Rewiring of the jasmonic acid signaling pathway during insect herbivory on Arabidopsis

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Differentiële jasmonzuursignalering tijdens insectenvraat op Arabidopsis

(met een samenvatting in het Nederlands)

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CHAPTER 1

General introduction

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Plants live in complex environments in which they intimately interact with a broad range of other organisms. Besides the plethora of deleterious interactions with pathogens and insect herbivores, relationships with beneficial microorganisms are frequent in nature as well, improving plant growth or helping the plant to overcome stress. The evolutionary arms race between plants and their enemies provided plants with a highly sophisticated defense system that, like the animal innate immune system, recognizes non-self molecules or signals from injured cells, and responds by activating an effective immune response against the invader encountered. Recent advances in plant immunity research underpin the pivotal role of cross-communicating hormones in the regulation of the plant's defense signaling network (Spoel and Dong, 2008; Pieterse et al., 2009). Their powerful regulatory potential allows plants to quickly adapt to their hostile environment and to utilize their resources in a cost-efficient manner. Plant enemies on the other hand, can hijack the plant's defense signaling network for their own benefit by affecting hormone homeostasis to antagonize the host immune response (Grant and Jones, 2009).

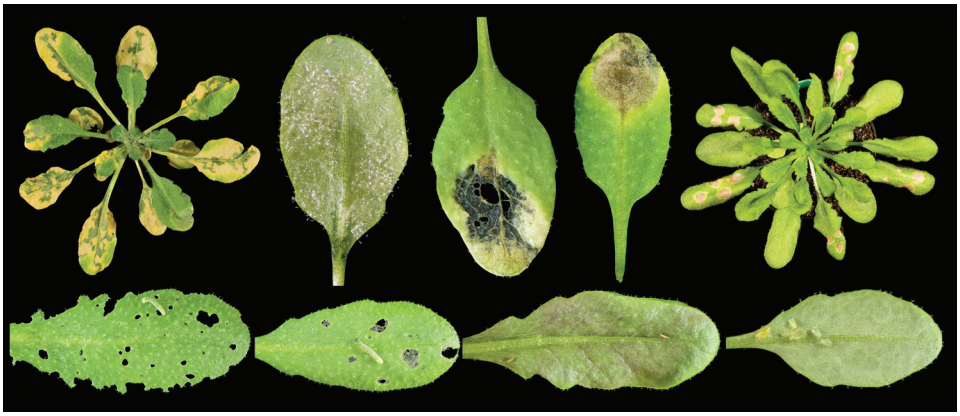


Figure 1: Plant attackers on Arabidopsis
From upper left to bottom right: Arabidopsis leaves attacked by the hemi-biotrophic bacterium *Pseudomonas syringae,* the biotrophic oomycete *Hyaloperonospora arabidopsidis*, the necrotrophic fungi *Botrytis cinerea, Alternaria brassicicola* and *Plectosphaerella cucumerina,* the tissue-chewing insect herbivores *Pieris rapae* and *Spodoptera exigua,* the cell-content feeding insect *Frankliniella occidentalis,* and the phloem-feeding insect *Myzus persicae.* Photos: Hans van Pelt.

Hormonal players in defense

Plant hormones are essential for the regulation of plant growth, development, reproduction, and survival. The importance of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) as primary signals in the regulation of plant defense is well established (Pozo et al., 2004; Van Loon et al., 2006b; Adie et al., 2007a; Loake and Grant, 2007; Von Dahl and Baldwin, 2007; Howe and Jander, 2008; Van der

Ent et al., 2009a; Vlot et al., 2009). Plant attackers (Fig. 1) possess different feeding and life styles. Biotrophic pathogens require a living host from which they subtly acquire nutrients, while necrotrophic pathogens kill host cells by producing toxins and proteolytic enzymes so that nutrients become available. Herbivorous insects feed by sucking from cell contents or phloem vessels or they chew on plant tissue. Plants respond with different defense strategies to different attackers. Interactions with biotic agents trigger changes in hormone concentration or sensitivity, which are responsible for a bouquet of hormone signaling events that steers adaptive plant responses specifically directed against the attacker. The composition and kinetics of the hormonal blend produced greatly influences the final outcome of the activated defense response (De Vos et al., 2005; Mur et al., 2006; Koornneef et al., 2008; Leon-Reyes et al., 2010a).

Salicylic acid

SA levels increase upon encounter of the plant with pathogenic bacteria, oomycetes, fungi, and viruses that in general have a (partial or hemi-) biotrophic life style (Malamy et al., 1990; Métraux et al., 1990; Vlot et al., 2009). SA is synthesized in the chloroplast and the bulk of pathogen-induced SA is produced from isochorismate through isochorismate synthase (ICS1), but also phenylalanine ammonia lyase (PAL) activity can lead to SA production from cinnamate (Mauch-Mani and Slusarenko, 1994; Wildermuth et al., 2001). Conclusive evidence for the requirement of SA in host defense came from experiments with transgenic tobacco and *Arabidopsis thaliana* (Arabidopsis) carrying the bacterial *NahG* gene that converts SA to catechol (Gaffney et al., 1993; Delaney et al., 1994), and the Arabidopsis *salicylic acid induction–deficient2* (*sid2*) mutant (Nawrath and Métraux, 1999), defective in ICS1 (Wildermuth et al., 2001). The hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv *tomato* and the obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* (formerly called *Peronospora parasitica*) were demonstrated to reach higher infection levels on NahG and *sid2* plants. Accumulation of high levels of SA is associated with the hypersensitive response (HR) that is activated by resistance (*R*) gene-mediated recognition of pathogen avirulence (Avr) factors, leading to localized cell death, which inhibits further growth of (hemi-)biotrophic pathogens (Weymann et al., 1995; Zhou et al., 1998).

To attenuate the SA signal, SA can be bound to glucoside to form SA-glucoside (SAG), from which SA can be released again when needed (Seo et al., 1995; Kawano et al., 2004). Furthermore, SA can be methylated to form methyl-SA (MeSA), which is highly volatile and part of the volatile blend emitted by insect-infested

plants (Van Poecke et al., 2001; Ament et al., 2004), in order to attract parasitoids that feed on the attackers of the plant (Zhu and Park, 2005). In this way, SA is part of the plants' indirect defense strategy. Plants that produce SA upon pathogen attack also emit MeSA (Shulaev et al., 1997; Koo et al., 2007; Attaran et al., 2009), which has been suggested to play a role in plant-to-plant communication (Shulaev et al., 1997), whereby neighboring plants sense a disease outbreak. In some studies MeSA is claimed to play a central role in systemic signaling leading to broad-spectrum systemic acquired resistance (SAR) (Park et al., 2007; Liu et al., 2011), whereas in other studies this is debated (Attaran et al., 2009).

SA induces alterations in the redox state, which leads to reduction of the inactive oligomeric form of NONEXPRESSOR OF PATHOGENESIS-RELATED (PR) GENES1 (NPR1) to the active monomeric form. Subsequently, active NPR1 monomers are translocated to the nucleus where they interact with TGACG motif binding (TGA) transcription factors (TFs) and WRKY domain binding (WRKY) TFs leading to SA-regulated defense gene expression (Després et al., 2000; Kinkema et al., 2000; Fan and Dong, 2002; Johnson et al., 2003; Mou et al., 2003; Wang et al., 2006). NPR1 is a key regulator of SA-mediated defense signaling (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997), controlling expression of *PATHOGENESIS-RELATED* (*PR*) genes (Van Loon et al., 2006a) and genes in the protein secretory pathway (Dong, 2004; Wang et al., 2005).

Jasmonic acid

JA is produced in plants that are mechanically damaged (Peña-Cortés et al., 1995; Howe et al., 1996; McConn et al., 1997). Damage is also inflicted by insects that feed on plant tissue and accordingly, levels of JA and other oxylipin derivatives (collectively called jasmonates (JAs)) increase in the plant upon infestation by insects (Baldwin et al., 1997; Halitschke et al., 2001; De Vos et al., 2005). Furthermore, JAs are also an important component of the plant's defense signal signature that is produced upon infection by necrotrophic pathogens (Penninckx et al., 1996; De Vos et al., 2005).

JAs are derived from chloroplast membrane lipids, such as linolenic acid, which are rapidly metabolized via the oxylipin biosynthesis pathway to form the JA precursors 12-oxo-phytodienoic acid (OPDA) and dinor (dn)-OPDA, respectively. After translocation to the peroxisome, OPDA and dn-OPDA undergo three steps of ß-oxidation resulting in the formation of JA (reviewed in Wasternack, 2007; Gfeller et al., 2010). Via the activity of the JA conjugate synthase JASMONIC

ACID RESISTANT1 (JAR1), JA can be readily conjugated to amino acids such as isoleucine (Staswick and Tiryaki, 2004), resulting in biologically highly active (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca et al., 2009).

JA-Ile is perceived by the JA receptor CORONATINE INSENSITIVE1 (COI1) (Katsir et al., 2008; Fonseca et al., 2009; Yan et al., 2009), an F-box protein that is part of the E3 ubiquitin-ligase Skip-Cullin-F-box protein degradation complex SCF^{COI1} (Xu et al., 2002). Binding of JA-Ile to COI1 facilitates binding to, and subsequent ubiquitin-mediated degradation of, jasmonate ZIM-domain (JAZ) proteins that are part of the JA receptor complex (Sheard et al., 2010) and in uninduced cells repress transcription of JA-responsive genes (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008). In the uninduced state, JAZ proteins bind to the basic helix-loop-helix (bHLH) leucine zipper TF MYC2, but upon activation of the SCF^{COI1} complex by JA-Ile, JAZ proteins are degraded and MYC2 is released, resulting in activation of JA-responsive gene expression (Chini et al., 2007). Apart from MYC2, also MYC3 and MYC4 were found to interact with JAZ proteins to mediate JA responses (Cheng et al., 2011; Fernandez-Calvo et al., 2011). Recently, Novel Interactor of JAZ (NINJA) and Groucho/Tup1-type co-repressor TOPLESS (TPL) were identified as part of the JAZ-repressor complex, in which JAZ-binding NINJA recruits TPL, which in turn represses transcription through the ET-responsive element binding factor-associated amphiphilic repression (EAR) motif (Pauwels et al., 2010) (see also Fig. 4).

COI1 plays a central role in JA signaling as is evident from the diverse phenotypic alterations of Arabidopsis *coi1* mutants (Feys et al., 1994; Xie et al., 1998), and the fact that expression of the majority of JA-responsive genes is completely blocked in *coi1* plants (Feng et al., 2003; Devoto et al., 2005; Wang et al., 2008b). Furthermore, mutant *coi1* is impaired in resistance to necrotrophic pathogens like *Alternaria brassicicola* and *Botrytis cinerea* (Penninckx et al., 1996; Thomma et al., 1998) and herbivorous insects like *Spodoptera exigua* (beet armyworm), *Spodoptera littoralis* (Egyptian cotton worm) and *Pieris rapae* (small cabbage white butterfly) (Mewis et al., 2005; Bodenhausen and Reymond, 2007; Van Oosten et al., 2008), highlighting the essential role of JA signaling in plant defense.

Apart from JA and JA-Ile, a myriad of other biologically active JA isoforms exist in plants. Conjugates with different amino acids are known, as well as the volatile form methyl-JA (MeJA) and hydroxyl-JA (Gfeller et al., 2010). Furthermore, the oxylipin pathway intermediate OPDA forms conjugates with glucosides resulting in arabidopsides (Hisamatsu et al., 2003; Hisamatsu et al., 2005), which may serve as direct anti-pathogenic compounds as well as a pool for slow jasmonate release (Kourtchenko et al., 2007). Whereas the myriad of JA isoforms underpins the

importance of this hormone in diverse plant processes, all their specific modes of action are not exactly known.

Downstream of COI1, different TFs regulate distinct JA-dependent defense responses. MYC2 and the APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF)-domain containing TFs ERF1 and ORA59 are main players in the regulation of JA-dependent defenses (Lorenzo et al., 2003; Anderson et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Onate-Sanchez et al., 2007; Kazan and Manners, 2008; Pré et al., 2008). The MYC2-branch of the JA pathway is associated with the wound-response and is thought to contribute to defense against insect herbivores (Lorenzo et al., 2004; Dombrecht et al., 2007), although it has also been reported to function in priming for enhanced pathogen defense (Pozo et al., 2008). The MYC2-branch regulates transcription of a large set of JA-responsive genes (Dombrecht et al., 2007), of which the insect resistance-related *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Liu et al., 2005) serves as a marker (Memelink, 2009). Two members of the AP2/ERF-family of TFs, ERF1 and ORA59, emerged as principal integrators of JA and ET signaling (Lorenzo et al., 2003; Pré et al., 2008). Both TFs activate the ERF-branch of the JA pathway and confer resistance to necrotrophic pathogens (Berrocal-Lobo et al., 2002; Pré et al., 2008). Activation of the ERF-branch results in transcription of a distinct set of JA/ET-responsive genes of which the antifungal *PLANT DEFENSIN1.2* (*PDF1.2*) serves as a marker (Penninckx et al., 1996; Penninckx et al., 1998; Memelink, 2009) (see also Fig. 4). So far, only MYC-type TFs have been shown to interact with JAZ repressor proteins (Chini et al., 2007; Cheng et al., 2011). However, the ERF-branch of the JA signaling pathway is still active in *myc2 myc3 myc4* triple mutants (Fernandez-Calvo et al., 2011), whereas the ERF-controlled defense responses are blocked in *coi1* (Lorenzo et al., 2003). This suggests that degradation of JAZ repressors is also a prerequisite for ERF-regulated defenses.

