemies of pests in monocultures. Loss of resistance can be due to (unintended) negative selection during breeding or to coincidence when there is no positive selection. Plants and their attackers have evolved together for hundreds of millions of years (Labandeira, 2005). To persist, plants evolved and maintained various defensive traits to prevent or limit attacks by phytophagous species by natural selection (Jones & Dangl, 2006; Karban & Baldwin, 1997). Owing to the diversity in selection pressures, i.e., due to diversity of pest species and to spatial and temporal environmental heterogeneity in natural ecosystems, wild plant species often are (genetically) diverse as well. This diversity is what natural selection acts on and from which new resistances arise. Therefore, the resistances in natural populations will be relatively up-to-date. In contrast, our cultivated crop species have undergone many years of artificial selection (domestication and breeding) for traits that often do not aid their survival chances but that improve the yield, edibility or ornamental value (Doebely et al., 2006; Meyer et al., 2012). During this process resistance traits often got lost, either by coincidence or because they were costly and counteracted the desired phenotypes (Rosenthal & Dirzo, 1997; Gols et al., 2008). Finally, since the overall genetic diversity within crop species is relatively low (Doebely et al., 2006; Shi & Lai, 2015), there are little genetic resources available for reintroducing resistances. The second reason why our crops suffer so much from pests is that in monocultures, and especially when pesticides are applied, the biodiversity of the local plant and animal community is low. This abolishes beneficial ecosystem services, including the biological control of pests by their natural enemies (Bianchi et al., 2006; Kleijn et al., 2009; Geiger et al., 2010).

Joint international governmental efforts to safeguard sustainable agricultural intensification are in part focused on enhancing biodiversity in agroecosystems, mostly by changing land-use management to restore diversified landscapes, but which strategy to follow is unclear (Tscharntke et al., 2012) and success is not guaranteed (Kleijn et al., 2006). Increasing pest resistance of crops represents another cornerstone of sustainable agricultural intensification (Lucas, 2010; Tester &
Langridge, 2010; Smith & Clement, 2012). Despite our impressive (bio)technological capabilities, increasing crop resistance is not an easy task either. Breeding for resistance is time-consuming, expensive and can come at the expense of yield. Moreover, often pest species will overcome the resistance (as I will explain in more detail later on). Many resistances rely on plant-produced chemicals or proteins that negatively affect the performance (survival, development, reproduction) of the herbivore or pathogen (Smith & Clement, 2012; Zhang et al., 2013c). One way to overcome a plant’s molecular resistance is to attenuate the production of the defensive chemicals and proteins via manipulation of the host plant’s molecular machinery. This strategy is known as ‘defense suppression’ and is thought to be used frequently by phytopathogens. Interestingly, since the first report by Musser et al. in 2002 on caterpillars that were found to suppress the accumulation of the toxin nicotine in tobacco, it has become clear that also several other herbivore species can suppress plant defenses (Kant et al., 2015). One can wonder if durable resistance of plants to defense-suppressing pests can be achieved using (additional) molecular resistance traits, because these may be sabotaged as well. Hence, we first need to understand how phytophagous species suppress defenses, such that we can identify which molecular targets we should modify, remove or reinforce to restore plant resistance to this special class of pests.

Among the herbivorous arthropods that are currently known to suppress plant defenses are several species of mites (Kant et al., 2015). Two of them, the two-spotted spider mite *Tetranychus urticae* and the closely related tomato red spider mite *Tetranychus evansi*, are the central players of this thesis. Mites from both species are a pest on commercially grown tomato (*Solanum lycopersicum*) plants, causing significant economic losses (Saunyama & Knapp, 2004; Meck et al., 2013). In this thesis I have explored the interaction between spider mites and cultivated tomato plants, in particular how these mites manage to suppress tomato defenses and how we can genetically engineer tomato plants to increase their resistance to such defense-suppressing mites. In this introduction I will first summarize the current knowledge on the molecular mechanisms underlying basic plant defenses to herbivores and pathogens. Then I will outline which types of counter-adaptations these attackers have evolved to overcome plant defenses and that allow them to persist as pest. Finally I will highlight some of the counter-counter adaptations found in plants and explain why these could be indicative of an arms race between plants and their parasites. After this general background knowledge section, I will introduce the experimental model system and finish with a brief outline of the thesis. In view of my thesis topic, I will limit my introduction on herbivory to insects and mites. Notably though, the majority of all herbivorous animal species belongs to these two groups (Schoonhoven et al., 2005; Walter & Proctor, 2013).
1.2 How do plants defend themselves?

Being sessile, plants cannot simply evade threats such as herbivores and pathogens, instead they have to face them and deal with them on the spot, either aboveground or belowground. As mentioned earlier, plants are definitely not passive victims (Van Loon, 2016), because they have evolved defensive traits that make them resistant to those attackers that cannot cope with these defenses. Yet, not all plant species have evolved the same defensive traits; although some are ubiquitous, they typically differ per plant family or order.

Plant defense mechanisms are incredibly diverse and can have functions beyond defense as well. Not all defensive traits involve deterring or harming the attacker, as many plants (additionally) have evolved to tolerate biotic stresses (Ney et al., 2012), for instance by increasing the allocation of resources to the roots upon herbivory on aboveground tissues to enable regrowth and reproduction after the attack (Schwachtje et al., 2006). Those defensive traits that are aimed at deterring or harming the attacker range from physical barriers, such as the waxy cuticle, recalcitrant cell walls, spines, thorns and (glandular) leaf hairs, to the production of toxic chemical compounds and antinutritive substances, and can further include traits that repel the attacker or attract natural enemies of herbivores, for example via the release of volatile compounds (Jones & Dangl, 2006; Howe & Jander, 2008). To deal with the diversity of biotic (and abiotic) stresses they encounter, all plant species together have evolved over 200,000 different compounds. The vast majority of these have no known function in the plant's primary metabolism (growth, development and reproduction) and are thus collectively referred to as secondary metabolites (Mithöfer & Boland, 2012). Some of the defenses are pre-formed and always present (i.e., constitutive defenses) and form the first layer of protection, while others are activated only upon pathogen infection or herbivory (i.e., induced defenses), mainly because they are energetically costly and/or autotoxic. Some types of the constitutive defenses get reinforced after herbivore or pathogen attack and other types are constitutively present as ‘pretoxin’ and are activated during herbivore feeding, e.g., by the gut environment of the herbivore (Kant et al., 2015). Additionally, defenses can be categorized as either direct or indirect, referring to their mode of action. Whereas direct defenses constitute plant traits that directly interfere with the attacker, for instance by killing it, slowing down its development or by repelling it (Howe & Jander, 2008; Spoel & Dong, 2012), indirect defenses rely on the action of other species, i.e., natural enemies of the attacker. These indirect defenses constitute the attraction and arrestment of natural enemies, for example via plant-produced volatiles, via provision of shelters (domatia) or via provision of alternative food (Heil, 2008). The dependence on natural enemies makes indirect defense the least reliable but also the most lethal of all plant defenses (Kant et al., 2009).
Different types of attackers elicit different defense responses. A first distinction can be made between pests that rely on living plant tissue for survival and reproduction (biotrophs), versus those that feed on dead tissue (necrotrophs) - whereas, for example, selective tissue death may hamper the first, it may facilitate the second (Glazebrook, 2005). A second distinction of defense responses is based on the (relative) mobility of the attacker. Whereas selective tissue death is effective against biotrophic pathogens, most herbivores can move away from such defenses. Resistance to mobile attackers thus relies on different defense mechanisms, such as deterrence, hampering movement and delaying development (Felton, 2005). Importantly, these direct defenses are usually augmented with indirect defense responses (Kant et al., 2015). Finally, herbivores feed from plants in diverse ways and their degree of sensitivity to direct plant defenses is diverse as well (Schoonhoven et al., 2005). Similarly, there is a great diversity of predators and parasitoids that prey on the herbivores and which may require distinct conditions (e.g., scent profiles, alternative food sources, shelters) in order to be attracted to the plant as well as to be arrested on it long enough to liberate it from its attackers. Hence, as a third distinction point, both direct and indirect defense responses are thought to be tailored to the type (Pieterse & Dicke, 2007; Ali & Agrawal, 2012), or possibly even species (Kahl et al., 2000) of the attacker, yet especially the latter has been proven difficult to verify experimentally (Ali & Agrawal, 2012).

1.3 Perception and recognition of the attacker
Considering that different types of attackers need to be dealt with in a specific way, resistance thus largely depends on the timely and proper recognition of an attacker’s identity, which is then followed by transduction of a specific signal to activate the most appropriate defenses at the most appropriate moment and location. How do (resistant) plants discriminate between attacker types? Two factors, that together represent a molecular signature, appear to give crucial hints to the plant’s detection machinery that point to the identity of the attacker: (i) the (type of) damage inflicted to the plant and (ii) specific compounds derived from the attacker or its feeding activity (Erb et al., 2012).

1.3.1 Damage inflicted by herbivores and pathogens
Pathogens and herbivores have acquired various ways to take up nutrients from plant tissue. The pest species among them often overexploit their host plant and thus are highly destructive when damaging or removing plant tissues. The feeding styles (sometimes referred to as feeding guilds) of phytophagous arthropods can generally be categorized as: (i) chewing, i.e., after cutting, scraping, tearing off or boring into plant tissue using mandibles; (ii) piercing-sucking, i.e., from vascular tissue or mesophyll cells using a stylet; (iii) siphoning/sponging, i.e., sucking or absorbing liquid/
liquefied plant material without piercing using a tubular mouthpart with or without a spongy labellum; or (iv) a combination of these (Labandeira, 1997; 1998). The different feeding styles, which can vary depending on the life stage of the herbivore (Schoonhoven et al., 2005), enable consumption of specific tissue types and cause qualitatively and quantitatively distinct damage patterns. It is believed that some of these elements may result in a ‘damage fingerprint’ that contains information on the herbivore guild or identity and which can be exploited by the plant to obtain a degree of adaptive tailoring of defense responses.