Ethylene

ET has been implicated in defense against various pathogens and pests (Broekaert et al., 2006; Van Loon et al., 2006b; Adie et al., 2007a; Von Dahl and Baldwin, 2007). ET levels increase upon infection with necrotrophic pathogens (Penninckx et al., 1998; Van Loon et al., 2006b) and in many plant species upon herbivory by insects (Von Dahl and Baldwin, 2007). ET is derived from the Methionine cycle. In the process of methionine formation, S-adenosylmethionine (AdoMet) is converted into methylthioadenosine (MTA) by the enzyme 1aminocyclopropane-1-carboxylate synthase (ACS). A by-product of this conversion is 1aminocyclopropane-1-

carboxylic acid (ACC), which is the precursor of ET (Bleecker and Kende, 2000). ACC is reduced by ACC oxidase (ACO), leading to the formation of ET. As a gas, ET rapidly diffuses and is perceived by a number of ET receptors (Hua et al., 1998). Upon recognition, the Raf-like protein kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) is inactivated, leading to activation of the TFs ETHYLENE INSENSITIVE3 (EIN3) and EIN3-likes (EILs) through action of ETHYLENE INSENSITIVE2 (EIN2) (reviewed by Stepanova and Alonso, 2009)). Upon their activation, a myriad of ET-regulated TFs are transcribed, such as *ERF1* and *ORA59* (Solano et al., 1998; Pré et al., 2008), leading to ET-regulated gene transcription.

In general, biotrophic pathogens are better able to infect mutants and transgenic plants affected in ET perception and signaling, whereas necrotrophic pathogens are less well able to grow on these plants (reviewed in Van Loon et al., 2006b). ET is involved in various physiological processes amongst which senescence. Since necrotrophic pathogens force a plant into a state of senescence, this might well be the basis of reduced performance of these attackers on ET mutants. Biotrophic pathogens require a living host and are therefore expected to grow better on non-senescing plants. Furthermore, expression of plant defensins, such as *PDF1.2*, depends on ET accumulation and perception (Penninckx et al., 1998). Hence, ET directly contributes to the defense response of the plant. The myriad of other processes in which ET is involved can also affect pathogenicity, which may explain unexpected effects of ET on disease resistance.

Abscisic acid (ABA)

ABA has been reported mainly to function in regulation of developmental processes, such as seed germination and senescence. But also responses to abiotic and biotic stresses have been shown to be regulated by ABA (Nambara et al., 1998; González-Guzmán et al., 2002; Anderson et al., 2004; López-Carbonell and Jáuregui, 2005; Melotto et al., 2006). Accumulation of ABA can be achieved through *de novo* biosynthesis. In the chloroplasts, β-carotene is converted in several enzymatic steps to neoxanthin and violacanthin. Several dioxygenases (NCEDs) are involved in reducing these compounds to xanthoxin. In the cytosol, xanthoxin is converted to abscisic aldehyde by ABA DEFICIENT2 (ABA2). Abscisic aldehyde is converted to ABA by aldehyde oxidase (AAO), which is activated through sulfuration by ABA3 (reviewed in Nambara and Marion-Poll, 2005). Alternatively, ABA glucose ester (ABA-GE), which is stored in the vacuole, is converted to ABA by ABA glucose hydrolase (AtBG1/BGLU18/At1g52400) (Lee et al., 2006).

An increase in ABA leads to closure of stomata and activation of genes encoding water-balance-restoring proteins, such as dehydrins (Close, 1997). Regulation of stomatal closure by ABA upon attack by bacterial pathogens that use the stomata as an entrance point to the plant apoplast has been demonstrated (Melotto et al., 2006). ABA is also involved in regulation of a second layer of defense against pathogens, namely deposition of callose-rich papillae, which encapsule the attacker in the apoplast, leading to arrest of the pathogen (Ton and Mauch-Mani, 2004; Kaliff et al., 2007; Luna et al., 2011). However, once the pathogen has entered the host, ABA can have a negative influence on disease resistance by interfering with defense signaling such as that regulated by SA (de Torres-Zabala et al., 2007; Mohr and Cahill, 2007; de Torres-Zabala et al., 2009), and by the ERF-branch of the JA signaling pathway (Anderson et al., 2004). Because of this, ABA production and signaling mutants display enhanced resistance to necrotrophic pathogens such as *Plectosphaerella cucumerina* and *B. cinerea* (Adie et al., 2007b; Sanchez-Rodriguez et al., 2009). The influence of ABA on diverse plant-pathogen interactions is documented in a series of nice reviews (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008; Ton et al., 2009).

ABA appears to play an important role in defense against insects as well. Peña-Cortés and colleagues (1995) showed that ABA-deficient tomato and potato plants do not respond to wounding with expression of *PROTEINASE INHIBITOR* (*PIN*), a well-known defense compound against insects (Peña-Cortés et al., 1995). In line with this, ABA-deficient tomato plants displayed reduced resistance to *S. exigua* (Thaler and Bostock, 2004). In Arabidopsis, a similar observation was made for resistance against *S. littoralis* (Bodenhausen and Reymond, 2007), which was growing better on the ABA biosynthesis mutant *aba2*. This may be caused by the positive influence that ABA normally exerts on activation of the MYC2-branch of the JA response, which is suggested to be involved in defense against insects (Anderson et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007).

Besides the aforementioned hormones, auxins, gibberellins, and brassinosteroids also emerged as hormonal signals with a role in plant-microbe interactions. In many cases, these hormones were shown to interact antagonistically or synergistically with the SA-JA-ET backbone of the plant immune signaling network thereby redirecting its defense output (Wang et al., 2007; Navarro et al., 2008; Campos et al., 2009; de Torres-Zabala et al., 2009). However, their importance in plant defense is less well characterized.

In order to activate the appropriate immune response that is tailored to the attacker, the plant has evolved pattern recognition receptors (PRRs) that recognize interaction with an alien organism (Jones and Dangl, 2006). PRRs are leucine-rich repeat receptors that in many cases contain a kinase domain or they can interact with proteins containing a kinase domain. Microbes are recognized by their so-called microbe-associated molecular patterns (MAMPs), which are essential for survival and can thus not be lost by the microbe to prevent recognition (Boller and Felix, 2009). MAMPs from pathogenic microbes are often referred to as pathogen-associated molecular patterns (PAMPs). Another group of elicitors recognized by PRRs are endogenous elicitors that are derived from the plant itself as damage-associated molecular patterns (DAMPs), which are released upon enzymatic plant cell wall or protein degradation by pathogens or herbivorous insects (Lotze et al., 2007; Heil, 2009). Perception of M/PAMPs and DAMPs by the PRRs initiates active defense responses, which hold the interacting organisms in check (Boller and Felix, 2009). The phenomenon of defense elicited by PAMPs is well known as PAMP-triggered immunity (PTI; Fig. 2) (Jones and Dangl, 2006).

A well-known example of a PAMP is flagellin of the bacterial flagellum, of which the core module flg22 was characterized as the recognized element. Flg22 is recognized by FLAGELLIN SENSING2 (FLS2) which, together with BRI1-ASSOCIATED KINASE1 (BAK1) initiates a signaling cascade upon recognition (Chinchilla et al., 2007). Another well-know PAMP is chitin, which is the main component of fungal cell walls. Chitin is sensed by CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) (Miya et al., 2007). An example of DAMPs that are induced upon injury of Arabidopsis are the peptide homologs AtPep1-7 that function as endogenous signals for neigbouring cells (Huffaker et al., 2006). The PRRs that bind AtPep1 to AtPep7 are named PEPR1 and PEPR2 and regulate the activation of defense responses upon AtPep perception (Krol et al., 2010; Yamaguchi et al., 2010). Intriguingly, the BAK1 receptor kinase was indispensable for full responsiveness to all AtPeps as well as for the MAMP flg22 (Krol et al., 2010), indicating that BAK1 may act as a general signaling partner for various PRRs.

Successful pathogens have found ways to breach this first line of PTI defense by disguising their PAMPs, as has been shown for Ecp6 of the fungal pathogen *Cladosporium fulvum*, which sequesters chitin that is released by the cell walls of the invading hyphae, leading to prevention of elicitation of host immunity (De Jonge et al., 2010). In addition, multiple reports describe that pathogens produce effectors that are secreted into the plant cell where they suppress important players in defense signaling pathways triggered during PTI, leading to effector-triggered susceptibility

(ETS) (Dodds and Schwechheimer, 2002; Jones and Dangl, 2006). In an ongoing arms race, plants have evolved a second line of defense in which resistance (R) proteins specifically recognize (either directly or indirectly) pathogen effector molecules, leading to effector-triggered immunity (ETI) (Jones and Dangl, 2006). For example, the AvrPto effector of the bacterial pathogen *P. syringae* interacts with BAK1, thereby interrupting defense signaling induced by flg22 in Arabidopsis (He et al., 2006; Shan et al., 2008). The R protein Pto binds to the avrPto effector, thereby activating ETI, which is often associated with a hypersensitive response (Xing et al., 2007). Besides pathogenic bacteria, also well-adapted fungal and oomycetal pathogens use a similar strategy of ETS (Ellis et al., 2007; Kamoun, 2007; Sohn et al., 2007; Baxter et al., 2010; Bos et al., 2010; Coates and Beynon, 2010).

Phenomena of DAMP-triggered immunity and possibly effector-triggered immunity in plant resistance to insects are not well established. There is debate about the perception of insects: is grazing, leading to DAMPs, the initial trigger for immunity (Heil, 2009) or is there also a role for compounds from oral secretions, such as fatty acid-amino acid conjugates (FACs), which are frequently referred to as herbivore-associated molecular patterns (HAMPs) (Felton and Tumlinson, 2008; Mithöfer and Boland, 2008)? The PEPR receptors are the only PRRs described to date that recognize potential insect elicitors (AtPeps which are DAMPs). Possibly, insect compounds (e.g. outer skeleton, oral secretion, saliva) can harbor conserved

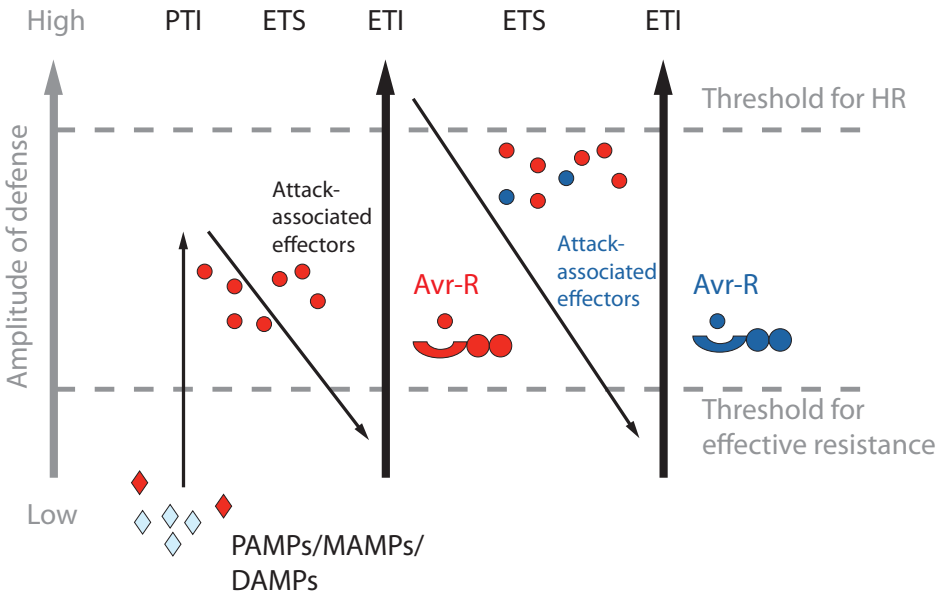


Figure 2: ZigZag model on evolutionary arms race between plant and attacker
Plant attack is recognized through recognition of pathogen/microbe/damage-associated molecular patterns (PAMPs/MAMPs/DAMPs), leading to PAMP-triggered immunity (PTI). Effectors evolved in the attacker, block recognition by the plant, leading to effector-triggered susceptibility (ETS). New recognition systems evolved in the plant to recognize microbial effectors, leading to effector-triggered immunity (ETI). New effectors and receptors evolved in an ongoing arms race between the plant and its attacker (adapted from Jones and Dangl, 2006).

patterns that are recognized in the first line of plant defense in analogy to PTI. However, an alternative explanation may be that DAMPs, which are damaged host proteins released by herbivory, are recognized as molecular patterns to elicit an immune response, in analogy to ETI (Lotze et al., 2007) and that compounds in oral secretions can function as effectors to suppress host defense signaling and that in some cases R proteins monitor this interference. Examples of host defense suppression by oral secretions are known through studies performed with caterpillars of which secretory glands were removed or ablated, leading to expression of a very different set of genes which thus is normally suppressed by the insects saliva (Musser et al., 2002; Musser et al., 2005; Delphia et al., 2006; Musser et al., 2006; Weech et al., 2008).