Whereas large caterpillars can completely defoliate a plant in a matter of days (Villanueva, 1998; Kessler & Baldwin, 2001), small pests, like mites, the larval stages of some insects, and microbial pathogens, usually do not cause much damage at the beginning of the infection/infestation. In fact, most bacteria cannot even penetrate through the cuticle, instead they enter the plant through natural surface openings like stomata or through wounds, after which they penetrate cells, withdraw plant-produced nutrients, proliferate and destroy plant tissues (Melotto et al., 2008). Fungi and oomycetes often use such natural openings as well, but many can also force their way in through the use of specialized infection structures and/or secretion of cell wall-degrading enzymes (Latijnhouwers et al., 2003; Kubicek et al., 2014). The damage inflicted by small pests may grow exponentially in parallel with the growth of the population.

1.3.2 Detection of damaged-self
Like most other multicellular organisms, plants are able to distinguish healthy from injured tissue by detecting damage-associated molecular patterns (DAMPs) and damage-associated events that function as ‘danger signals’ (Heil & Land, 2014). Wounding disintegrates host cell walls, membranes and organelles, which results in the release of fragmented cell wall and membrane components, plus all intracellular molecules, into the extracellular space (apoplast). Consequently, these molecules may interact with enzymes that are separated from each other in intact cells but not anymore in disrupted cells. Thus, numerous and structurally diverse delocalized and/or newly formed molecules can in principle serve as DAMPs. Wounding additionally disturbs the osmotic and pH balances. For most of the damage-associated molecular patterns and events, however, it is not known (yet) how specific they are and whether they are functional danger signals (Boller & Felix, 2009; Heil & Land, 2014).

To date, receptors for three types of DAMPs have been identified and they all belong to the class of surface-localized pattern recognition receptors (PRRs), which are equivalent to the mammalian immune system-associated Toll-like receptors (TLRs) (Zipfel, 2014). One example of a DAMP (with a characterized receptor) are the oligagalacturonides, which are oligosaccharides that are produced when the major
1.3.3 Detection of non-self: microbial pathogens

Besides perception of damaged-self, PRRs also function as sentinels to detect non-self molecules, i.e., those derived from pathogens and herbivores (Figure 1.1) (Zipfel, 2014). In case of the former, PRRs specifically bind (small parts of) highly abundant, but indispensable microbial compounds, which are referred to as microbe-associated molecular patterns (MAMPs). Multiple MAMPs derived from either bacteria, fungi or oomycetes have been described (Boiler & Felix, 2009) and for some the corresponding plant receptor has been identified as well (Zipfel, 2014). Probably the best-known example is the bacterial MAMP flagellin and its plant receptor FLS2. Flagellin is the main constituent of the bacterial flagellum: a lash-like appendage that is primarily used for motility via rotary movement. The extracellular domain of Arabidopsis receptor FLS2 binds a conserved 22 amino acids fragment of flagellin (flg22), which ultimately induces defense responses that increase the plant’s resistance to bacteria (Gómez-Gómez & Boller, 2000; Chinchilla et al., 2007). Functional FLS2 orthologs have been described in phylogenetically distant plant species, indicating that flagellin recognition is highly conserved (Boiler & Felix, 2009).

1.3.4 Detection of non-self: arthropod herbivores

Analogous to pathogens and MAMPs, herbivores are most likely recognized by plants via the PRR-mediated detection of so-called herbivore-associated molecular patterns (HAMPs) (Wu & Baldwin, 2010; Acevedo et al., 2015; Schmelz, 2015). Although no HAMP-receptor pair has been identified yet, the existence of HAMPs is well-established and several HAMPs have been characterized at the molecular level (see below). HAMPs can emanate from: the saliva, oral secretions, salivary and ventral eversible gland secretions, regurgitant, the cuticle, secreted waste products (e.g., frass, honeydew), pheromones, nuptial gifts, oviposition-associated secretions, or eggs (Acevedo et al., 2015; Schmelz, 2015).

Several of the characterized HAMPs constitute plant-derived compounds that have been modified by herbivore-derived enzymes as a result of feeding. The fatty acid-amino acid conjugates (FACs) likely are the best example of this class of HAMPs. Multiple insect species produce FACs, i.e., most (if not all) caterpillar larvae of moths and butterflies (Lepidoptera), yet also some crickets (Orthoptera) and the fruit fly Drosophila melanogaster (Diptera) (Yoshinaga et al., 2007). As indicated by...
Their name, FACs are conjugates of two moieties; the backbone is a C18 or C16 fatty acid and attached is either glutamate (Glu) or glutamine (Gln) (Alborn et al., 1997; Halitschke et al., 2001). The fatty acid moiety is of plant origin, i.e., linolenic acid (18:3) or linoleic acid (18:2) released from membranes by lipases or wounding, while the amino acid is of herbivore origin (Paré et al., 1998; Yoshinaga et al., 2008). Both compounds are conjugated in the herbivore’s gut (Paré et al., 1998), possibly to enhance its nitrogen assimilation efficiency (Yoshinaga et al., 2008). In the case of lepidopteran larvae, FACs can be regurgitated onto the damaged leaf tissue while feeding (external digestion) and this induces defense responses, among them the emission of volatile compounds that attract natural enemies of the herbivore (Alborn et al., 1997; Halitschke et al., 2001). Accordingly, the application of purified FACs to wounded tissue of various plant species consistently increased the release of volatiles by these plants (Halitschke et al., 2001; Schmelz et al., 2009). The regurgitant of grasshoppers (Orthoptera), i.e., close relatives of crickets, does not contain FACs, instead these herbivores produce caeliferins, which are sulfated fatty acids (C15-C20) that also trigger the emission of parasitoid-attracting volatiles when applied to wounded maize (Zea mays) seedlings (Turlings et al., 1993; Alborn et al., 2007). How caeliferins are produced and where the precursors originate from is not clear (Alborn et al., 2007). Furthermore, direct and indirect defense responses were induced in cowpea (Vigna unguiculata) plants after wounded tissue was treated with inceptins (Schmelz et al., 2006; 2007). Inceptins are small peptides (11-13 amino acids) with a disulfide bond, that were originally part of the conserved plant chloroplastic ATP synthase enzyme, but that have been cleaved off in the herbivore’s gut by digestive enzymes (Schmelz et al., 2006; 2007). Like with FACs, the oral secretions of larvae of various Lepidoptera species contain inceptins (Schmelz et al., 2012) and some species produce both HAMPs while feeding.

Herbivore eggs are correlated with future herbivory, hence not surprisingly, oviposition and egg-associated cues have been found to take effect as HAMPs that trigger defense responses that, for instance, eliminate eggs or the larvae (after they have hatched) via recruitment of parasitoids (Hilker & Fatouros, 2015). Eggs deposited onto the leaf surface of Brussels sprout (Brassica oleracea) plants by female large and small cabbage white butterflies (Pieris brassicae and Pieris rapae, respectively) are covered with reproductive gland-derived secretions that induce indirect defenses (Fatouros et al., 2008; 2009). The HAMPs responsible for the defense induction are the nitrile benzyl cyanide (for P. brassicae) and the aromatic indole (for P. rapae). These compounds are included in the nuptial gift from the male butterflies, which transfer them to females as anti-aphrodisiac when they mate (Fatouros et al., 2008; 2009). Finally, eggs of the pea weevil (Bruchus pisorum) and the cowpea weevil (Callosobruchus maculatus), deposited on pods of pea (Pisum sativum) cultivars,
induce the tumor-like growth of undifferentiated cells (neoplasms), which represent a physical barrier for hatched larvae that want to enter the pod to feed. Here, long chain (C22-C24) $\alpha,\omega$-diols that are esterified at one or two oxygens with 3-hydroxypropanoic acid, named bruchins, were extracted from adult female weevils and these were demonstrated to induce neoplasm formation when applied to pea pods exogenously (Doss et al., 2000).

Indications for the involvement of PRRs in the induction of defense responses upon herbivory come from various experiments. First, the FAC volicitin, N-(17-hydroxylinoleoyl)-L-Gln, was demonstrated to bind a plasma membrane-localized protein that was isolated from maize leaves (Truitt et al., 2004). Second, in arabidopsis, the induction of direct defenses upon exogenous application of $P$. brassicae egg extracts was shown to partially depend on the PRR LecRK-1.8 (Gouhier-Darimont et al., 2013).

Third, in coyote tobacco plants ($Nicotiana attenuata$) a PRR, LecRK1, significantly contributes to the induction of defense responses upon, and concomitant resistance to, herbivory by chewing tobacco hornworm ($Manduca sexta$) caterpillars (Gilarthori et al., 2011).

Fourth, introgression of a gene cluster encoding three rice ($Oryza sativa$) LecRK PRRs into a susceptible rice variety conferred enhanced resistance to the brown plant hopper ($Nilaparvata lugens$), a piercing-sucking phloem-feeder (Liu et al., 2015).

Lastly, resistance of arabidopsis to the green peach aphid ($Myzus persicae$), also a piercing-sucking phloem-feeder, depends on BAK1 (Prince et al., 2014), which is a co-receptor that is required for downstream signaling by several PRRs (Zipfel, 2014). Infiltration of $M$. persicae-derived extracts into arabidopsis leaves induced defense responses similar to those observed after a flg22 treatment, but were independent of known PRRs (Prince et al., 2014). These results therefore suggest the existence of an uncharacterized PRR that binds a $M$. persicae-derived HAMP and which requires BAK1 for signaling. However, the unknown PRR might also have bound a MAMP from the $M$. persicae extract, because most aphid species harbor one or more endosymbiotic microorganisms (Douglas, 1998; Oliver et al., 2010). Thus, although HAMPs are expected to be perceived by PRRs, experimental evidence is indirect and alternative or additional mechanisms might exist (Acevedo et al., 2015).