From recognition to signaling

How the perception of danger signals, through recognition of M/PAMPs, DAMPs or effectors, leads to the induction of hormone-regulated signaling and thus activation of defense responses is to a large extent still unknown. Current evidence indicates that different types of attackers trigger specific defense responses (Thomma et al., 1998; De Vos et al., 2005). Among the earliest physiological responses are changes in ion fluxes across the plasma membrane, in which an increase in influx of H^+ and Ca^{2+} and concomitant efflux of K^+ is typical. One of the roles of Ca^{2+} in defense is the activation of calcium-dependent protein kinases. In addition, MAMPs induce an oxidative burst, in which reactive oxygen species function as antibiotic agents, cause cell wall cross-linking or act as secondary stress signals. Another relatively fast response involves activation of Mitogen Activated Protein (MAP) kinase signaling cascades in which a series of phosphorylation events results in activation of TFs (reviewed in Boller and Felix, 2009). Plant responses are tightly regulated and MAPK signaling is an important first discriminator in response activity. Therefore, negative regulators of plant defense are also found in MAPK cascades. Mutant *mpk4* contains high basal levels of SA and constitutively expresses SA-regulated *PR* genes, indicating that MPK4 is a modulator of SA levels (Brodersen et al., 2006). Apart of modulating levels of SA, MPK4 also modulates JA signaling, since activation of JA-dependent defenses is no longer possible in the *mpk4* mutant (Petersen et al., 2000). Mutant *edr1* (*enhanced disease resistance 1*) is mutated in a *MAPKKK* (Frye et al., 2001) and displays faster and stronger activation of the SA response upon induction (Van Hulten et al., 2006).

Defense mechanisms in plants

Plants possess a range of constitutive defenses. Plant epidermis and cutical wax not only serve as barriers against loss of water, they also preclude microorganisms from entering the leaf. Furthermore, trichomes and thornes are efficive weapons in lowering attractiveness to herbivores. Inside the plant, constitutive chemical defenses are present. So-called two component systems, whereby two compounds are stored in separate plant compartments and one of these compounds is modified upon contact with the other compound, is widespread in plants (reviewed in Morant et al., 2008). A well studied example is found in brassicacious species and involves glucosinolates and myrosinase. Cell disruptions bring glucosinolates in contact with myrosinase resulting in toxic break-down products such as nitriles and thiocyanates. These toxins are detrimental towards insects and pathogens (Hopkins et al., 2009).

Furthermore, inducible defense pathways contribute to non-host resistance against opportunistic attackers. JA-insensitive Arabidopsis *coi1* mutants were found to be subject to herbivory by saprophagues arthropods that normally only feed on seeds and dead plant tissue (Farmer and Dubugnon, 2009). A similar phenomenon was observed in lipoxygenase-silenced wild tobacco *(Nicotiana attenuata*), that attracted not only more tobacco-adapted insects, but also herbivore species that normally do not feed on tobacco (Kessler et al., 2004). JA production-impaired Arabidopsis triple mutant *fad3 fad7 fad8* was highly sensitive to feeding by common saprophagous fungal gnat (*Bradysia impatiens*), whilst wild-type neighboring plants were largely left unaffected (McConn et al., 1997). ET-insensitive tobacco Tetr plants (transformed with mutant ET receptor allele *etr1–1* of Arabidopsis) were found to be spontaneously killed by a soil-borne *Pythium* fungus that was unable to infect wild-type plants (Knoester et al., 1998).

Many defenses are inducible and not constitutive, which is most likely related to the costs involved in expressing defense (Simms and Fritz, 1990; Walters and Heil, 2007). Energy that is used in defense cannot be invested in growth or reproduction. Once defense is inducible, it will only cost energy at the moment of pathogen or insect attack. Plants constitutively expressing SA-dependent defenses, such as mutant *cpr1* (*constitutive expressor of PR genes1*), display strong growth retardation and have dwarfed phenotypes (Bowling et al., 1994; Van Hulten et al., 2006). The same phenomenon is observed in plants constitutively displaying JA-dependent defenses, such as *cev1* (Ellis and Turner, 2001).

Whereas pathogens feed from the inside of plant tissue and need to enter their host to successfully subtract nutrients, insects ingest from the outside of the plants. They either feed by tapping sap from phloem vessels, like aphids and whiteflies, feed from single cells by sucking out cell content, such as thrips and spider mites, or feed by chewing on plant parts, like larvae from different lepidopteran species such as small cabbage white (*Pieris rapae*). Because of this difference in the dimension of feeding, also different defense strategies are required. Most defense responses directed against insects are regulated through JA. Plants rely heavily on distortion of the insect's digestion once attacked. This can be regulated via lowering of nutritive value of the consumed plant tissue, such as induction of polyphenol oxidases (PPOs), enzymes that catalyze the oxidation of phenolics to quinines. These quinines crosslink to the nucleophilic side chain of proteins and free amino acids, decreasing digestability and, thereby, nutritive value (Kessler and Baldwin, 2002). A similar mode of action is deployed by amino acid deaminases (Chen et al., 2005), which are able to degrade amino acids in the insects midgut, lowering uptake of essential food compounds by the insect. Other ways of interfering with digestion by insects are directed against enzymes involved in food assimilation, such as proteinases. Insect feeding is known to induce transcription of genes encoding proteinase inhibitors (PINs), which are directly interfering with nutrition of the insect (Lawrence and Koundal, 2002).

Apart from interfering with digestion, insect feeding induces a myriad of plant toxins that are directed at killing or severely impairing the insect, thereby decreasing the chance of major damage caused by the insect. Well known toxins are glucosinolates found in brassicaceous species, alkaloids, such as jacobine, in *Jacobeae* species, and ricin in *Ricinae* species, but far more toxins are being produced in plants. The downside of producing anti-nutritive or toxic compounds is that this provides a new niche on which insects can specialize. Enzymes are produced in the insect's midgut, that are involved in detoxifying plant toxins. Important detoxifying enzymes are cytochrome P450s, which can be induced when the insect experiences toxins. Larvae of the corn earworm (*Helicoverpa zea*) have been shown to produce several cytochrome P450s upon the ingestion of plant derived SA and JA molecules, resulting in upregulation of these detoxification enzymes at the same time that the plant is upregulating production of toxic compounds (Li et al., 2002b). Larvae of *P. rapae* contain a nitrile specifier protein in their guts, which has similar properties as plant-derived epithiospecifier protein (ESP) in directing glucosinolate breakdown towards relatively low-toxic nitriles at the expense of isothiocyanates (Wittstock et al., 2004). Isothiocyanates were found to be toxic to *P. rapae* larvae if applied to artificial diets (Agrawal and Kurashige, 2003), illustrating the need of this nitrile specifier protein in the insects gut. Other insects use plant-

derived toxins to their own benefit by storing these compounds, exploiting the toxins' detrimental properties against potential preditors (Ishiguro et al., 2001). For example, cinnabar moth (*Tyria jacobaeae*) larvae and adults use alkaloids of *Jacobeae* species to defend themselves against predators (Dempster, 1982; Van Zoelen and Van der Meijden, 1991), whereas cabbage aphids (*Brevicoryne brassicae*) use glucosinolates from brassicaceous species to defend themselves against ladybirds (Pratt et al., 2008).

There are many more mechanisms by which plants can lower the susceptibility for insects. One of these mechanisms relies on repelling the insects or by reducing edibility through the production of toxic compounds as mentioned above. Furthermore, plants release volatile compounds upon insect feeding, that are perceived by parasitoids and used as signals to trace their prey. The detrimental herbivores can be directly consumed by the attracted parasitoids, but there are also examples of parasitoid insects that use the other insect's body as incubator for their eggs and food source for their larvae (Dicke et al., 1990; Turlings et al., 1990; Turlings et al., 1993; Geervliet et al., 1994). A whole different strategy is to tolerate damage inflicted by insects, mostly accompanied by reallocation of nutrients to storage organs followed by regrowth after completion of the life cycle of the insect (Stowe et al., 2000).

Also adjusting the plant-life cycle to avoid growth during the most active season of herbivores can be considered as an important strategy to prevent consumption. Arabidopsis is a good example of this avoiding strategy, with many accessions whose seeds germinate in autumn and who complete their life cycle in spring. Despite this avoidance strategy, Arabidopsis still needs to have defense mechanisms against insects in place, because presence of *P. rapae* and other lepidopterans has been reported on Arabidopsis in the field (Yano and Ohsaki, 1993; Mauricio, 1998) and *P. rapae* is able to complete its life cycle on Arabidopsis (Harvey et al., 2007). Accordingly, Arabidopsis is very well capable of responding to different types of insect herbivores and the molecular responses overlap to a large extent with those observed in other plant species (Reymond et al., 2004; Van Poecke and Dicke, 2004; De Vos et al., 2005; Pieterse and Dicke, 2007; Browse, 2009). Together this makes this model species well suited to study the molecular biology of plant-insect interactions.

Induced resistance against pathogens and pests

As a response to different environmental stimuli, plants can acquire systemic resistance in distal plant parts that provides protection against subsequent pathogen or insect attack. This phenomenon was first observed in tobacco plants infected by

tobacco mosaic virus (TMV) (Ross, 1961). Necrotic lesions caused by a second infection of the same plant remained much smaller than those due to the primary infection, showing that the pathogen was contained faster in the induced plants. The phenomenon was called systemic acquired resistance (SAR) and was shown to depend on an intact SA signaling pathway and to have a broad spectrum effect against diverse pathogens (Vernooij et al., 1994; Van Loon, 1997; Mauch-Mani and Métraux, 1998; Durrant and Dong, 2004). A similar phenomenon was observed upon herbivory, where caterpillars gained less weight on plants that had been infested before (Green and Ryan, 1972; Agrawal and Kurashige, 2003; De Vos et al., 2006a). This type of induced resistance is referred to as wound induced resistance (WIR) and is largely dependent on JA signaling (Kessler and Baldwin, 2002; Howe and Jander, 2008). In addition, systemic resistance can also be induced by beneficial microbes, such as mycorrhizal fungi (Pozo and Azcon-Aguilar, 2007) and plant growth-promoting rhizobacteria, which is referred to rhizobacteria-mediated induced systemic resistance (ISR) with a broad spectrum effect against pathogens and pests. ISR functions independently of SA, but instead requires a functional JA and ET response pathway (Pieterse et al., 1996; Van Wees et al., 1997; Pieterse et al., 1998; Van Wees et al., 2008). Chemicals that activate defense signaling pathways like the SA mimic benzothiadiazole (BTH) can also induce resistance (Conrath et al., 2006).

Priming in hormone-regulated plant defense

It is generally believed that hormone-regulated induced defense responses evolved to save energy under enemy-free conditions, since they only involve costs when defenses are activated upon pathogen or insect attack (Walters and Heil, 2007). These costs arise from the allocation of resources to defense and away from plant growth and development. Trade-offs between plant growth rate and expression of disease resistance have been well documented (Walters and Heil, 2007) and support the hypothesis that plant growth and defense are regulated by a network of interconnecting signaling pathways. Priming for enhanced defense adds another layer of complexity to the way by which plants can adapt to their biotic environment (Conrath et al., 2006; Frost et al., 2008). In primed plants, defense responses are not activated directly by the priming agent, but are accelerated only following perception of biotic or abiotic stress signals, resulting in an enhanced level of resistance (Conrath et al., 2006). The primed state can be induced biologically by beneficial rhizobacteria (Van Wees et al., 2008), mycorrhizal fungi (Pozo and Azcon-Aguilar, 2007), pathogens (Cameron et al., 1999), and insect herbivores (Engelberth et al., 2004; De Vos et al., 2006a), but also chemically, for example, by

exogenous application of low doses of SA (Mur et al., 1996), JA (Kauss et al., 1994) or β-aminobutyric acid (Ton et al., 2005). By studying the costs and benefits of priming in Arabidopsis, it was shown that the fitness costs of priming are lower than those of constitutively activated defenses, such as expressed in the constitutive SAR expressing mutant *cpr1* (Van Hulten et al., 2006). Intriguingly, the fitness benefits of priming outweighed its costs under pathogen pressure, suggesting that priming functions as an ecological adaptation of the plant to respond faster to its hostile environment (Van Hulten et al., 2006; Walters et al., 2008).

Typically, priming is characterized by accelerated defense-related gene expression once primed plants are attacked by a pathogen or an insect, resulting in an enhanced level of resistance against the invader. This enhanced resistance is effective against a broad spectrum of attackers. The molecular basis of priming is poorly understood, but recent advances in research on priming demonstrate that the accumulation of latent defense-related TFs (Van der Ent et al., 2009c), MAP kinases (Beckers et al., 2009) and secondary metabolites such as azelaic acid (Jung et al., 2009) and volatile organic compounds (Engelberth et al., 2004; Heil and Silva Bueno, 2007; Ton et al., 2007) can play a role in the establishment of the primed state.