1.4 Signal transduction upstream of induced defenses

How DAMPs and HAMPs are perceived is poorly documented, but for MAMPs this process is understood much better and the processing of signals emanating from DAMP/HAMP detection is thought to be similar. Perception of an attacker-associated ligand, i.e., a MAMP, and associated di- or multimeric receptor complex formation lead to (auto)phosphorylation of the cytoplasmic kinase domain of the receptor and/or co-receptor, thereby generating an active receptor complex (Macho & Zipfel, 2014). What follows is a cascade of molecular signaling events (Tena et al., 2011;
that ultimately induces various defense responses in the local attacked tissue as well as in non-attacked (systemic) tissues, which together are referred to as pattern-triggered immunity (PTI) (Figure 1.1) and are presumed to mediate resistance to the majority of all pathogens and herbivores (Spoel & Dong, 2012; Campos et al., 2014; Macho & Zipfel, 2014).

1.4.1 Early signaling events
Our knowledge on early signaling events is largely restricted to plant-microbe interactions. An activated receptor phosphorylates cytoplasmic proteins, thereby altering the conformation of these proteins and concomitantly their activity, (sub)cellular localization and/or stability. Phosphorylation (by kinases) usually activates a protein, while dephosphorylation (by phosphatases) inactivates it. Proteins that are phosphorylated by PRRs often are kinases themselves or are proteins that interact with other kinases, but can also be membrane-localized ion channels/transporters (Tena et al., 2011; Macho & Zipfel, 2014). In agreement with the latter, ligand perception by PRRs rapidly triggers transmembrane ion fluxes (Nürnberger et al., 1994; Jabs et al., 1997), most importantly the influx of calcium into the cytosol. Furthermore, three distinct, yet interconnected, protein kinase signaling branches that are critical for plant immunity, are
activated directly or indirectly by PRRs: (i) receptor-like cytoplasmic kinases (RLCKs) (Lin et al., 2013), (ii) mitogen-activated protein kinases (MAPKs) (Meng & Zhang, 2013), and (iii) calcium-regulated kinases (FIGURE 1.1) (Seybold et al., 2014).

Further downstream, but still detectable within a few minutes after wounding, pathogen attack or herbivory, is the increased production of reactive oxygen species (ROS), predominantly superoxide radicals ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which is known as the oxidative burst. ROS are cytotoxic, as they covalently bind proteins, lipids, DNA and RNA, which alters the functionality of these molecules. Under non-stress conditions, ROS are produced as well, i.e., as a byproduct of cellular metabolism, however their cytotoxic effects are minimal, because ROS-scavenging enzymes and non-enzymatic antioxidants are highly abundant in plant tissues. The oxidative burst is triggered by calcium and RLCKs, which activate ROS-producing enzymes (FIGURE 1.1) (Suzuki et al., 2011). Not only do ROS act as second messengers in plant immunity (Orozco-Cárdenas et al., 2001), they also function directly in wound-healing (by crosslinking of cell wall fragments) and in resistance to pathogens and possibly herbivores (Kerchev et al., 2012; Suzuki & Mittler, 2012; Baxter et al., 2014). Notably, the oxidative burst is an immune response that is conserved across the tree of multicellular life (Suzuki & Mittler, 2012; Nathan & Cunningham-Bussel, 2013).

Wounding, pathogen attack and herbivory additionally trigger the rapid production of nitric oxide (NO) (Delledonne et al., 1998; Bricchi et al., 2010) and most likely also its radical derivatives, collectively named reactive nitrogen species (RNS). Together, ROS and NO are important regulators of induced defense responses against biotrophic pathogens, including the selective tissue death response (Scheler et al., 2013). The role of NO (and other RNS) in defense against herbivores is less well understood, but NO appears to have an effect on the concentrations of defense-associated phytohormones (which function further downstream in defense signaling; see below) and their signaling properties (Mur et al., 2013; Scheler et al., 2013).

The transmembrane ion fluxes and ROS production, decrease the electrochemical gradient that normally exists between the interior (negatively charged) and exterior (positively charged) of the plant cell. To this event is referred to as depolarization of the plasma transmembrane potential (FIGURE 1.1). The change in the electrical potential of a damaged cell is sensed by its neighboring cells and activates their ion channels too. The result is a cell-to-cell propagation of surface potential changes, i.e., an electrical signal, that activates defenses in systemic tissues. The amplitude and duration of the depolarization of the plasma transmembrane potential depend on the stimulus and thus appear to confer some degree of specificity to the signal (Wildon et al., 1992; Zimmermann et al., 2009; Krol et al., 2010; Bricchi et al., 2012; Mousavi et al., 2013; Gilroy et al., 2014; Salvador-Recatala et al., 2014; Zimmermann et al., 2016). In arabidopsis, the electrical signal generated by feeding (chewing) of...
an Egyptian cotton leafworm (Spodoptera littoralis) travels through the plant at an average speed of nearly 6 cm per minute (Mousavi et al., 2013).

1.4.2 Phytohormones
The early cellular signaling agents modulate the concentration of various phytohormones, i.e., small molecules that regulate processes not only locally but also distal from their site of production (Pieterse et al., 2009), by regulating the enzymes involved in their biosynthesis, (in)activation or degradation (Tsuda & Somssich, 2015; Zebelo & Maffei, 2015). Two phytohormones in particular are of key importance to plant immunity: salicylic acid (SA) and jasmonic acid (JA) (FIGURE 1.1) (Erb et al., 2012; Pieterse et al., 2012). Although often the concentration of both hormones (differentially) increases in response to biotic stresses, generally only one of them is required to activate the appropriate defense responses and resist the attacker, while the other might, for instance, act to prevent secondary infections/infestations. In this respect, it is predominantly SA that accumulates in response to attack by biotrophic pathogens and (relatively) immobile arthropods (like feeding cell-inducing nematodes and phloem-feeding aphids and whiteflies) in order to induce defenses (Kaloshian & Walling, 2005; Vlot et al., 2009). In contrast, upon attack by necrotrophic pathogens and more mobile herbivores (such as caterpillars, beetles and spider mites), it is mainly JA that accumulates and regulates defenses (Glazebrook, 2005; Howe & Jander, 2008). More complex are responses to hemibiotrophic pathogens, which initially have a biotrophic lifestyle, but later on during the infection switch to a necrotrophic lifestyle, thereby (sequentially) inducing SA and JA-regulated defenses (Spoel et al., 2003).

1.4.2.1 Salicylic acid biosynthesis and signaling
In plants, chorismate, the end-product of the Shikimate pathway, can be used as substrate in two biochemical pathways that lead to the production of salicylic acid (i.e., 2-hydroxybenzoic acid). For many plant species though, it is currently not known which pathway is used under which condition(s). Furthermore, for both pathways some biosynthetic enzymes remain to be identified and/or characterized (Widhalm & Dudareva, 2015). In the first pathway chorismate is converted into isochorismate by isochorismate synthase (ICS) and subsequently to SA by an unknown protein, putatively one with pyruvate lyase activity (Wildermuth et al., 2001). This pathway takes place entirely in the chloroplast, from which SA is exported (Serrano et al., 2013). The second pathway is longer and starts with the conversion of chorismate into prephenate by chorismate mutase, followed by the production of phenylalanine (Phe) via arogenate. Phenylalanine ammonia lyase (PAL) then turns Phe into trans-cinnamic acid (TCA), which can be converted to SA via either ortho-coumaric acid or benzoic acid. Phenylalanine is primarily produced in the chloroplast and
exported to the cytoplasm, where SA synthesis can be completed (Figure 1.1), but depending on the biosynthetic route, this might also require peroxisomal or mitochondrial enzymes and corresponding interorganellar metabolite transport. After production, SA can be further modified, e.g., by glycosylation, methylation, hydroxylation, or conjugation to amino acids, albeit often for unknown reasons (Seyfferth & Tsuda, 2014; Widhalm & Dudareva, 2015). However, the methylated form of SA (MeSA) is volatile and can induce SA responses in systemic tissue (Park et al., 2007) and its release (in sufficient quantities) is required for the attraction of predatory mites to tomato plants that are infested with spider mites (Ament et al., 2010).

Signal transduction via SA is complex and not completely understood. However, as is the case for the majority of phytohormones, SA signaling depends on the ubiquitin-proteasome system (Seyfferth & Tsuda, 2014). This system deploys a multienzyme complex (CRL) to polyubiquitinate a designated protein, thereby targeting it for degradation by a proteasome complex (Petroski & Deshaies, 2005). The main molecular players specific for SA signaling are the transcriptional co-activator NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and its paralogs NPR3 and NPR4, as well as TGA transcription factors that activate the transcription of defense-associated genes (i.e., in the nucleus) in the presence of SA and NPR1 (Seyfferth & Tsuda, 2014). Each of the NPR proteins can function as a CRL adapter subunit that determines which protein(s) will be degraded, i.e., by binding them (Furniss & Spoel, 2015). Which proteins are targeted for degradation by the CRL-NPR1 complex (CRL3NPR1) is unknown (Furniss & Spoel, 2015). Surprisingly though, NPR1 is itself a substrate for CRL3NPR3/NPR4 (Fu et al., 2012). It is of note that although NPR1 is indispensable for plant immunity as transcription of virtually all SA-responsive genes depends on NPR1, paradoxically its proteosomal degradation is required for SA signal transduction and plant immunity as well (Spoel et al., 2009) (not shown in Figure 1.1 for reasons of clarity). In arabidopsis, all three NPRs have been identified as receptors of SA (but not SA-derivatives), albeit they have different binding affinities (Fu et al., 2012; Wu et al., 2012). In addition, binding of SA has a distinct effect on each of the NPRs. The interaction between NPR1 and SA induces conformational changes in NPR1 that turn it into an active transcriptional co-activator (Wu et al., 2012). Binding of SA by NPR3 promotes the NPR1-NPR3 interaction, thereby targeting NPR1 for degradation. Yet, binding of SA by NPR4 has the opposite effect, as it disrupts the NPR1-NPR4 interaction, thereby preventing the degradation of NPR1 (Fu et al., 2012). Still, the role of SA in the regulation of immune responses is even more complicated, because SA additionally controls the monomerization of NPR1 in the cytoplasm, the subcellular localization of NPR1 (cytoplasm or nucleus) and the DNA binding affinity of TGA transcription factors (Figure 1.1) (Spoel & Loake, 2011). In short, this means that the NPR1-mediated transcription of defense-associated genes is most highly
induced at intermediate SA concentrations, whereas high SA concentrations do not promote transcription of SA-responsive genes, but instead are correlated with the selective tissue death response (Seyfferth & Tsuda, 2014).

The majority of defense-associated SA-responsive genes codes for pathogenesis-related (PR) proteins. Seventeen classes of PR proteins have been described in plants, yet not all have been characterized in detail. Most PR proteins have antimicrobial activities, for instance due to their ability to break down pathogen cell walls (e.g., by β-1,3-glucanases, chitinases, lysozymes), disrupt pathogen membranes (e.g., by defensins, thaumatins) or degrade pathogen RNA (by ribonucleases). PR proteins can also negatively affect the performance of herbivores, for example by interfering with their digestive abilities (e.g., by defensins). Different plant-attackers (including herbivores) have been shown to trigger the production and subsequent secretion of distinct sets of PR proteins (Van Loon et al., 2006; Spoel & Dong, 2012).

1.4.2.2 Jasmonic acid biosynthesis and signaling

The biosynthetic pathway leading to the production of JA was elucidated already in the late 1980s (Vick & Zimmerman, 1987), after which most of the responsible enzymes have been identified and characterized. Yet, how JA at the molecular level confers the downstream activation of defense responses has only become clear in the past decade (Wasternack & Hause, 2013). Importantly, it was demonstrated that not JA but its isoleucine (Ile) conjugate, JA-Ile, regulates the expression of ‘JA-responsive’ defense-associated genes (Thines et al., 2007).

The biosynthesis of JA-Ile (FIGURE 1.1) starts with the cleavage of linolenic or linoleic acid from galactolipids of chloroplast membranes by stimulus-specific phospholipases (Bonaventure et al., 2011). Lipoygenase (LOX) enzymes then catalyze the dioxygenation of these polyunsaturated fatty acids, forming hydroperoxides (Wasternack, 2007). To generate JA, the molecular oxygen needs to be inserted in the C-13 position of linolenic acid and this is done by dedicated 13-LOXs, yielding 13(S)-hydroperoxyoctadecatrienoic acid (13-HPOT) (Wasternack & Hause, 2013). A 13-allene oxide synthase (13-AOS) converts 13-HPOT into an unstable allene oxide, which rapidly forms the more stable 12-oxo-phytodienoic acid (OPDA) by action of allene oxide cyclase (AOC) (Wasternack, 2007). These initial biochemical reactions take place inside the chloroplast. The next reaction however, the reduction of OPDA, which is catalyzed by OPDA reductase 3 (OPR3), occurs inside the peroxisome (Strassner et al., 2002) and thus implies interorganellar transport of OPDA. How OPDA, or perhaps a modified form of OPDA, is exported from the chloroplast and imported into the peroxisome is still largely unclear. The compound that is generated by OPR3, i.e., 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8), is subjected to three successive β-oxidation cycles that shorten its carboxylic acid
side-chain to ultimately give rise to OPC-2; i.e., (+)-7-iso-jasmonic acid (Wasternack, 2007), hereafter referred to as ‘JA’. The final biosynthetic step comprises conjugation of JA to Ile, thereby producing (+)-7-iso-jasmonyl-L-isoleucine, hereafter referred to as ‘JA-Ile’. This reaction is catalyzed in the cytoplasm by one or more jasmonate-amido synthetase-encoding JASMONATE RESISTANT 1 (JAR1) enzyme(s) (Staswick & Tiryaki, 2004; Kang et al., 2006; Suza et al., 2010).

Except for JA-Ile, other JA-amino acid conjugates can be formed by JAR1 as well (Staswick & Tiryaki, 2004), but bioactivity of these compounds has not been shown (Thines et al., 2007). Furthermore, both JA and JA-Ile are subjected to many other (sequential) biochemical modifications, like hydroxylation, carboxylation, glycosylation and methylation. Again, none of these modifications yields a biologically active jasmonate, however they probably mediate JA responses indirectly by regulating JA-Ile and precursor pools (Wasternack & Hause, 2013).

Under non-stress conditions, JA and JA-Ile levels in leaf tissue are generally low or undetectable (e.g., see Suza et al., 2010 for data on tomato plants). Concurrently, the transcription of JA-responsive genes takes place at an equally low rate (Figure 1.1). This is because transcription factors that can activate the transcription of these genes, such as MYC2 and its paralogs MYC3 and MYC4 (Kazan & Manners, 2013), are functionally repressed by jasmonate ZIM-domain (JAZ) proteins at low JA-Ile concentrations (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Zhang et al., 2015a). JAZ proteins physically interact with MYCs, which impairs the ability of MYCs to recruit proteins that are required for activation of transcription (Zhang et al., 2015a). In addition, JAZ proteins indirectly inhibit transcription by interacting with co-repressor TOPLESS or TOPLESS-related proteins, which recruit(s) genuine transcriptional repressors (Pauwels et al., 2010; Kazan & Manners, 2012).

Wounding, triggers the rapid and massive increase of JA and JA-Ile concentrations (see Suza et al., 2010 for data on tomato plants). Moreover, in N. attenuata plants it was demonstrated that the exogenous application of oral secretions of M. sexta caterpillars to a wound, or even more specifically the application of FACs present in these oral secretions, resulted in higher JA and JA-Ile concentration increases than when the wound was supplemented with water only (i.e., as a control treatment) (Wu et al., 2007). This indicates that HAMPs -when detected- enhance the JA response.

Probably via diffusion, JA-Ile ends up in the nucleus, where it acts as a molecular glue by promoting the interaction between its two co-receptors, CORONATINE INSENSITIVE 1 (COI1) and JAZ proteins, in a concentration-dependent manner (Thines et al., 2007; Sheard et al., 2010). JA-Ile induces conformational changes in both proteins that mediate the release of JAZ from MYC and instead anchor it to COI1 (Sheard et al., 2010; Zhang et al., 2015a). Since COI1 is the substrate-picking adapter protein of the CRL-COI1 complex (SCF COI1), the JA-Ile-mediated interaction of COI1...
and JAZ targets the latter for polyubiquitination and subsequent proteosomal degradation (Chini et al., 2007; Thines et al., 2007). In short, JA-Ile thus triggers the breakdown of JAZ proteins, thereby de-repressing MYC transcription factors and enabling transcription of JA-responsive genes (Figure 1.1) (Wasternack & Hause, 2013).

The transcriptional activation of JA-responsive genes leads to the accumulation of proteins and secondary metabolites that play a role in the defensive processes against herbivores and pathogens (Campos et al., 2014; Kant et al., 2015). The secondary metabolites, which can roughly be categorized as phenolics, terpenoids, or nitrogen-containing compounds (although conjugates also exist), contribute to direct as well as indirect defense responses. Notably, several of the defensive proteins have antinutritive properties and specifically take effect inside the herbivore's digestive tract, thus after ingestion of plant tissue (Felton, 2005). Proteinase inhibitors (PIs) are the best-studied class of such defensive proteins. In the herbivore's gut, some of the plant's PIs take effect as inhibitors of digestive proteases that otherwise cleave ingested plant proteins to generate free amino acids, which are required for the herbivore's growth, development and reproduction. The reduced availability of essential amino acids resulting from PI activity can significantly impair the herbivore's performance (Ryan, 1990). Plant-produced enzymes that degrade free amino acids in the herbivore's gut, such as threonine deaminases (TDs) and arginases (Chen et al., 2005; Gonzales-Vigil et al., 2011), or that inactivate digestive proteases by cleaving them, such as leucine aminopeptidases (LAPs) (Lomate et al., 2013), can have a similar effect on the herbivore. Plants further produce polyphenol oxidase (PPO) enzymes, which catalyze the oxidation of phenolic compounds, thereby generating quinones. Quinones are highly reactive, as they can instantly modify the structure (and thus function and/or mobility) of random molecules by adding carbon-based side chains (via alkylation) or by cross-linking them to other molecules or to each other to form polyphenolics. Although the evidence for the defensive role of PPOs is not rock solid, it is believed that quinones that enter (or are produced in) the herbivore's gut can impair digestive processes by damaging enzymes, membranes or DNA (Constabel & Barbehenn, 2008). In addition, PPOs and their phenolic substrates can also be found in glandular leaf hairs (trichomes) of some plant species. The movement of small herbivores on the plant's surface ruptures these glandular trichomes, mixes PPOs with their substrates, and the instantly generated sticky phenolic polymers can entrap the herbivore or hamper its movement (Constabel & Barbehenn, 2008). There are several other classes of plant defensive proteins that interfere with herbivore digestion, like certain cysteine proteases (Pechan et al., 2002), lectins (Macedo et al., 2004) and defensins (Shiau et al., 2006).

Finally, JA can enhance the production of extrafloral nectar (a food source) to promote indirect defenses (Heil, 2008) and can initiate morphological changes, such as
increasing the density of (glandular) trichomes on new leaves (i.e., those that will emerge in the future) (Traw & Bergelson, 2003; Maes & Goossens, 2010). Glandular trichomes are a mechanical barrier and can produce many of the above mentioned secondary metabolites and defensive proteins, and thus are an important source for resistance (Glas et al., 2012). Not all JA-responses are activated simultaneously; some proteins and metabolites are for instance produced within minutes, while it can take several days/weeks before an effect on the trichome density can be detected.

1.4.2.3 Other hormones and crosstalk
Plant immunity is not solely regulated by SA and JA, in fact probably all hormones are somehow and to some degree involved. In line with this it has been established that ethylene (ET) (Van der Ent & Pieterse, 2012), abscisic acid (ABA) (Ton et al., 2009), gibberellins (GAs) (Daviere & Achard, 2016), cytokinins (Naseem et al., 2014), auxins (Kazan & Manners, 2009) and brassinosteroids (Lozano-Duran & Zipfel, 2015) can all play significant roles in plant resistance to herbivores and/or pathogens, because genetic or (bio)chemical interference with their biosynthesis or signaling pathways made plants either more susceptible or more resistant. The most severe defense-phenotypes, though, resulted from impairment of the SA (e.g., Delaney et al., 1994) and JA (e.g., Howe et al., 1996) pathways, hence their primary role in plant immunity. By contrast, all other hormones primarily function in growth and development.