The range of effectiveness of priming depends on the way in which priming is induced (Van der Ent et al., 2009b). Priming by activators of SAR is mainly functional against pathogens that are counteracted by SA-dependent defenses, e.g. biotrophic pathogens (Ton et al., 2002). ISR functions mainly against pathogens that are sensitive to JA-dependent defenses (Ton et al., 2002). For instance, ISR is associated with priming for enhanced JA-responsive gene expression (Van Wees et al., 1999; Hase et al., 2003; Pozo et al., 2008) and as a result is effective against necrotrophic pathogens, such as *A. brassicicola, B. cinerea, and P. cucumerina* (Ton et al., 2002; Van der Ent et al., 2008), and insect herbivores, such as *S. exigua* (Van Oosten et al., 2008). ISR is also associated with priming for enhanced callose deposition at the site of pathogen entry, resulting in enhanced protection against pathogens such as the oomycete *H. arabidopsidis* (Van der Ent et al., 2008). BABA-IR depends on SA and ABA and is not only effective against a broad range of biotic stresses (Siegrist et al., 1998; Zimmerli et al., 2000; Ton and Mauch-Mani, 2004; Hodge et al., 2005), but also to salt and osmotic stress (Jakab et al., 2005).

Hormone crosstalk in plant defense

Hormone crosstalk is a process in which different hormone signaling pathways act antagonistically or synergistically, thereby providing a powerful regulatory potential

to flexibly tailor the plant's adaptive response to a variety of environmental cues. Crosstalk between SA, JA, and ET signaling pathways emerged as an important regulatory mechanism of plant immunity (Spoel and Dong, 2008; Grant and Jones, 2009; Pieterse et al., 2009). Many studies have demonstrated that endogenously accumulating SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent resistance over JA-dependent defense (Koornneef and Pieterse, 2008). ET often plays a modulating role in this respect (Leon-Reyes et al., 2009; Zander et al., 2009; Leon-Reyes et al., 2010a). However, depending on the plant species and strategy of the attacker, JA can also antagonize the SA pathway. For instance, in wild tobacco (*Nicotiana attenuata*) it was demonstrated that the JA and ET bursts triggered by herbivore-derived elicitors, impede the SA burst resulting in tuning of the SA-JA signal interaction and hence the defense responses of the plant to herbivory (Diezel et al., 2009). Recently, ABA, auxins, gibberellins, and brassinosteroids emerged as forces on the battle field as well. In many cases, these hormones interact antagonistically or synergistically with the SA-JA-ET backbone of the plant immune signaling network, thereby redirecting its defense output (Wang et al., 2007; Navarro et al., 2008; Yasuda et al., 2008; Campos et al., 2009; de Torres-Zabala et al., 2009; Ton et al., 2009; De Vleesschauwer et al., 2010; Jiang et al., 2010) (Fig. 4).

TF MYC2 is a key regulator in the JA pathway. Together with partially redundant MYC3 and MYC4, it is the only TF so far that has been shown to interact with JAZ repressor proteins which are repressing all JA responses (Fernandez-Calvo et al., 2011). Therefore, hormone crosstalk at the level of the JA response is likely to occur on the level of MYC regulation. Indeed, ET was shown to inhibit *MYC2* gene expression (Anderson et al., 2004), although used concentrations of ET were relatively high (200 ppm), whereas ABA has been shown to induce expression of *MYC2* (Anderson et al., 2004; Lorenzo et al., 2004). In general, the JA response can be divided into two branches. The MYC2-branch is co-regulated by ABA and results in transcription of genes such as *VSP2*, implicated in defense against insects (Liu et al., 2005). The ERF-branch is co-regulated by ET (Lorenzo et al., 2003) and involves a number of JA- and ET-regulated ERF-type TFs which act in a redundant mode. Activation of the ERF-branch results in transcription of genes such as *PDF1.2*, implicated in defense against pathogens (Penninckx et al., 1998). MYC2 recognizes a G-box motif (CACGTG), which is also present in the promoter of *ERF1*, providing in a mechanism for MYC2 to suppress the ERF-branch (Dombrecht et al., 2007). ERF TFs recognize GCC-box motifs (AGCCGCC) (Fujimoto et al., 2000; Zarei, 2007). These two branches act in a mutually antagonistic mode (Lorenzo et al., 2004). Whether both branches influence each other on the level of these binding motifs, or whether other mechanisms play a role remains to be investigated.

Hormone crosstalk in a changing environment

While the importance of hormones in the hardwiring of the plant immune signaling circuitry is evident, little is known about how environmental factors feed into the regulatory network. Light signaling has been shown to strongly influence hormone-regulated defenses. For instance, Arabidopsis plants display an altered sensitivity to JA-dependent defenses under conditions of crowding and shade, which is regulated via the photoreceptor phytochrome (Moreno et al., 2009; Robson et al., 2010). This results in a tradeoff between resource investment in JA-dependent defense and increased growth to out-compete neighbors (Moreno et al., 2009). Crosstalk between ABA-mediated drought stress and JA-dependent defense against necrotrophic pathogens in Arabidopsis was recently shown to be regulated by the TF OCP3 (Ramirez et al., 2009). Interestingly, SA was shown to antagonize both adaptive responses (Spoel et al., 2007; Yasuda et al., 2008). However, in the adaptive response of Arabidopsis to heat stress, SA and JA seem to act together (Clarke et al., 2009), highlighting the complexity of the regulatory processes involved in the adaptive response of plants to their continuously changing environment.

Decoy tactics

While hormone crosstalk may provide the plant with a powerful regulatory potential to finely tune its defense, crosstalk is also a possible target for plant attackers to manipulate the immune signaling network for their own benefit. In recent years, several nice examples of pathogens and insect herbivores that hijack specific hormone-regulated signaling pathways to redirect the immune response have been described (reviewed in Robert-Seilaniantz et al., 2007; López et al., 2008; Grant and Jones, 2009). For instance, the wilting pathogen *Fusarium oxysporum* has been shown to take control of the JA pathway in Arabidopsis, thereby enhancing Fusarium wilt-disease symptoms that lead to plant death (Thatcher et al., 2009). The bacterial pathogen *P. syringae* was shown to promote disease by altering the host's auxin and ABA physiology through the injection of specific virulence effectors into the host cell (Chen et al., 2007; de Torres-Zabala et al., 2007; de Torres-Zabala et al., 2009) (Fig. 4). *P. syringae*-induced ABA levels appeared to suppress SA-biosynthesis and action, resulting in enhanced susceptibility to this pathogen (de Torres-Zabala et al., 2007; de Torres-Zabala et al., 2009). In addition, *P. syringae* produces the virulence factor coronatine, which acts as a molecular mimic of biologically the most active form of JA, JA-Ile. Coronatine suppresses SA-dependent defenses, thereby also contributing to the suppression of the host's immune response to this pathogen (Uppalapati et al., 2007; Katsir et al., 2008). Interestingly, coronatine directly binds

to the JA receptor COI1 and is much more active than JA-Ile (Katsir et al., 2008; Yan et al., 2009), exemplifying the ingenious ways by which pathogens can take control over the plant's hormone signaling network to suppress host immunity.

Insects have been demonstrated to exert similar decoy tactics. For instance, nymphs of the phloem-feeding silverleaf whitefly (*Bemisia tabaci*) were shown to induce the SA pathway and suppress the JA pathway resulting in a faster development of the nymphs (Zarate et al., 2007). A similar phenomenon was observed with caterpillars from *S. exigua*. Elicitors from salivary excretions of this herbivore suppressed effectual JA-dependent defenses through the activation of the SA pathway (Weech et al., 2008; Diezel et al., 2009). Recently, elicitors from insect eggs were found to activate the SA pathway in Arabidopsis at the site of oviposition. As a result, JA-dependent defenses were suppressed resulting in an advantage for the newly hatched offspring that fed from the undefended tissue (Bruessow et al., 2010; Fig. 3).

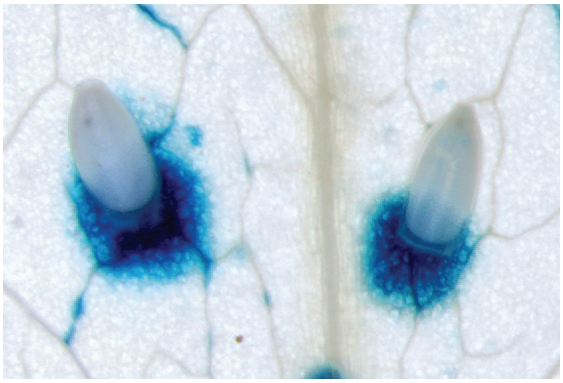


Figure 3. Activation of SA defenses by caterpillar eggs.
Eggs of the cabbage white butterfly *(Pieris rapae)* activate the SA-responsive *PR1::GUS* reporter gene at the site of oviposition. Activation of the SA pathway suppresses JA-dependent herbivore resistance, providing an advantage for newly hatched larvae of generalist herbivores that feed from this de-immunized tissue (Bruessow et al., 2010). Photo: Hans Van Pelt.

Multispecies interactions: juggling the good, the bad, and the ugly

In nature, plants often deal with simultaneous or subsequent invasion by multiple pathogens and insects. In a field study with *Brassica oleracea* plants, it was demonstrated that early season herbivory significantly affected plant defense responses to secondary herbivores and the development of their populations (Poelman et al., 2008). Gene expression analyses implicated a dominant role for plant hormones in the regulation of this process. Also in Arabidopsis, pathogen and insect attack have been shown to affect secondary interactions with antagonists. For example, induction of the SA pathway by *P. syringae* suppressed JA signaling and rendered infected leaves more susceptible to the necrotrophic fungus *A. brassicicola* (Spoel et al., 2007). Similarly, prior inoculation with the biotrophic downy mildew pathogen *H. arabidopsidis* suppressed JA-mediated defenses that were activated upon feeding by caterpillars of the small cabbage white *P. rapae* (Koornneef et al., 2008). In Lima

bean it was demonstrated that infestation with phloem-feeding whiteflies (*B. tabaci*) negatively affected indirect plant defenses that are triggered by spider mites (*Tetranychus urticae*), resulting in a reduced attraction of predatory mites (Zhang et al., 2009). This effect could be mimicked by SA and involved suppression of JA biosynthesis, indicating that the effect of SA-JA crosstalk extends to plant immune responses against organisms from different trophic levels.

In the soil, plant roots interact with an enormously diverse community of microbes, insects and worms, which can influence the defense status of the aerial plant parts. For instance, belowground infestation by the western corn rootworm (*Diabrotica virgifera virgifera*) was shown to affect the aboveground hormone signature of maize and the level of aboveground resistance against herbivores and necrotrophs (Erb et al., 2009). Belowground interactions are frequently beneficial in nature. Because beneficial microbes are also recognized as alien organisms, active interference with the plant immune signaling network is fundamental for the establishment of intimate mutualistic relationships. In many cases, SA, JA and ET emerged as the dominant regulators in this process (Van Wees et al., 2008; Gutjahr and Paszkowski, 2009; Van der Ent et al., 2009a; López-Ráez et al., 2010a; Pineda et al., 2010). During the beneficial interaction of Arabidopsis roots with the endophytic fungus *Piriformospora indica*, components of the ET pathway were implicated in the balance between beneficial and non-beneficial traits of this symbiosis (Camehl et al., 2010). Suppression of ET signaling was also observed in mycorrhizal tomato plants (López-Ráez et al., 2010a). In addition, plant growth-promoting *Pseudomonas fluorescens* bacteria were shown to actively suppress ET-dependent MAMP-induced responses in the roots of Arabidopsis (Millet et al., 2010), presumably to avoid being recognized as an uninvited guest. Colonization of roots by these beneficial rhizobacteria induces ISR (Pozo et al., 2008; Van Oosten et al., 2008; Van der Ent et al., 2009c). The list of examples by which beneficial microbes recruit hormone signaling pathways to establish a mutualistic interaction is growing rapidly. However, our understanding of how plants regulate their immune signaling network to maximize both profitable and protective functions during simultaneous interactions with the good, the bad, and the ugly is still limited.

Unraveling the hows and whys of hormone crosstalk in plant defense

In recent years, several players in the interactions between hormone-regulated defense signaling pathways have been described (reviewed in Pieterse et al., 2009). These include transcriptional (co)regulators such as NPR1, DELLAs, WRKYs, TGAs, MYC2, and ERFs. Unexpected connections between the gene

regulatory networks of these factors start to emerge. For example, TGA TFs that were previously implicated in the regulation of SA-dependent defenses, were also shown to be essential activators of induced defenses that are triggered by the combined action of JA and ET (Zander et al., 2009). However, upon simultaneous activation of the SA, JA and ET pathways, TGAs act together with MYC2 to suppress JA/ET-dependent defenses (Zander et al., 2009), again demonstrating the delicate interrelationship between these hormones. While the players in hormone crosstalk become more and more identified, it is clear that various posttranslational protein modifications add to the complexity of the hormone-regulated immune signaling network (Spoel et al., 2010). Exciting developments in hormone signaling research underpin the central role of the ubiquitin proteasome system in this respect, as this regulatory protein machinery is involved in hormone perception, de-repression of hormone signaling pathways, degradation of hormone specific TFs, and regulation of hormone biosynthesis (Santner and Estelle, 2010).

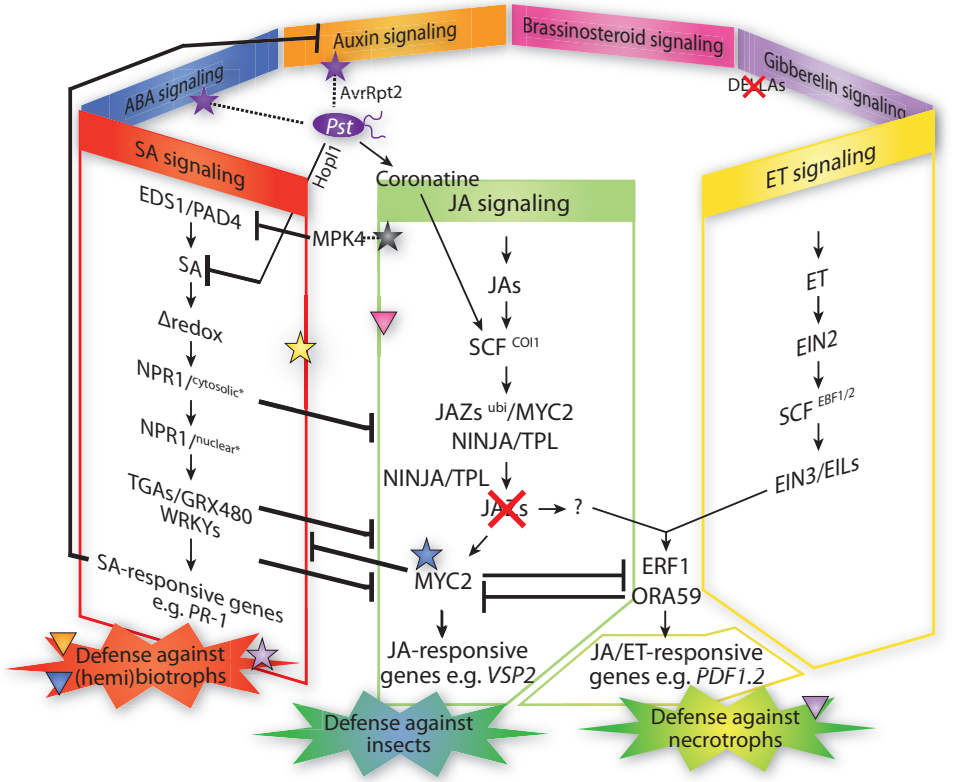


Figure 4: Working model of defense related hormone signaling
Cross-communication between hormone signaling pathways provides the plant with a large regulatory capacity that may tailor its defense response to different types of attackers. On the other hand, pathogens such as *P. syringae (Pst)* produce effector proteins (for example coronatine, HopI1 and AvrRpt2) that manipulate the signaling network to suppress host immune responses and promote virulence. The SA, JA and ET signaling pathways represent the backbone of the defense signaling network, with other hormonal signaling pathways feeding into it. Arrows; induction, blocked lines; repression, triangles and stars (colors correspond to hormone pathways); negative and positive effect, respectively. Modified from: Pieterse et al. (2009).

Recent genomics research has revealed that the signaling networks that are activated in response to mutualists, pathogens and insects overlap, indicating that the regulation of the adaptive response to these organisms is finely balanced between protection against aggressors and acquisition of benefits. Major challenges for future research lie in understanding how complex multi-dimensional signal interactions are regulated during the interaction of plants with single or multiple organisms, and why they steer the immune response toward a particular defense output.

OUTLINE OF THE THESIS

Microbial pathogens and insects have developed different invading strategies to obtain nutrients from plants and the plants possess a whole suite of defensive strategies to effectively counteract the plethora of harmful organisms in their environment. Previously, De Vos *et al.* (2005) showed that plants respond to different types of attackers with unique hormonal signal signatures, which translates into unique changes in the transcriptome. For instance, the tissue-chewing insect herbivore *P. rapae*, the leaf content feeding insect Western flower thrips *F. occidentalis* and the necrotrophic fungal pathogen *A. brassicicola* all induced production of JA, but activated a very different set of JA-responsive genes in Arabidopsis plants (De Vos et al., 2005). The notion that transcriptional changes in response to diverse JA-inducing insect herbivores and pathogens show limited overlap, suggested that the context in which the JA signal is perceived is crucial in tuning the final outcome of the JA response. Recent advances in plant immunity research underpin the pivotal role of cross-communicating hormones in the regulation of the plant's defense signaling network (Pieterse et al., 2009). Because JA signaling is highly sensitive to modulating actions by other hormones, such as ET, ABA and SA, we set out to investigate the molecular and biological basis of differential JA responses. To this end, we started with the observation that feeding by *P. rapae* on Arabidopsis leaves, leads to stimulation of the MYC2-branch of the JA pathway, whereas the ERF-branch was not activated (De Vos et al., 2005), and asked the questions: 1) how are the differential JA responses regulated during the *P. rapae*-Arabidopsis interaction?; 2) what is the role of other hormones in this phenomenon?; 3) do differential JA responses contribute to resistance to the insect, or is it a decoy tactic of the herbivore to suppress effective defenses?; 4) to what extent are differential JA responses expressed systemically in undamaged tissues of *P. rapae*-infested plants?; and 5) does suppression of the MYC2-branch lead to enhanced fitness of plants grown in the field when under attack by a necrotrophic pathogen?

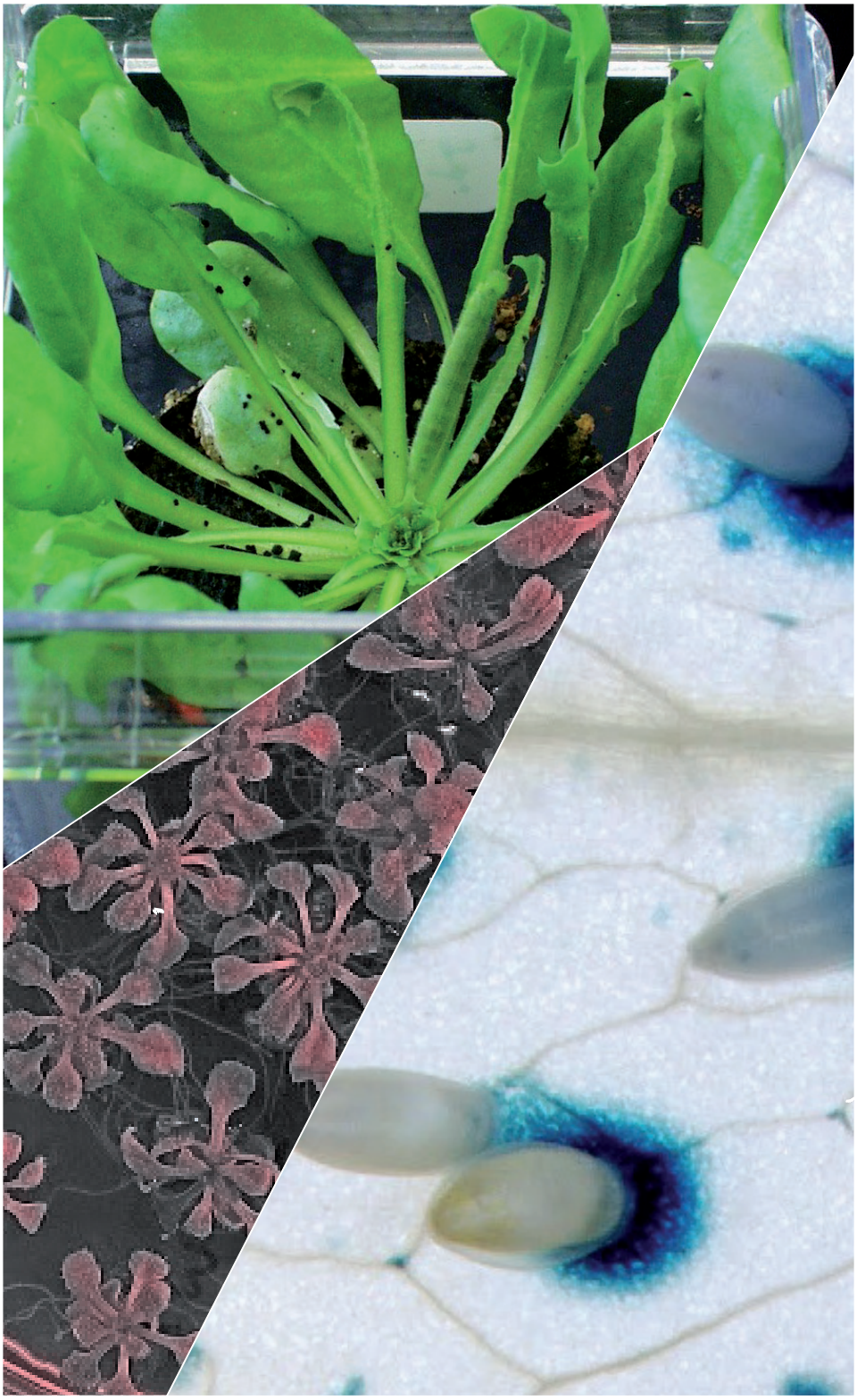
In Chapter 2, we made use of Arabidopsis genotypes that are affected in the MYC2- or ERF-branch of the JA pathway to demonstrate that during *P. rapae* feeding activation of the MYC2-branch is prioritized over the ERF-branch, which is suppressed in a MYC2-dependent manner. Although this had no major effect on growth of the larvae in no-choice assays, two-choice assays revealed that *P. rapae* larvae preferred to feed from plants that expressed the ERF-branch of the JA pathway. Interestingly, application of larval oral secretion into wounded leaf tissue stimulated the ERF-branch of the JA pathway, suggesting that compounds in the oral secretion have the potential to manipulate the plant response toward the preferred ERF-regulated branch of the JA response. Our results suggest that by activating the MYC2-branch, plants rewire the JA signaling pathway to prevent stimulation of the herbivore-preferred ERF-branch of the JA pathway.

In Chapter 3, we examined the role of ABA, ET and SA in the rewiring of the differential JA response as observed during herbivory by *P. rapae.* By examining hormone levels and using mutant and pharmacological approaches we demonstrated that ABA plays an essential role in stimulation of the MYC2-branch and the concomitant repression of the ERF-branch of the JA pathway during the interaction of Arabidopsis with *P. rapae*, which highlights the important modulating role of ABA in the hormone-regulated defense signaling network in plants under attack by harmful organisms.

In Chapter 4, we investigated whether the activation of differential JA responses by insect herbivory spread to systemic tissues. We provide evidence that herbivory does not lead to direct activation of JA-dependent defenses in systemic undamaged leaves, but instead induces priming of the MYC2-branch of the JA pathway, in this tissue.

In Chapter 5, the MYC2-impaired mutant *jin1*, that is primed for enhanced expression of JA-responsive genes of the ERF-branch upon pathogen attack, was subjected to a study in the field. The performance of the constitutively primed mutants *jin1* and *edr1* (primed for SA reponses) was determined after inoculation with the necrotrophic fungus *P. cucumerina* and the biotrophic oomycete *H. arabidopsidis*. Our data suggest that wild-type plants in the field obtained priming levels that were comparable to those of *jin1* and *edr1*, possibly caused by naturally occurring factors, like e.g. ISR-inducing rhizobacteria. Interestingly, we also observed that infection by the biotrophic pathogen *H. arabidopsidis* can enhance the fitness of infected plants.

In Chapter 6, the results presented in this thesis are discussed in view of the current status of knowledge on plant defense signaling.



CHAPTER 2

Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory

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ABSTRACT

Plant defenses against insect herbivores and necrotrophic pathogens are differentially regulated by different branches of the jasmonic acid (JA) signaling pathway. In Arabidopsis, the basic helix-loop-helix leucine zipper transcription factor MYC2 and the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain transcription factor ORA59 antagonistically control these distinct branches of the JA pathway. Feeding by larvae of the specialist insect herbivore *Pieris rapae* activated *MYC2* transcription and stimulated expression of the MYC2-branch marker gene *VSP2,* while it suppressed transcription of *ORA59* and the ERF-branch marker gene *PDF1.2.* Mutant *jin1* and *jar1-1* plants, which are impaired in the MYC2-branch of the JA pathway, displayed a strongly enhanced expression of both *ORA59* and *PDF1.2* upon herbivory, indicating that in wild-type plants the MYC2-branch is prioritized over the ERF-branch during insect feeding. Weight gain of *P. rapae* larvae in a no-choice setup was not significantly affected, but in a two-choice setup the larvae consistently preferred *jin1* and *jar1-1* plants, in which the ERF-branch was activated, over wild-type Col-0 plants, in which the MYC2-branch was induced. In MYC2- and ORA59-impaired *jin1-1*/RNAi-*ORA59* plants this preference was lost, while in *ORA59*-overexpressing *35S:ORA59* plants it was gained, suggesting that the herbivores were stimulated to feed from plants that expressed the ERF-branch rather than that they were deterred by plants that expressed the MYC2-branch. The feeding preference of the *P. rapae* larvae could not be linked to changes in glucosinolate levels. Interestingly, application of larval oral secretion into wounded leaf tissue stimulated the ERF-branch of the JA pathway, suggesting that compounds in the oral secretion have the potential to manipulate the plant response toward the caterpillar-preferred ERF-regulated branch of the JA response. Our results suggest that by activating the MYC2-branch of the JA pathway, plants prevent stimulation of the ERF-branch by the herbivore, thereby becoming less attractive to the attacker.