From this, it becomes clear that growth and defense responses are interconnected (Denance et al., 2013; Huot et al., 2014), which is established via dynamic interactions of (hormonal) signaling pathways, termed ‘crosstalk’ (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). For example, when under attack by a herbivore, JA accumulates and elicits defense responses but simultaneously represses auxin and GA signaling (Huot et al., 2014). This likely allows plants to temporarily prioritize defense over growth, because it mediates (among others) the redirection of metabolic fluxes to sustain defense responses at the expense of growth (Ferrieri et al., 2015). As the name implies, crosstalk is bidirectional; indeed auxin and GA also feedback on JA signaling to minimize tradeoffs on growth (Huot et al., 2014).

Crosstalk can affect all components of a signaling pathway, i.e., upstream and downstream of phytohormone accumulation, yet it appears that especially core transcriptional regulators of distinct signaling pathways control each other's production and/or activity by physically interacting with each other. This has for instance been shown for JAZs and MYCs from the JA pathway, which interact with EIN3 and EIL1 from the ET pathway and DELLAs from the GA pathway (Song et al., 2014). An additional layer of complexity is added by the fact that crosstalk can be antagonistic as well as synergistic, depending on the hormone's relative concentrations (Mur et al., 2006) and order of induction (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012).
Crosstalk does not only happen between growth-related pathways and defense-related pathways, but also between the defense-related pathways alone, in general in a reciprocal antagonistic manner. Here the function may be to tailor the overall defense responses to distinct attackers (Thaler et al., 2012). However, SA-JA crosstalk during PTI signaling is not exclusively antagonistic (Figure 1.1) and also contributes to the defense-response in synergism (Kim et al., 2014), reminiscent of the synergistic effects that SA and JA can have on defense responses when added artificially to plants (Mur et al., 2006). The SA and JA pathways probably can crosstalk without the assistance of other hormones, although so far only one mechanism has been described: here the transcript and protein abundance of transcription factor ORA59, a positive regulator of the JA pathway in Arabidopsis, are under negative control of SA (Caarls et al., 2015). More is known about the SA-JA crosstalk mediated by other hormones. ET, for example, acts as a regulator of both the SA and JA pathway (Van der Ent & Pieterse, 2012). In Arabidopsis, ET enhances the transcription of SA-responsive PR-genes, thus working in concert with SA. Yet, after infection with necrotrophic pathogens ET acts synergistically with JA to antagonize SA. In turn, attack by chewing herbivores triggers the accumulation of JA and ABA, after which the latter antagonizes the ET pathway/response (Broekgaarden et al., 2015) and likely the SA response too, because ABA was recently found to promote the CRL3NPR3/NPR4-mediated degradation of NPR1 (Ding et al., 2016). In N. attenuata, the feeding of M. sexta caterpillars triggers the increased accumulation of JA plus ET, and both hormones antagonize the production of SA (Dziezel et al., 2009). ET additionally mediates the inhibition of nicotine biosynthesis, which might be indicative of a plant adaptive response, i.e., downregulation of a costly and incompatible direct defense response to facilitate the more effective JA-regulated indirect defense, because nicotine is toxic to natural enemies of M. sexta, while the caterpillar itself is insensitive to it (Kahl et al., 2000; Voelckel et al., 2001). In contrast to M. sexta, feeding by beet armyworm (Spodoptera exigua) caterpillars results in the increased accumulation of JA plus SA. Here, SA antagonizes the production of ET and partially JA (Dziezel et al., 2009). Although the JA levels were lower in S. exigua-damaged plants than in M. sexta-damaged ones as a consequence of the SA-JA crosstalk (Dziezel et al., 2009), the JA levels were sufficiently high to induce the production of PIs and nicotine as effective anti-herbivore defenses (Steppuhn & Baldwin, 2007). Taken together, the outcome of the SA-JA crosstalk mediated by other hormones can vary with plant and attacker species, and may serve to tailor defense responses accordingly (Thaler et al., 2012).

1.5 Counter-adaptations by herbivores and pathogens
The same traits that enable plants to resist attackers, pose selection pressure on these attackers to overcome them. Consequently, pathogens and herbivores have evolved
varies ways to cope with plant defenses. Among them, three distinct -yet not mutually exclusive- mechanisms can be distinguished (Alba et al., 2011; Kant et al., 2015).

The first mechanism is avoidance of defenses. This is a behavioral strategy and may apply especially to herbivores, because it requires a certain degree of mobility as well as physical and chemical sensory perception abilities (Dicke, 2000). Using elegant field experiments, it was shown that Empoasca spp. leafhoppers, which normally rarely feed from N. attenuata plants, did so abundantly when the plants were impaired in their ability to synthesize or perceive JA-Ile and hence to mount JA-mediated defense responses (Kessler et al., 2004; Kallenbach et al., 2012). Avoidance also manifests itself at a smaller scale, i.e., at the plant organ level. Larvae of the cotton bollworm (Helicoverpa armigera), for instance, feed on leaves and fruits of tomato plants, but they avoid nutritious flowers. This was in part explained by the several hundred-fold higher activity of antinutritive PIs in flower tissue as compared to the other tissues (Damle et al., 2005). When feeding from arabidopsis plants, H. armigera larvae moved away faster from leaves that had been fed on by conspecifics shortly before than from distant non-attacked leaves of similarly treated plants, but this behavior was not observed on mutant myc2 plants with an impaired JA-signaling ability, indicating that the caterpillars avoid contact with JA-mediated defenses and try to stay ahead of them (Perkins et al., 2013). This behavior was also found for M. sexta caterpillars on N. attenuata plants (Paschold et al., 2007; Stork et al., 2009). Zooming in further, at the within-leaf scale, H. armigera larvae preferentially ate the inner laminar tissue of arabidopsis leaves, but not the peripheral tissue or midvein, because the latter tissues contained the highest concentration of toxic secondary metabolites (i.e., glucosinolates) (Shroff et al., 2008). Furthermore, phloem-feeding insects, especially whiteflies, have evolved ‘stealthy’ feeding modes, meaning that they carefully navigate their stylet through the plant tissue to reach the phloem sieve element, thereby minimizing wound signaling and associated defense responses (Walling, 2008).

The second mechanism is insensitivity to defenses. Insensitivity (here equivalent to resistance) can result from adaptations that quantitatively reduce the exposure or sensitivity to plant-produced secondary metabolites or defensive proteins (Kant et al., 2015). Exposure can be limited via detoxification, toxin sequestration, and/or toxin disposal mechanisms. This requires the (concerted) action of detoxification enzymes and metabolite transporters. Some herbivores have acquired one or more genes coding for such proteins from microorganisms via horizontal gene transfer. For example, probably a few hundred million years ago, the ancestor of modern spider mites acquired a gene from bacteria that was originally involved in the biosynthesis of the amino acid cysteine, but which has since been co-opted by mites to detoxify hydrogen cyanide (a secondary metabolite generated by certain plants upon wounding) and this allows them to feed from cyanogenic plants (Wybouw et al., 2014).
Alternatively, microorganisms that are associated with herbivores (e.g., in their digestive tract) can neutralize harmful plant substances ingested by their host (Hammer & Bowers, 2015). Finally, the sensitivity to plant-produced secondary metabolites and defensive proteins can be reduced/abolished by mutation of the amino acid(s) that is/are targeted by the toxin. As such, a single amino acid substitution can already significantly reduce toxin sensitivity (Holzinger & Wink, 1996; Zhen et al., 2012).

The third mechanism is manipulation of defenses. Considering the highly conserved defense-signaling mechanisms, strong dependence on SA and JA for immunity, and the apparent crosstalk between these pathways, one might expect that plant-attackers can disarm their host by manipulating conserved hubs or mechanisms in these signaling networks. Indeed, in some cases pathogens have been shown to do this. For example, the hemibiotrophic bacterial pathogen \textit{Pseudomonas syringae} pathovar \textit{tomato} DC3000 (\textit{Pst DC3000}) secretes ‘coronatine’, a structural mimic of JA-Ile, which promotes (i) bacterial entry by opening the stomata, and (ii) bacterial growth by binding to COI1, turning JA responses into overdrive, thereby antagonizing SA biosynthesis and its downstream defense responses (Mittal & Davis, 1995; Zheng et al., 2012).

Nonetheless, phytopathogens that interfere with the onset or effectiveness of plant defense responses usually target multiple host components and (therefore) secrete more than one molecule to target these (Mukhtar et al., 2011; Wessling et al., 2014). Such secreted molecules, i.e., all proteins and small molecules that are encoded by the genome of the attacker but that function at the interface with the host plant or inside plant cells to alter host cell structure and function, are termed ‘effectors’ (Hogenhout et al., 2009). Returning to the example; besides coronatine \textit{Pst DC3000} produces another 39 effector molecules, while 19 isolated strains of \textit{P. syringae} together were found to produce in total 64 different effectors (Baltrus et al., 2011) that target at least 17 distinct plant defense-related proteins (Lindeberg et al., 2012). The hemibiotrophic oomycete pathogens \textit{Phytophthora sojae} and \textit{Phytophthora ramorum} were even predicted to secrete as many as 1464 and 1188 proteins, respectively, during their interaction with the host plant and several hundreds of them are thought to be involved in host defense suppression (Tyler et al., 2006; Jiang et al., 2008). The ability to suppress plant defenses seems to have evolved several times independently and is widespread among bacteria (Macho & Zipfel, 2015), fungi (Lo Presti et al., 2015), oomycetes (Bozkurt et al., 2012), viruses (Pumplin & Voinnet, 2013) and nematodes (Goverse & Smant, 2014). Arthropod herbivores, too, can suppress plant defenses (Kant et al., 2015) and this topic will be discussed in more detail later on.