INTRODUCTION

Plants possess a powerful innate immune system by which they recognize non-self molecules or signals from injured cells, and respond by activating an effective defense response (Jones and Dangl, 2006; Howe and Jander, 2008). Defense mechanisms that are induced upon attack by herbivorous insects involve direct defenses such as the production of proteinase inhibitors and glucosinolates that target essential physiological processes in the insect, and indirect defenses such as the emission of volatiles that attract parasitoids and predators of the herbivores that feed on the plant (Howe and Jander, 2008; Dicke et al., 2009; Hopkins et al., 2009). The plant hormone jasmonic acid (JA) and its oxylipin derivatives (collectively called here jasmonates (JAs)) are key players in the regulation of induced plant responses against herbivory (Koo and Howe, 2009). JAs also play important roles in plant defense against necrotrophic pathogens (Glazebrook, 2005; Laluk and Mengiste, 2010). However, the JAs-controlled responses to necrotrophs and insect herbivores seem to be regulated via different branches of the JA signaling pathway (Lorenzo and Solano, 2005; Kazan and Manners, 2008).

Upon pathogen or insect attack, JAs are rapidly synthesized via the oxylipin biosynthesis pathway (Wasternack, 2007; Gfeller et al., 2010). Through the JA conjugate synthase JAR1, JA can be readily conjugated to amino acids such as isoleucine (Staswick and Tiryaki, 2004), resulting in biologically highly active jasmonoyl-isoleucine (JA-Ile) (Fonseca et al., 2009). The F-box protein COI1 (CORONATINE INSENSITIVE 1) functions as a JA-Ile receptor in the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI1} (Yan et al., 2009). Binding of JA-Ile to COI1 leads to degradation of JASMONATE ZIM-domain (JAZ) transcriptional repressor proteins via the proteasome (Chini et al., 2007; Thines et al., 2007). Consequently, the physical interaction of JAZ proteins with transcriptional activators, leading to repression of JA signaling in resting cells, is broken in JA-stimulated cells, which results in the activation of a large number of JA-responsive genes (Chini et al., 2007; Memelink, 2009).

Transcriptional changes in response to diverse JA-inducing pathogens and insect herbivores show limited overlap, suggesting that the context in which the JA signal is perceived is crucial in tuning the JA response (De Vos et al., 2005; Pauwels et al., 2009). The plant hormones ethylene (ET), abscisic acid (ABA), and salicylic acid (SA), which in various combinations and concentrations can be part of the signal signature that is produced upon pathogen or insect attack, emerged as important differential regulators of the JA response (Leon-Reyes et al., 2009; Pieterse et al., 2009; Leon-Reyes et al., 2010a). While SA generally acts antagonistically on the JA response (Koornneef et al., 2008; Koornneef and Pieterse, 2008; Verhage et al.,

2010), ET and ABA each co-regulate a different branch of the JA response (Adie et al., 2007a; Kazan and Manners, 2008).

JA and ET are often simultaneously produced upon infection by necrotrophic pathogens (De Vos et al., 2005). In *Arabidopsis thaliana* (Arabidopsis), JA and ET signaling act synergistically on the expression of defense-related genes, such as *PLANT DEFENSIN1.2* (*PDF1.2*) (Penninckx et al., 1998). Two members of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of transcription factors (TFs), ERF1 and ORA59, emerged as principal integrators of JA and ET signaling (Lorenzo et al., 2003; Pré et al., 2008). Both TFs activate the ERF-branch of the JA pathway and confer resistance to necrotrophic pathogens (Berrocal-Lobo et al., 2002; Pré et al., 2008). RNAi-*ORA59* plants were demonstrated to be completely blocked in JA/ET-induced *PDF1.2* expression, indicating that ORA59 is required for this response. Another important master regulator of JA-responsive gene expression is the basic helix-loop-helix leucine zipper TF MYC2 (originally called JIN1 for JASMONATE INSENSITIVE1). On the one hand, MYC2 acts in concert with ABA signaling in negatively regulating the ERF-branch of the JA pathway (Anderson et al., 2004; Lorenzo et al., 2004). On the other hand, MYC2 functions as a transcriptional activator of genes in the MYC2-branch of the JA pathway, including the JA marker gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Dombrecht et al., 2007). The MYC2-branch of the JA pathway is associated with the wound-response and is thought to contribute to defense against insect herbivores (Lorenzo et al., 2004; Dombrecht et al., 2007; Fernandez-Calvo et al., 2011), although it has also been demonstrated to play a role in priming for enhanced pathogen defense (Pozo et al., 2008).

Continuing co-evolution between plants and herbivores has provided the latter with mechanisms to avoid, suppress, or eliminate host defenses (Schoonhoven et al., 2005; Pieterse and Dicke, 2007; Kant et al., 2008). Larvae of the specialist insect herbivore *Pieris rapae* (small cabbage white butterfly) feed exclusively on crucifers. In the field, they have been reported to feed on many brassicaceous species, including Arabidopsis (Yano and Ohsaki, 1993). *P. rapae* larvae are well adapted to the induced defenses of brassicaceous species. For instance, *P. rapae* produces an enzyme that redirects the myrosinase-driven conversion of glucosinolates from the toxic isothiocyanates towards the less noxious nitriles. By detoxifying the herbivory-triggered 'mustard oil bomb', *P. rapae* caterpillars efficiently avoid exposure to these highly toxic chemicals (Wittstock et al., 2004). However, Arabidopsis still posesses additional direct defenses that are active against this specialist herbivore as demonstrated by elevated resistance in systemic undamaged plant parts induced upon prior feeding by *P. rapae* (De Vos et al., 2006a). Herbivory by *P. rapae* leads to increased production of JAs and extensive reprogramming of the expression of JA-

responsive genes, many of which are associated with plant defense (Reymond et al., 2000; Reymond et al., 2004; De Vos et al., 2005). Transcriptional changes inflicted by mechanical wounding or insect feeding overlap only marginally (Reymond et al., 2000), suggesting that insect-derived cues play an important role in the modulation of the plant's transcriptional response to herbivory.

Ever since the seminal paper by Ehrlich and Raven (Ehrlich and Raven, 1964), ecologists have intensely debated the arms race between plants and herbivorous insects (Thompson, 1994; Schoonhoven et al., 2005). However, knowledge of the underlying mechanisms is still relatively limited in comparison to the well-studied mechanisms involved in the arms race between pathogens and their host plants (Jones and Dangl, 2006). Induced plant responses to insect herbivory consist of a mix of direct and indirect plant defenses aimed at limiting insect performance, and of insect-mediated plant responses that favor the insect herbivore. Here, we investigated the response of Arabidopsis to herbivory by the specialist *P. rapae*. We demonstrate that oral secretions of *P. rapae* induce the ERF-branch of the JA pathway, whereas feeding larvae activate the antagonistic counterpart of the JA response that is regulated by MYC2. We establish that activation of the MYC2-branch is favorable for the plant, not because it would actively deter caterpillars that feed on the plant, but because it suppresses the ERF-branch of the JA pathway for which the caterpillars have a preference. This "hide-the-candy" strategy of the plant sheds new light on the mechanisms involved in the evolutionary arms race between plants and their herbivorous enemies.

RESULTS

Herbivory activates the MYC2-branch and suppresses the ERF-branch of the JA pathway

The transcriptional response of Arabidopsis to *P. rapae* feeding is predominantly regulated via the JA signaling pathway (Reymond et al., 2004; DeVos et al., 2005). To investigate the significance of the MYC2- and the ERF-branch of the JA pathway in this response, we monitored the expression of the marker genes *PDF1.2* (ERF-branch) and *VSP2* (MYC2-branch). Caterpillars of larval stage L1 were allowed to feed on leaves of Arabidopsis wild-type Col-0 plants for 24 h, after which they were removed (Fig. 1A). Damaged leaves of *P. rapae*-infested plants were harvested for gene expression analysis. Caterpillar feeding activated *VSP2* in infested tissue (from 6 h onwards; Fig. 1B), which correlated with the induced expression pattern of *MYC2. PDF1.2* was only mildly induced in herbivore-damaged leaves (24 h onwards).

To investigate whether herbivory-mediated activation of the MYC2-branch of the JA pathway was associated with a concomitant suppression of the ERF-branch, we monitored the expression of *PDF1.2* and *VSP2* in *jin1* mutants that are impaired in MYC2 function (Anderson et al., 2004; Lorenzo et al., 2004). In Col-0 plants, 24 h of *P. rapae* feeding resulted in the accumulation of high levels of *VSP2* mRNA, which slowly declined back to basal levels 24 h after removal of the caterpillars (Fig. 1C). In MYC2-defective *jin1-1, jin1-2, jin1-7,* and *jin1-10* mutants, the level of *VSP2* mRNA was strongly reduced, confirming that MYC2 is an important regulator of *VSP2* transcription. Interestingly, the *jin1* mutants accumulated high levels of *PDF1.2* mRNA after 24 h of herbivory, which slowly declined after removal of the *P. rapae* larvae. Figure 1D displays the average relative expression levels of *PDF1.2* and *VSP2* in Col-0 and *jin1* mutants during 24 h of insect feeding (0-24 h) and up to 3 d after removal of the herbivores (24-72 h). Together, these results indicate that in wild-type plants feeding by *P. rapae* activates the expression of *VSP2* while it suppresses *PDF1.2* transcription, and that in MYC2-impaired *jin1* mutants these expression patterns are swopped. Mutant *jar1-1* plants, which are impaired in the production of bioactive JA-Ile (Staswick and Tiryaki, 2004) and as a result display reduced MYC2-dependent transcriptional activity, showed a similar *PDF1.2* expression pattern as the *jin1* mutants (Fig. 1C), corroborating the role of MYC2 in antagonizing the ERF-branch of the JA pathway during herbivory.

Role of ORA59 in herbivore-mediated suppression of the ERF-branch of the JA pathway

Since the AP2/ERF-type TF ORA59 is a major regulator of the ERF-branch of the JA pathway (Pré et al., 2008), we monitored the expression of *ORA59* in *P. rapae*-infested Col-0 and *jin1-2* plants. Figure 2A shows that herbivore-induced *ORA59* expression was significantly higher in *jin1-2* than in Col-0 plants. To

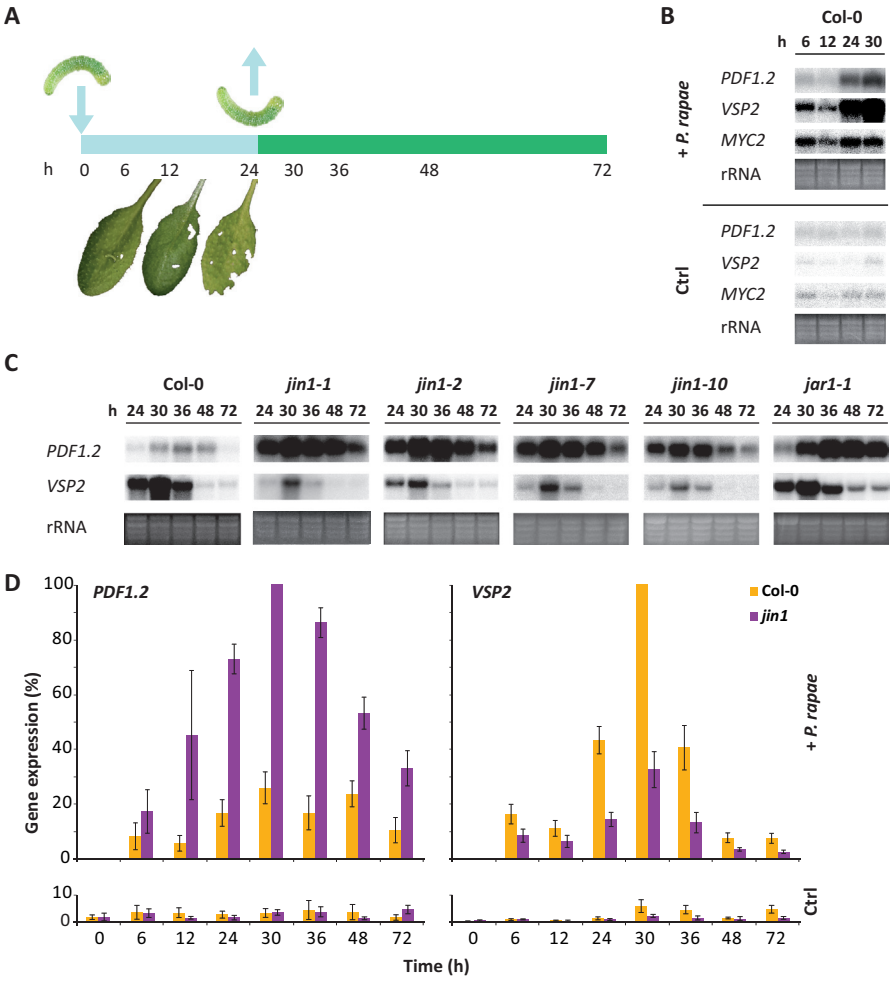

Figure 1: Effect of MYC2 on the differential JA response during herbivory by *P. rapae*.
(A) Experimental setup for the gene expression analyses of the Arabidopsis-*P. rapae* interaction. First-instar larvae of *P. rapae* were allowed to feed on 5-week-old Arabidopsis plants, after which they were removed. The time scale shows the harvest time points that were used in the different experiments. (B) Northern blot analysis of JA-responsive *PDF1.2* (marker for ERF-branch), *VSP2* (marker for *MYC2*-branch), and *MYC2* gene expression in leaves of non-treated control (Ctrl) and *P. rapae*-infested wild-type Col-0 plants. (C) Northern blot analysis of *PDF1.2* and *VSP2* transcription in Col-0, and mutant *jin1* and *jar1-1* plants. To check for equal loading, rRNA bands were stained with ethidium bromide. (D) Signal intensities of *PDF1.2* and *VSP2* mRNA on the northern blots of multiple experiments were quantified using a phosphor imager and plotted relative to the highest level (set at 100%). Error bars represent ±SE.