Recent technological advances such as high throughput DNA/RNA sequencing, bioinformatics and protein-protein interaction assays in combination with advances in genetic manipulation of plants and the genome-wide availability of gene-knockout mutants (such as T-DNA insertion lines in arabidopsis) have facilitated the identifica-
tion and characterization of dozens of microbial effector molecules and their in planta targets (e.g., Mukhtar et al., 2011). Roughly, effectors can be functionally categorized into six groups: (i) metabolites, such as hormone mimics (Zheng et al., 2012) and toxins (Duke & Dayan, 2011) which affect host metabolic processes; (ii) enzymes, which catalyze the modification of host metabolites/proteins, for instance to detoxify secondary metabolites (Okmen et al., 2013); (iii) nucleotide-binding effectors, like transcription factors (Kay et al., 2007) that manipulate host gene transcription; (iv) DNA that is translocated into the genome of the host and that codes for pathogenesis-promoting proteins (Bourras et al., 2015); (v) small RNAs that use the host’s antiviral RNA interference (RNAi) machinery to alter transcription or translation of host genes/gene transcripts (e.g., Weiberg et al., 2013); and (vi) protein-binding effectors, which change the activity, (sub)cellular localization and/or abundance of host proteins (e.g., King et al., 2014).

1.6 Counter-counter-adaptations by plants

In reaction to the effector molecules used by phytophagous species to subvert PTI, plants have evolved intracellular nucleotide-binding site/leucine-rich-repeat (NLR) proteins that detect effectors or their actions (i.e., modification of host proteins) and subsequently re-establish plus amplify PTI responses, using existing and relayed signaling pathways, thereby inducing more robust and powerful defenses to resist the attack, collectively referred to as effector-triggered immunity (ETI) (Spoel & Dong, 2012; Cui et al., 2015). These effector-specific NLR proteins are encoded by genes that are referred to as R-genes (resistance genes), whereas many of the effector’s target proteins are encoded by so-called S-genes (susceptibility genes) (Van Schie & Takken, 2014). Unlike PTI, ETI is typically associated with a hypersensitive response, which is characterized by the rapid programmed cell death of attacked and neighboring cells (here previously referred to as ‘selective tissue death’), supplemented with the massive production of PR proteins and secondary metabolites in the surrounding tissue (Spoel & Dong, 2012; Cui et al., 2015).

Sometimes it is difficult to strictly discriminate between PTI and ETI responses, as the MAMP/HAMP-PRR versus effector-NLR dichotomy does not always hold (Cook et al., 2015). For example, the effector Avr4, secreted by the fungus Cladosporium fulvum to protect its cell wall from degradation by tomato-secreted chitinases (Van den Burg et al., 2006), is not perceived inside the plant cell by an NLR protein, but in the apoplast by a PRR complex, which nevertheless results in ETI (Liebrand et al., 2013).

Just like PTI, ETI puts selection pressure on phytophagous species to overcome the associated defense responses, thereby continuing the arms race between plants and their parasites. Effectors and R-genes were long believed to evolve in a gene-for-a-gene like fashion, but this model appears to be too simplistic given our current knowl-
edge of effectors, their in planta targets, and the connections within the protein interaction networks downstream of perception (Mukhtar et al., 2011; Wessling et al., 2014).

1.7 Suppression of plant defenses by arthropod herbivores
The majority of defense-suppressing arthropod species have evolved traits -or associations with microorganisms- that enable them to exploit the SA-JA antagonism (Kant et al., 2015); i.e., they induce harmless SA responses, thereby suppressing the harmful JA-mediated defense responses. This has been demonstrated for the silver-leaf whitefly (Bemisia tabaci) when feeding from arabidopsis (Zarate et al., 2007; Zhang et al., 2013a,b) and tomato (Su et al., 2015), for the western flower thrips (Frankliniella occidentalis) feeding from arabidopsis (Abe et al., 2012), for the Colorado potato beetle (Leptinotarsa decemlineata) on tomato (Chung et al., 2013), for the mealybug Phenacoccus solenopsis feeding from tomato (Zhang et al., 2015b) and probably also cotton (Gossypium hirsutum) (Zhang et al., 2011), for larvae of the beet armyworm (S. exigua) feeding from arabidopsis (Weech et al., 2008) and N. attenuata (as outlined earlier; Diezel et al., 2009), and finally for eggs deposited by large cabbage white (P. brassicae) butterflies and Egyptian cotton leafworm (S. littoralis) moths (Bruessow et al., 2010). There are indications that aphids can also exploit the SA-JA antagonism (Mewis et al., 2005; Schwartzberg et al., 2014), but this seems to differ considerably per aphid and host plant species (Jaouannet et al., 2014). It is important to mention that induced SA responses are not necessarily harmless. This was exemplified by Zhang et al. (2013a), who showed that although B. tabaci whiteflies indeed benefitted from suppressed direct JA-mediated defenses, the indirect defenses were still functional, because the whitefly parasitoid Encarsia formosa has evolved to locate its prey by responding to specific SA-induced volatiles.

The SA responses that are triggered by deposited P. brassicae and S. littoralis eggs appear to be initiated by the perception of egg or oviposition-derived HAMPs (Gouhier-Darimont et al., 2013). Yet, how the SA signaling pathway is activated by the herbivores is not always clear. Analogous to microorganisms and nematodes, herbivores can secrete effectors to manipulate their host plant (Hogenhout & Bos, 2011; Kant et al., 2015). Contrary to pathogen effectors, hardly anything is known about herbivore effectors, let alone about their in planta targets. The discrepancy between our knowledge on pathogen and herbivore effectors can be explained by several not mutually exclusive factors: (i) traditionally phytopathology has been much more molecular biology driven than entomology, especially because microbes are easier to propagate and to experimentally manipulate; (ii) suppression of defenses by pathogens is experimentally much easier to observe (i.e., hypersensitive response or not) than suppression of metabolites or gene transcription by herbivores; (iii) some of the identified defense-suppressing arthropods feed only from non-model plant
species for which less genetic and molecular tools are available; (iv) arthropod genomes are larger than those of microbes, which makes genome and transcriptome sequencing to identify effectors more expensive and complicated and therefore lagging behind; and (v) molecular tools to genetically manipulate arthropods are largely limited to a few (non-herbivorous) model species.

At least one effector responsible for the induced SA responses (to exploit the SA-JA antagonism) has been identified: the salivary enzyme glucose oxidase (GOX). Actually, GOX was the first effector isolated from a plant-eating arthropod, i.e., the corn earworm (*Helicoverpa zea*), and was shown to impair the JA-mediated production of nicotine in tobacco (*N. tabacum*) plants (Musser et al., 2002). Feeding by *S. exigua* larvae on the closely related *N. attenuata* plants brings the salivary GOX into contact with foliar glucose, this generates H$_2$O$_2$ and leads to an increased accumulation of SA in the attacked leaf (Diezel et al., 2009). The saliva of lepidopteran larvae commonly contains GOX (Eichenseer et al., 2010). In addition, GOX has been found in the saliva of aphids (Hemiptera) (Harmel et al., 2008), while the salivary glands of thrips (Thysanoptera) contained GOX transcripts (Stafford-Banks et al., 2014), suggesting that insects from different orders (and feeding guilds) may use GOX to suppress JA defenses via the SA-JA antagonism. Yet, whether suppression of nicotine production by GOX in tobacco depends on the SA-JA antagonism is still not sure (Musser et al., 2005). In fact, the suppression of wound-inducible arabidopsis genes by *S. littoralis* and *P. brassicae* caterpillars is not mediated by GOX, nor does it depend on COI1 (JA signaling) or ICS1 (SA biosynthesis) (Consales et al., 2012), indicating that GOX does not always have the same effect in plants and moreover that multiple mechanisms to suppress defenses (co-)exist in herbivores, just like with pathogens.

Accordingly, apart from GOX, the saliva of *H. zea* larvae contains ATP hydrolyzing enzymes that reduce the amount of extracellular ATP (a DAMP; Tanaka et al., 2014) in tomato tissue while feeding, which reduces DAMP-mediated JA defense responses (Wu et al., 2012). Furthermore, multiple aphid effectors have been demonstrated to promote aphid performance by (simultaneously) interfering with distinct plant defense responses (e.g., phloem clogging, ROS production, hormonal signaling, cell wall reinforcement, secondary metabolite production), unfortunately their *in planta* interactors remain elusive (Will et al., 2007; Mutti et al., 2008; Bos et al., 2010; Atamian et al., 2013; Pitino & Hogenhout, 2013; Elzinga et al., 2014; Naessens et al., 2015). Similarly, effectors have been isolated from the saliva of Hessian fly (*Mayetiola destructor*) larvae (Chen et al., 2008), which are a destructive pest on wheat (*Triticum* spp.), as the larvae suppress defenses and hamper plant growth and development (Liu et al., 2007). In this case though, it was recently shown that the largest *M. destructor* gene family encodes secreted proteins that can interact with the plant CRL complex and operate as its substrate-picking subunit (Zhao et al., 2015). This suggests that, anal-
ogous to certain pathogens, *M. destructor* larvae can take control over the plant ubiquitin-proteasome system and target (yet to be identified) host proteins for degradation (Zhao et al., 2015).

Besides via herbivore-derived GOX, SA accumulation and signaling (to exploit the SA-JA antagonism) can be triggered by herbivore-associated microorganisms. For example, infection of *arabidopsis* with *Tomato spotted wilt virus* improved the performance of the virus’s arthropod vector, *F. occidentalis* thrips, by inducing SA accumulation to suppress JA-regulated plant defenses (Abe et al., 2012). In addition, the oral secretions of *L. decemlineata* beetle larvae were found to harbor a rich ensemble of (gut-derived) non-phytopathogenic bacteria, some of which triggered an increased production of SA when deposited onto damaged tomato leaf tissue during larval feeding, potentially due to the detection of MAMPs, thereby suppressing JA-defenses to which the larvae are susceptible (Chung et al., 2013). Interestingly, non-phytopathogenic bacteria that are associated with herbivores, but for which the interaction with plant tissue is not immediately evident, can also be involved in suppression of JA-mediated anti-herbivore defenses. This was demonstrated to occur via the SA-JA antagonism for the endosymbiotic *Hamiltonella defensa* bacteria of *B. tabaci* whiteflies (Su et al., 2015). These endosymbionts are generally confined to specialized host cells (bacteriocytes) and are unlikely to be secreted, nevertheless their presence altered the content of the whitefly’s saliva such that it induced much stronger SA responses when applied to wounded tomato tissue (Su et al., 2015).

In other cases, herbivore-associated microorganisms have been found to assist suppression of JA-defenses independent from the SA-JA antagonism. *Macrosteles quadrilineatus* leafhoppers, for example, vector phytopathogenic phytoplasmas (bacteria) that, after being transmitted to the host plant, secrete an effector named SAP11, which inhibits JA accumulation by binding and destabilizing transcription factors that promote the transcription of the JA biosynthesis gene *LOX2* (Sugio et al., 2011). As another example, the protein βC1, encoded in the genome of *Tomato yellow leaf curl China virus*, can disrupt JA signaling via interaction with at least two plant transcription factors; AS1 and MYC2. AS1 is a negative regulator of a subset of defense-associated genes and βC1 reinforces the negative regulation (Yang et al., 2008). MYC2 is a master regulator of JA-regulated defense responses and binding by βC1 prevents MYC2 from activating the transcription of these genes (Li et al., 2014). Together, in virus-infected plants the βC1 protein causes the reduced production of JA and its downstream products such as volatile and non-volatile secondary metabolites, which concurrently enhances the attraction and performance of *B. tabaci* whiteflies, the virus’s insect vector (Zhang et al., 2012; Luan et al., 2013; Li et al., 2014). Some viruses related to *Tomato yellow leaf curl virus* and that are exclusively vectored by *B. tabaci* produce the protein C2 that interferes with the activity of CRL complexes, including
SCFCOI1, thereby disrupting downstream JA responses. The performance of *B. tabaci* may therefore be higher on host plants infected with either of these viruses than on uninfected plants (Lozano-Duran et al., 2011). Furthermore, an infection with *Turnip mosaic virus* reduced plant resistance to *M. persicae* aphids predominantly by attenuating ET signaling, but in part also through the JA pathway (Casteel et al., 2015). Finally, the endosymbiotic and non-phytopathogenic *Wolbachia* bacteria associated with larvae of the western corn rootworm (*Diabrotica virgifera virgifera*) have been reported to mediate the suppression of both SA and JA-regulated defense genes in attacked maize roots. The suppression, though, was not associated with an increased performance of the herbivore (Barr et al., 2010). Moreover, another, independent research group failed to reproduce these results and firmly concluded that *Wolbachia* bacteria do not affect plant defense responses to *D. v. virgifera* larvae (Robert et al., 2013). Despite this controversy, overall it seems clear that herbivores can benefit from associating with microorganisms that suppress JA-mediated plant defenses. The microorganisms, in turn, might benefit from the improved performance of their vector/host by being dispersed more frequently and/or by becoming more abundant within the herbivore’s population. Considering the abundance and diversity of plant-attacking arthropods and microorganisms, such alliances might be widespread in nature (Frago et al., 2012; Zhu et al., 2014).

Like insect herbivores, several species of plant-eating mites are able to suppress plant defenses. Since defense-suppressing spider mites are the central players of this thesis, they will be introduced in more detail. First, using a tomato mutant that is unable to increase JA production in response to wounding or herbivory (i.e., *defenseless-1*; Howe et al., 1996) and a transgenic tomato plant that constitutively expresses the Prosystemin-encoding gene and therefore displays enhanced constitutive and induced JA defenses (i.e., 35S::Prosystemin; McGurl et al., 1994), Kant et al. (2008) demonstrated that within a natural population of the two-spotted spider mite (*T. urticae*) three types of spider mite individuals occur, each of which coping with defenses differently. The first type were individuals that were resistant to induced JA defenses: this type does not play a role in this thesis. The second type was found to induce JA defenses and to be susceptible to these defenses (from hereon called ‘inducers’). The third type was also susceptible, yet capable of suppressing induced JA defenses (from hereon called ‘suppressors’). Hence, suppressor mites (like resistant mites) were able to maintain a much higher reproductive performance on wild type tomato plants than inducers (Kant et al., 2008). Why these types co-occur in natural populations is unknown. Induction and suppression of JA defenses could be shown at the level of gene transcription as well as protein activity. Inducer mites elicited much higher transcript levels of a wound-induced PI-encoding gene (*WIPI-II*, a marker gene for JA defenses) in tomato than suppressors (Kant et al., 2008). Moreover, chymotrypsin PI activity significantly increased
in leaflets after infestation with inducer mites, but not after infestation with suppressor mites. The same pattern was observed for the emission of the two volatiles methyl-salicylate (MeSA) and 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene (TMTT), which are under control of JA (Ament et al., 2004) and are important for the attraction of predatory mites (Ament et al., 2004, 2010; De Boer et al., 2004). Later, the closely related spider mite species *T. evansi* (the tomato red spider mite) was also shown to suppress tomato defenses (Sarmento et al., 2011). Feeding by *T. evansi* did not lead to significantly increased transcript levels of WIPI-II and the PR protein-encoding gene PR-P6 (used as marker for SA defenses), nor did the blend of volatiles emitted by *T. evansi*-infested tomato plants differ from that of uninfested control plants (Sarmento et al., 2011). This suggested that *T. evansi* mites suppress both JA and SA-regulated direct defenses and volatile emission, although predatory mites still preferred infested plants over uninfested plants. On top of that, the PI activity of *T. evansi*-infested leaflets was significantly lower than that of uninfested control leaflets (Sarmento et al., 2011), indicating that *T. evansi* can suppress defenses stronger than *T. urticae* and/or that *T. evansi* accomplishes suppression in a different manner. Unpublished data from the Kant et al. (2008) study on *T. urticae* suppressor mites showed that these mites suppress SA-responses as well, albeit less strong than *T. evansi*. Recently, another spider mite species, *T. ludeni*, was found to suppress tomato defenses to a magnitude comparable to that by *T. evansi* (Godinho et al., 2016). A fourth mite species that suppresses plant defenses is the tomato russet mite (*Aculops lycopersici*), an eriophyid (Glas et al., 2014). The superfamilies Eriophyoidea (including the russet mites) and the Tetranychoidea (including the spider mites) belong to the Prostigmata, a suborder of the Trombidiformes (i.e., the ‘sucking mites’ or ‘true mites’) and are phylogenetically quite distant and morphologically different. Russet mites suppress JA-defenses and induce SA defenses while feeding on tomato. Unexpectedly though, the suppression of JA-responses by russet mites occurs independently of SA-JA crosstalk, because it remains intact on transgenic tomato plants that are unable to accumulate SA (Glas et al., 2014), i.e., due to the constitutive expression of a bacterial salicylate hydroxylase (*nahG*) gene (see Delaney et al., 1994). Interestingly, when an inducer spider mite is co-introduced to a russet mite infested leaflet, JA-responses remain suppressed on wild type plants but not on NahG plants. This apparent contradiction is explained by the fact that their combined induced SA-responses add up and that this doubled SA-response suppresses the induction of JA-defenses by the spider mites. Hence, during this interaction the SA-JA antagonism is not the (primary) cause of suppression of JA-responses by russet mites, but occurs as a consequence of it when a second species is introduced (Glas et al., 2014).

Recently our lab identified several spider mite secreted proteins that can account for the suppression of SA defenses and, possibly, also of JA defenses (Jonckheere
et al., submitted; Villarroel et al., 2016). However, how these salivary effectors establish suppression is not yet known.

1.8 The experimental system

The data presented in this thesis has been obtained from experiments using cultivated tomato and the two species of spider mites introduced previously: *T. urticae* and *T. evansi* (Figure 1.2).

Cultivated tomato (*S. lycopersicum*) belongs to the family of Solanaceae (> 3000 species) and originates from the Andes in South-America, where its wild relatives can still be found. The domestication of tomato occurred in two steps; first and foremost in subtropical Central-America, from where it was brought to Europe in the 15th century, where domestication continued at a greater pace. Intense breeding for agricultural, commercial and nutrition-related traits commenced in the 20th century and has since given rise to more than 7,500 cultivated varieties (including many hybrids) that produce tomatoes in various sizes, colors and shapes and concurrently have different tastes (Doebley et al., 2006; Bai & Lindhout, 2007). For this thesis three cultivars were used: Castlemart, Micro-Tom and Moneymaker. The former was used for mite-infestation experiments in all chapters. Cultivar Castlemart (LA2400) is a field variety reported for the first time in 1978 in Canada, but is probably older and produces 30-40 relatively large fruits per plant. Cultivar Castlemart is the genetic background of

![Figure 1.2](image1.png)  
**Figure 1.2** Adult female spider mites (*Tetranychus* spp.) from each of the four strains used for this thesis research. (a) *T. urticae* Santpoort-2; (b) *T. urticae* DeLier-1; (c) *T. evansi* Viçosa-1 with two eggs; and (d) *T. evansi* Algarrobo-1. The white scale bars indicate 0.5 mm.
the JA-biosynthesis mutant defenseless-1 (def-1) and the 35S::Prosystemin transgenic plants, which are also used in this thesis research. The Micro-Tom cultivar (LA3911) is a miniature tomato (max. 15-25 cm tall) due to a mutation in at least three genes (Marti et al., 2006), has a relatively fast generation time and produces about 40 small tomatoes per plant. Micro-Tom plants were only used in Chapter 5. Cultivar Moneymaker (LA2706) is an old (± 1910) British greenhouse variety that was commercially grown for decades until the 1980s when better (more resistant) varieties were introduced. Moneymaker remains very popular with home gardeners, because it is easy to grow and each plant produces 40-60 medium-sized tomatoes. This variety was only used in Chapter 5. Using an Agrobacterium tumefaciens-based tissue transformation protocol, genetic modification of tomato plants is relatively easy to achieve. In fact, the transgenic tomato cultivar ‘Flavr-Savr™’ that was marketed in 1994, was the first food-crop for which transgenic fruits were commercially available (Bai & Lindhout, 2007). In 2012, another commercial cultivar, Heinz 1706, was the first cultivated tomato from which the genome was completely sequenced (Tomato Genome Consortium, 2012). Those of another 54 followed shortly after (100 Tomato Genome Sequencing Consortium, 2014). Thus, a wealth of genetic information and tools are available for tomato nowadays (see www.solgenomics.net).