further investigate the role of ORA59 in the suppression of the ERF-branch of the JA pathway during *P. rapae* feeding, we analyzed herbivore-induced transcription of *PDF1.2*, *VSP2*, *ORA59* and *MYC2* in *ORA59*-silenced RNAi-*ORA59* plants (Pré et al., 2008). As expected, *ORA59* and *PDF1.2* transcript levels were virtually undetectable in herbivore-infested RNAi-*ORA59* plants (Fig. 2B). Conversely, *MYC2* and *VSP2* levels were enhanced in RNAi-*ORA59* plants in comparison to Col-0, suggesting that in wild-type plants ORA59 attenuates the MYC2-branch of the JA pathway during insect herbivory. In *jin1-1* plants, *ORA59* and *PDF1.2* mRNAs accumulated to high levels after *P. rapae* feeding, again indicating that the MYC2-mediated suppression of the ERF-branch was alleviated in this mutant. Silencing of *ORA59* in the *jin1-1* mutant background (*jin1-1*/RNAi-*ORA59*) strongly suppressed the *P. rapae*-induced expression levels of *ORA59* and *PDF1.2* that were apparent in the *jin1-1* single mutant. Together, these results point to a model in which the suppression of the ERF-branch of the JA pathway by MYC2

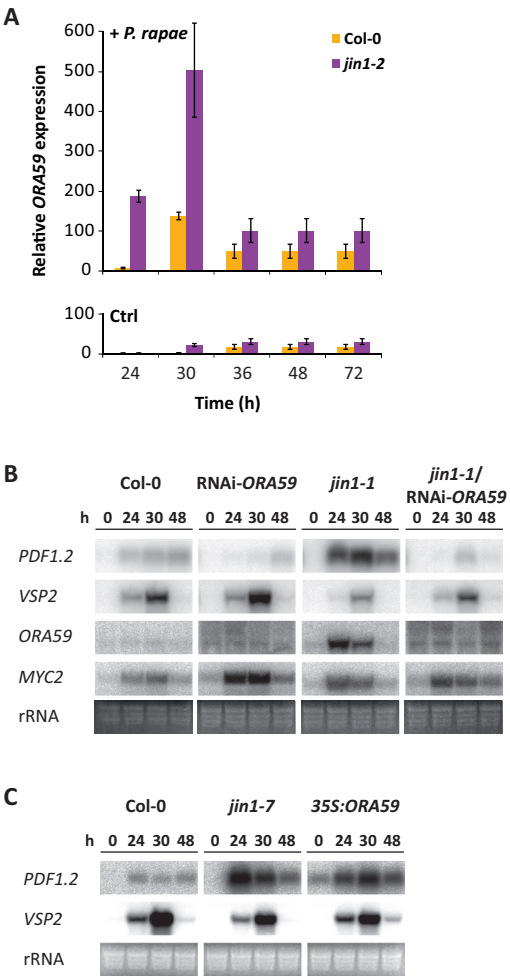

Figure 2: Effect of ORA59 on the differential JA-response during *P. rapae* feeding.
(A) RT-qPCR analysis of *ORA59* transcript levels (relative to At1g13320) in non-treated and *P. rapae*-infested Arabidopsis Col-0 and *jin1-2* plants at 24, 30, 36, 48 and 72 h after removal of *P. rapae* caterpillars that had fed on the tissue for 24 h. Transcript levels relative to non-treated Col-0 at t=24 h, which was set at 1, are plotted. (B) Northern blot analysis of *PDF1.2, VSP2, ORA59,* and *MYC2* transcript levels in Col-0, RNAi-*ORA59, jin1-1* and *jin1-1*/RNAi-*ORA59* plants at different time points after *P. rapae* feeding. (C) Northern blot analysis of *PDF1.2* and *VSP2* transcript levels in Col-0, *jin1-7* and *35S:ORA59* plants at different time points after *P. rapae* feeding. To check for equal loading, rRNA bands were stained with ethidium bromide. The experiments were repeated with similar results.

during insect herbivory acts through an antagonistic effect of MYC2 on *ORA59* transcription. To verify this hypothesis we analyzed the expression of *PDF1.2* and *VSP2* in *P. rapae*-infested *35S:ORA59* plants in which any suppressive effect on the *ORA59* promoter would be bypassed by the *35S*-driven overexpression of *ORA59*. Figure 2C shows that *P. rapae* feeding induced the expression of *PDF1.2* in *35S:ORA59* plants to the same level as that observed in *jin1-7* plants, indicating that in wild-type plants repression of *ORA59* is a limiting factor for the herbivore-induced expression of the ERF-branch. These results support the notion that MYC2-mediated suppression of the ERF-branch of the JA pathway during *P. rapae* feeding is likely to be caused by suppression of *ORA59* transcription, rather than by suppression of ORA59 activity.

Effect of the MYC2- and the ERF-branch of the JA pathway on insect performance in a no-choice assay

To investigate whether prioritization of the MYC2-branch over the ERF-branch during herbivory affects insect performance, we assessed growth of *P. rapae* larvae over a 10-day feeding period. Feeding of *P. rapae* L1 instar larvae on the JA receptor mutant *coi1-1*(*gl1*) (in glabrous Col-5(*gl1*) background) resulted in a moderate but significant increase in weight gain of the caterpillars (Fig. 3A), confirming previous findings that JA signaling is involved in resistance against grazing by *P. rapae* caterpillars (Reymond et al., 2004; Bodenhausen and Reymond, 2007; Van Oosten et al., 2008). As shown previously (Reymond et al., 2004), *P. rapae* larvae gained significantly more weight on glabrous Col-5(*gl1*) than on Col-0, which has a normal trichome phenotype (Fig. 3B). However, neither the *jin1-1*(*gl1*) mutation in the glabrous Col-5(*gl1*) background, nor the *jin1-7* mutation in the Col-0 background had an effect on the performance of *P. rapae* (Fig. 3B and 3C). Also on mutant *jar1-1* and the *ORA59*-silenced lines RNAi-*ORA59*#4 and RNAi-*ORA59*#9 (Pré et al., 2008) growth of *P. rapae* was not majorly affected, although at 4 d after infestation the larvae gained significantly more weight on *jar1-1* than on Col-0 (Fig. 3D and 3E). Together, these results indicate that the COI1-regulated JA-dependent defenses that negatively affect growth of the specialist herbivore *P. rapae,* are not seriously impaired in genotypes affected in either the MYC2- or ERF-branch of the JA pathway.

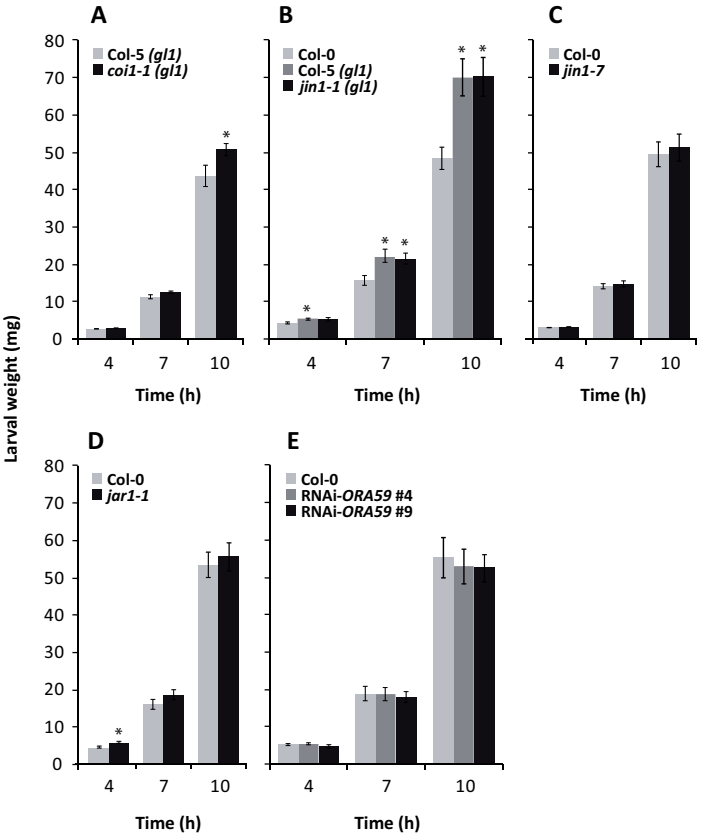

Figure 3: Growth of *P. rapae* larvae on Arabidopsis genotypes that are impaired in the JA response.
Growth of first-instar larvae of *P. rapae* was assessed on *coi1-1(gl1)* (A), *jin1-1(gl1)* (B), *jin1-7* (C), *jar1-1* (D) and RNAi-*ORA59*#4 and #9 (E). The genotypes were compared with their respective trichome-containing (Col-0) and trichome-less (Col-5*(gl1)*) wild-type backgrounds. Larval fresh weight was determined after 4, 7, and 10 days of feeding. The values represent means (±SE) of ±45 larvae per genotype. Asterisks indicate statistically significant differences in comparison to similarly-treated wild-type plants (Dunnett's pairwise multiple comparison *t* test, $P<0.05$).

Effect of the MYC2-branch of the JA pathway on insect preference in a two-choice assay

One of the ways by which plants can prevent loss of tissue during herbivory is by activating defenses that deter the attacker. We investigated the effect of the MYC2-branch of the JA pathway on the feeding preference of *P. rapae* caterpillars in a two-choice assay. To this end, four plants were placed in a two-choice arena (n=20) consisting of two plants per genotype (Fig. 4A). The plants in each arena were in physical contact with each other to allow the caterpillars to freely move from one plant to the other. At the start of the assay, two first-instar larvae were placed

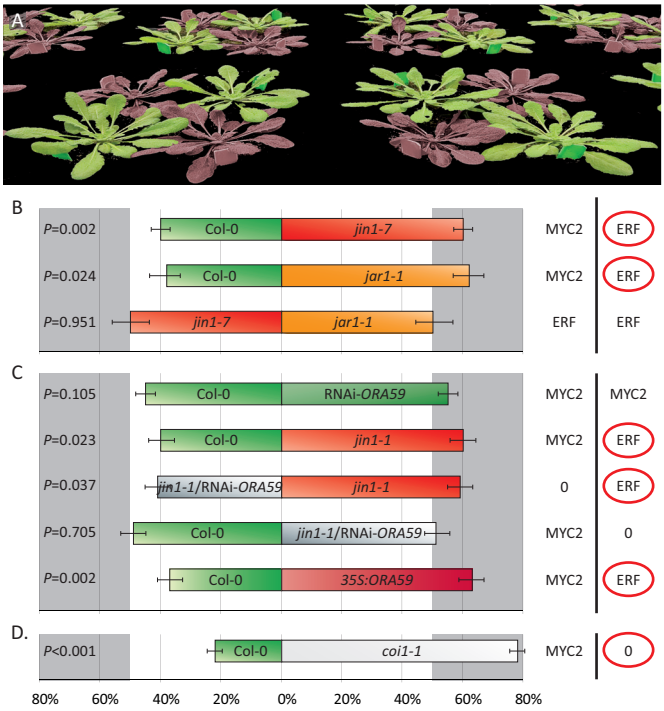

Figure 4: Effect of the MYC2- and ERF-branch of the JA pathway on the preference of *P. rapae* in two-choice tests.
In the two-choice arena, two first-instar larvae of *P. rapae* were placed on each plant (A). Each two-choice arena (*n*=20) consisted of two 6-week-old plants of each genotype (total 8 larvae per arena). After 4 days the number of caterpillars on each genotype was determined. The preference of *P. rapae* larvae was tested in combinations with wild-type Col-0 plants and genotypes that are affected in the MYC2-branch of the JA pathway (B), genotypes that are affected in the ERF-branch and/or the MYC2-branch of the JA pathway (C), and mutant *coi1-1* plants that are completely blocked in JA signaling (D). The right panel displays which branch of the JA pathway is predominantly activated in the corresponding genotypes that are displayed in the left panel. The "0" indicates that neither of the branches of the JA pathway was activated. Displayed are the average percentages (±SE) of the distribution of the *P. rapae* larvae over the two genotypes (X-axis). Statistically significant differences from the 50% percentile were analyzed using a Student's *t*-test. In cases of statistically significant differences (Student's *t*-test; *P*<0.05), the preferred branch of the JA pathway is marked with a red circle. Experiments were repeated with similar results.

on each plant (8 larvae per arena) and after 4 days the number of caterpillars per plant genotype was determined in 20 independent two-choice arenas. MYC2-branch-related mutants *jin1-1*, *jin1*-7, and *jar1-1* accommodated significantly more *P. rapae* larvae at the end of the two-choice assay than did Col-0 plants (frequency distribution of the larvae over mutant and wild-type plants: 60:40%, 60:40%, and 62:38%, respectively; Fig. 4B and 4C). When comparing *jin1*-7 with *jar1-1* in the two-choice test, the frequency distribution of the larvae over both mutants was similar (50:50%; Fig. 4B). These results suggest that the MYC2-branch of the JA pathway regulates defense responses that affect the feeding preference of *P. rapae* larvae, and that the *jin1* and the *jar1* mutation affect this MYC2-dependent trait to a similar extent.