Field and greenhouse-grown tomato plants are frequently attacked by spider mites (Migeon et al., 2010). For *T. urticae*, tomato is only one of its many host plants, as it can feed from and reproduce on over 1100 plant species of over 140 families worldwide (Migeon et al., 2010). This implies *T. urticae* behaves like a true generalist, although it is not known if all individuals of the species can do so or if the species is composed of multiple (host) races. Notably, such extremely polyphagous plant-attacking species are rare in nature (Barrett & Heil, 2012). Most herbivores and pathogens are relatively specialized, i.e., they are either oligophagous or monophagous and hence feed from only a few closely related plant species or a single species, respectively (Barrett & Heil, 2012). Although specialization is not necessarily caused by physiological constraints, it often has imposed these, and therefore specialized species usually perform poorly on non-host plants, if at all. On their preferred host plants though, specialists typically have a higher reproductive performance than generalists, because of behavioral, physiological and/or metabolic adaptations (Ali & Agrawal, 2012; Barrett & Heil, 2012). *Tetranychus evansi* is less polyphagous than *T. urticae*, and has been reported from 136 plant species belonging to 37 plant families (Migeon et al., 2010). Nevertheless, this mite species preferentially infests Solanaceae, mainly tomato, tobacco, potato or eggplant, and is therefore often referred to as oligophagous and a Solanaceae-specialist (Navajas et al., 2013). *Tetranychus evansi* originates from subtropical regions in Brazil, but has invaded large parts of the world in the past half a century and is responsible for massive losses of
Spiders belong to the family Tetranychidae, which consists of about 1,200 species, and adults thus have four pairs of legs (Helle & Sabelis, 1985). Female spiders reach about 0.5 mm in length, while males are a bit smaller and less voluminous, which makes the sexes easy to distinguish by eye. Adults use an approximately 150 μm long pair of stylets (grouped together to form a single hollow tube) to pierce through the cuticle, epidermis and eventually cell wall of spongy or palisade parenchyma cells (Park & Lee, 2002) to inject saliva. Subsequently they can suck up the cellular content in a matter of seconds, probably not by using the stylets but their mouth, aided by a muscle called the pharynx that functions as a pump (Helle & Sabelis, 1985). Emptied cells appear as white (chlorotic) spots on the leaf surface and feeding damage can therefore easily be quantified (Kant et al., 2004). In adult females, most of the food is converted into eggs that -relative to their body- are fairly large (diameter 0.2 mm; Figure 1.2c) and depending on the host plant quality, temperature and humidity they can produce up to 15 eggs per day. Eggs can develop into egg-producing females within six days (Gotoh et al., 2010), but under our experimental conditions (25°C) it takes about 2 weeks. When unfertilized, the eggs develop into haploid males, while fertilized eggs usually develop into females, which are diploid (Helle & Sabelis, 1985). Experimentally, a genotype can be ‘conserved’ by back-crossing a female with her sons into a ‘line’ or ‘strain’. Mite populations grow exponentially, hence under optimal conditions just a few females can give rise to a population of thousands of individuals within a few weeks (Sarmento et al., 2011). The increase in population size is reflected in the damage that is inflicted to the host plant (Figure 1.3), consequently plants often die when they are overexploited. With increasing population size another characteristic of spider mite infestations becomes apparent; the plant gets wrapped in a silken web (Figure 1.3), which is produced by the mites to protect them from the environment (e.g., predators) and to facilitate their movement (Helle & Sabelis, 1985). The genome of T. urticae has been sequenced and annotated (Grbić et al., 2011).

To prevent spider mite pest-outbreaks, crop plants are frequently sprayed with pesticides (Van Leeuwen et al., 2015). Unfortunately, spider mites rapidly develop resistance mechanisms to pesticides, which severely hampers their chemical control (Van Leeuwen et al., 2010). Alternatively, natural enemies of spider mites, such as predatory mites, can be used to protect crops, especially those grown in greenhouses (Van Lenteren, 2011). For T. evansi though, natural enemies are not (yet) commercially available, and therefore this mite species is a potential threat for greenhouse-grown crops in European countries where pesticide usage is restricted and will be banned increasingly in the near future.
FIGURE 1.3 Infestation of tomato (*Solanum lycopersicum*) plants with "suppressor" or "inducer" spider mites results in distinct damage and infestation phenotypes. Shown are photos of tomato plants (cv. Micro-Tom) at different time points (consecutive weeks) after an infestation with either suppressor *Tetranychus evansi* Viçosa-1, suppressor *T. urticae* DeLier-1 or inducer *T. urticae* Santpoort-2 mites. Uninfested plants are shown as a reference on the left. Plants were 21 days old when their second leaf was infested with 150 adult female mites. Photos in each row were taken at the same time point (after infestation). Note that *T. evansi* infested plants were overexploited within 4 weeks of the infestation moment.
1.9 Thesis outline

In this thesis I present the research I have performed to elucidate how a tomato plant responds to suppression of its defenses by *T. urticae* and *T. evansi* mites with the aim to genetically alter tomatoes such that defenses cannot be suppressed any longer. In **CHAPTER 2** we present data obtained from mites we collected from natural populations of both mite species. We fixed the standing variation of these populations in the form of so-called ‘iso-female lines’ by crossing individual females back with their sons for several generations. Subsequently, by using *def-1*, wild type and *35S::Prosystemin* tomato plants we could estimate the relative abundance of JA-defense-suppressing *T. urticae* individuals. In the laboratory we used one of these defense-suppressing iso-female *T. urticae* lines, referred to as DeLier-1 (from The Netherlands) and two *T. evansi* lines referred to as Vicosa-1 (from Brazil) and Algarrobo-1 (from Spain) for the remaining experiments in this thesis (FIGURE 1.2). We included a *T. urticae* iso-female line obtained previously that cannot suppress defenses, referred to as Santpoort-2 (from The Netherlands), as a benchmark inducer line (FIGURE 1.2). We used the three suppressor lines to analyze tomato defense-related responses, i.e., levels of jasmonates, SA and transcripts of various defense-associated genes, and compared these with the responses to inducer mites until the infested leaflet died (a week). Mites from both species were found to suppress defenses downstream from phytohormone accumulation and independent from the SA-JA antagonism. These experiments also showed that suppression by *T. evansi* is powerful enough to co-suppress the JA and SA responses induced by the inducer mites sharing the same leaflet. However, suppression by *T. urticae* DeLier-1 on a leaflet co-infested with inducer mites results only in significant suppression of the SA-responses.

Whereas the former experiments were done using whole (infested) leaflets as sampling unit, in **CHAPTER 3** we investigated the spatial and temporal complexity of defense responses at the within-leaflet scale. We non-destructively divided leaflets into three sectors perpendicular to the midvein (tip, middle and bottom) and infested the middle and/or tip section with either suppressor or inducer mites. Next, phytohormone and gene expression analyses as well as mite performance assays were carried out to determine the local and leaflet-systemic effects of defense induction and suppression on heterospecific competitors. Here we found that induction of defenses by spider mites is very local, i.e., mostly restricted to the feeding site. Moreover, we observed a puzzling phenomenon: in leaflets infested with *T. urticae* and *T. evansi* in different sectors the magnitude of suppression by *T. evansi* became higher than when infested with *T. evansi* only and this hyper-suppression could not be explained by SA-JA crosstalk.

In **CHAPTER 4** we asked if mite-associated bacteria may be involved in suppression of defenses. Hence, we analyzed and characterized the bacterial community associated with suppressor and inducer *T. urticae* mites and found that they differ for some
well-known mite endosymbionts, i.e., *Wolbachia, Cardinium* and *Spiroplasma*. We treated mites with antibiotics to remove these endosymbionts in order to assess their influence on mite performance, on the spider mite transcriptome and on the induction/suppression of defenses in the plant. We conclude that endosymbionts may impact on the gene expression of their host as well as on plant defense responses induced by mites. These processes can be interconnected and the phytohormone data sometimes contradicted the transcript levels of marker genes, making these data hard to interpret. Judging from the reproductive performance of mites with or without endosymbionts we conclude that *Wolbachia* has the characteristics of a mutualistic symbiont while *Cardinium* has the characteristics of a parasite.

In Chapter 5 we asked if mites that suppress defenses also induce (non-defense related) tomato genes and, if so, if the promoters (i.e., regulatory DNA fragment that precedes the protein-coding region) of these genes could be used to engineer inducible resistance in response to suppressor mite feeding. To do so, we first compared the genome-wide transcriptomic responses of tomato plants to feeding by suppressor and inducer mites using microarrays and RNA-sequencing. From this dataset we selected tomato genes that are rapidly and significantly induced by suppressor mites, but are not expressed under uninfested control conditions. We subsequently isolated and cloned the promoter region of some of these genes and fused them upstream of either tomato *Proystemin* to engineer an inducible JA-defense or the fungal avirulence gene *Avr4* to engineer an inducible hypersensitive response. Tomato plants were then genetically transformed with one of the generated constructs with the aim to restore defense responses against suppressor mites. My results suggest that some of the selected promoters indeed can be used to re-engineer inducible defenses.

Finally, in Chapter 6 I discuss how the data we have collected contributes to our understanding of mite-induced plant defenses and to the nature of defense suppression. Also in the discussion I present some preliminary data on the plant’s primary metabolism during induction and suppression in order to emphasize that plant resistance may not solely result from plant defenses, or alternatively, that suppressors might manipulate more than defense responses. Furthermore, I discuss how my results can be used to engineer novel resistances in cultivated crops.

**References**


CHAPTER 1 | GENERAL INTRODUCTION


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