P. rapae larvae prefer to feed on plants that express the ERF-branch of the JA pathway

Because genetic obstruction of the MYC2-branch of the JA pathway alleviates the suppression of the ERF-branch, the preference of the *P. rapae* larvae for *jin1* over Col-0 plants may be caused by 1) a deterring effect of the MYC2-branch that is activated in Col-0 plants upon herbivory, or 2) an feeding stimulatory effect of the ERF-branch that is induced in *jin1* plants. To test this, we compared the following Arabidopsis genotypes, which were demonstrated to be affected in MYC2- and/or ERF-regulated gene expression (Figs. 1 and 2), in the two-choice assay: Col-0 (MYC2-branch on, ERF-branch off), *jin1-1* (MYC2-branch off, ERF-branch on), RNAi-*ORA59* (MYC2-branch on, ERF-branch off), *35S:ORA59* (MYC2-branch off, ERF-branch on), and *jin1-1*/RNAi-*ORA59* (both branches off). Figure 4C shows that mutant *jin1-1* plants accommodated significantly more caterpillars than did *jin1-1*/RNAi-*ORA59* plants (frequency distribution 59:41%), suggesting that *P. rapae* larvae preferred feeding from plants that expressed the ERF-branch of the JA pathway, rather than that they were deterred by plants that expressed the MYC2-branch. Col-0 and *jin1-1*/RNAi-*ORA59* accommodated similar numbers of *P. rapae* larvae (frequency distribution 49:51%), indicating that activation of the MYC2-branch of the JA-pathway in Col-0 did not make the plants less preferred than *jin1-1*/RNAi-*ORA59* plants in which neither of the two branches were activated. The frequency distribution of the caterpillars over RNAi-*ORA59* and Col-0 plants did not significantly differ (frequency distribution 55:45%), but the small trend towards RNAi-*ORA59* supports that the larvae were not deterred by stronger activation of the MYC2-branch in RNAi-*ORA59* plants (Fig. 2B). *ORA59*-overexpressing *35S:ORA59* plants (Pré et al., 2008) accommodated significantly more caterpillars then did Col-0 plants (frequency distribution 63/37%), confirming that *P. rapae* larvae prefer feeding from plants that express the ERF-branch of the JA pathway upon herbivory.

Interestingly, the JA receptor mutant *coi1-1*, which is fully blocked in the capacity to express either the MYC2-branch or the ERF-branch of the JA pathway, accommodated more *P. rapae* larvae in the two-choice assay than did wild-type Col-0 (Fig. 4D). In Col-0 the ERF-branch is suppressed by the MYC2-branch but still displays low levels of *ORA59* and *PDF1.2* expression upon *P. rapae* feeding. Nevertheless, *P. rapae* larvae preferred *coi1-1* over Col-0 plants. This suggests that besides the feeding stimulatory effect that is mediated by the ERF-branch, a COI1-dependent deterring component affects *P. rapae* performance in this system as well.

Oral secretion of *P. rapae* stimulates the ERF-branch of the JA pathway

The observed attraction of *P. rapae* larvae towards plants expressing the ERF-branch of the JA pathway (Fig. 4B and C), prompted us to investigate whether *P. rapae* has ways to actively stimulate this favorable branch of the JA response. To this end, we simulated caterpillar feeding by mechanically damaging the leaves with three needle pricks per leaf and applying oral secretion of *P. rapae* into the wounds. Wounding transiently activated *VSP2* transcription, which peaked at 6 h after wounding and leveled off to undetectable levels at 24 h after wounding (Fig. 5A), confirming previous findings that wounding activates the MYC2-branch of the JA pathway (Lorenzo et al., 2004). Mechanical damage alone did not activate the ERF-branch marker *PDF1.2* (Fig. 5A). However, when oral secretion of *P. rapae* was introduced in the wounds, *PDF1.2* was strongly activated at 24 and 48 h after the treatment (Fig. 5B). These results suggest that elicitors in the oral secretion of the caterpillars have the potential to activate the ERF-branch of the JA pathway, possibly to improve the quality for consumption.

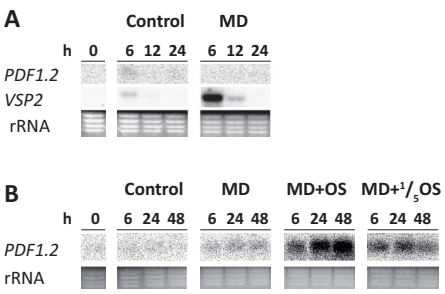

Figure 5: Oral secretion of *P. rapae* activates the ERF-branch of the JA pathway in mechanically-damaged plants. Northern blot analysis of *PDF1.2* and *VSP2* transcript levels at different time points after mechanical damage (MD) (A) or MD in combination with treatment with water, or undiluted or 5-fold diluted oral secretion (OS) of *P. rapae* at the sites of MD (B). Wounds were created by puncturing 3 small holes (1 mm diameter) in each leaf using a sterile needle. Subsequently, 1 µL of water or OS was applied to each wound. To check for equal loading, rRNA bands were stained with ethidium bromide. The experiments were repeated with similar results.

P. rapae feeding preference is not affected by plant glucosinolate composition

Glucosinolates have been demonstrated to influence the behavior of *P. rapae* (Schoonhoven and van Loon, 2002). To investigated whether the observed preference of *P. rapae* larvae for plants expressing the ERF-branch of the JA pathway is associated with a change in glucosinolate composition, first-instar *P. rapae* larvae were allowed to feed for 4 days, after which damaged plant tissue was collected and

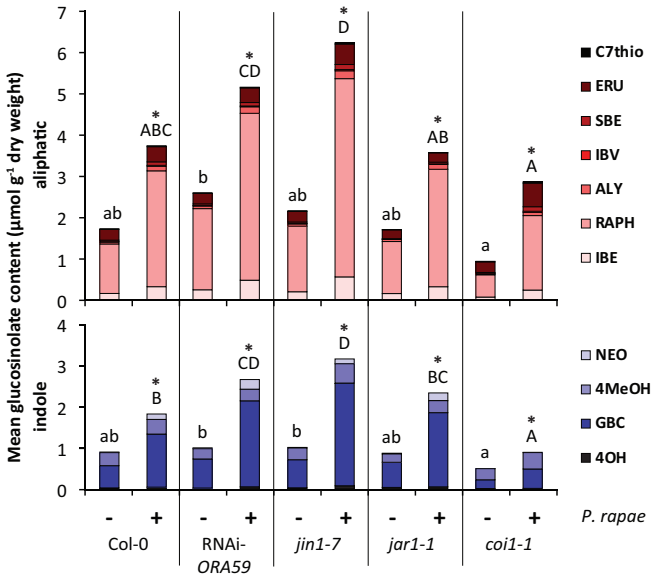

Figure 6: Glucosinolate content in different Arabidopsis genotypes after 4 days of feeding by *P. rapae*.
Contents of aliphatic and indole glucosinolates of 6-week-old Col-0, *coi1-1, jin1-7, jar1-1* and RNAi-*ORA59* plants after 4 days of feeding by first-instar larvae of *P. rapae* (+). Controls (-) show glucosinolate contents in non-infested plants. Asterisks indicate statistically significant differences between total constitutive and induced glucosinolate contents within a genotype (Student's *t*-test; P<0.05). Different letters indicate statistically significant differences in total glucosinolate contents between wild-type Col-0 and the JA signaling mutants and transgenics that received the same treatment (Tukey Post-hoc test; *P*<0.05). C7thio, Methyl-thioheptyl; ERU, Glucoerucin; SBE, Glucosiberin; IBV, Glucoiberverin; ALY, Glucoalyssin; RAPH, Glucoraphanin; IBE, Glucoiberin; NEO, Neo-glucobrassicin; 4MeOH, 4-Methoxy-glucobrassicin; GBC, Glucobrassicin; 4OH, 4-Hydroxy-glucobrassicin.

Table 1: Fold-difference of glucosinolate levels in non-treated and *P. rapae*-induced JA signaling mutants and transgenics relative to wild-type Col-0 plants[1].

		aliphatic							indole			
		IBE	RAPH	ALY	IBV	SBE	ERU	C7thio	4OH	GBC	4MeOH	NEO
- caterpillar	*coi1-1*	0.5	0.5	0.5	0.7	0.8	1.0	1.3	0.4	0.4	0.9	0.2
	jin1-7	1.2	1.3	1.2	0.9	1.0	1.0	1.2	1.1	1.3	0.9	1.0
	jar1-1	1.0	1.1	1.0	0.4	0.4	0.8	0.2	1.5	1.1	0.6	1.3
	RNAi-*ORA59*	1.5	1.6	1.4	1.1	1.1	1.0	0.8	0.9	1.3	0.8	1.4
+ caterpillar	*coi1-1*	0.8	0.6	0.7	1.3	1.3	1.6	1.3	0.3	0.4	1.1	0.1
	jin1-7	1.7	1.7	1.7	1.4	1.5	1.3	1.3	1.8	1.9	1.3	0.9
	jar1-1	1.0	1.0	1.1	0.4	0.5	0.6	0.2	1.2	1.4	0.8	1.4
	RNAi-*ORA59*	1.5	1.4	1.4	1.0	1.0	1.0	0.7	1.2	1.6	0.8	1.8

[1] Green filled cells indicate statistically significant higher induced levels than in Col-0, whereas red filled cells indicate statistically significant lower induced levels of the indicated glucosinolate compound (Dunnett's pairwise multiple comparison *t*-test, *P*<0.05).

analyzed for glucosinolate content. Total glucosinolate levels were measured in both infested and non-infested Arabidopsis plants. In Col-0, *coi1-1*, *jin1-7*, *jar1-1*, and RNAi-*ORA59* plants, caterpillar feeding significantly induced the accumulation of aliphatic and indole glucosinolates (Fig. 6). The constitutive levels of both the

total aliphatic and indole glucosinolates, as determined in non-infested plants, were found to be similar in all plant genotypes tested (Fig. 6). However, the basal levels of three out of four indole glucosinolate forms (4OH, GBC, and NEO) were reduced in *coi1-1* and a decrease of one of the indole glucosinolate forms (4MeOH) was also found for *jar1-1* (Table 1). Although the *P. rapae*-induced levels of specific aliphatic and indole glucosinolate forms differed between Col-0 and the different genotypes tested (Table 1), no consistent pattern was observed that correlated with the preference of the *P. rapae* larvae for Arabidopsis genotypes that activated the ERF-branch of the JA pathway after herbivory (*jin1*-7 and *jar1-1*) over the ones that activated the MYC2-branch (Col-0 and RNAi-*ORA59*). For example, the levels of four aliphatic and three indole glucosinolates were significantly enhanced in *jin1*-7 over Col-0 (Table 1), whereas in *jar1-1* the majority of these glucosinolates were not different from Col-0 or accumulated even to significantly lower levels after *P. rapae* feeding. Nevertheless, *jin1*-7 and *jar1-1* were similarly attractive to *P. rapae* larvae in the two-choice assays (Fig. 4B). Together, these results demonstrate that the differences observed in glucosinolate content are not causally related to the feeding preference of the caterpillars in the two-choice assays.

JA-insensitive *coi1-1* plants had lower constitutive levels of aliphatic and indole glucosinolates than wild-type Col-0 plants, and also the *P. rapae*-induced levels were lower for a number of the glucosinolate compounds (Fig. 6; Table 1). It can, thus, not be ruled out that the difference in preference of *P. rapae* larvae for Col-0 and *coi1-1* (Figure 4) is partly due to the differences in glucosinolate production in these genotypes.

DISCUSSION

MYC2 and ORA59 shape plant defenses upon herbivory

The JA signaling pathway differentially regulates plant defenses in response to insect herbivores and necrotrophic pathogens. Here, we investigated the molecular basis and biological function of the differential JA response as triggered in Arabidopsis by the specialist insect herbivore *P. rapae.* Feeding by *P. rapae* activated the expression of the MYC2-branch of the JA pathway (exemplified by the activation of the TF gene *MYC2* and the marker gene *VSP2*), while the ERF-branch, which is typically activated in response to necrotrophic pathogens, was repressed (exemplified by the suppression of the ERF TF gene *ORA59* and the marker gene *PDF1.2*). In MYC2-impaired *jin1* plants, this herbivore-induced differential JA response was redirected towards the ERF-branch (Fig. 1). Also in JA-Ile-compromised *jar1-1*

plants, in which the MYC2-branch of the JA pathway is impaired (Chini et al., 2007; Thines et al., 2007; Chung et al., 2008), this change in JA-responsive gene expression pattern could be observed, indicating that activation of the MYC2-branch coincides with suppression of the ERF-branch during insect feeding. Using knockdown RNAi-*ORA59* and overexpression *35S:ORA59* lines, we provide evidence that the antagonism between the MYC2- and the ERF-branch during insect herbivory is regulated at the level of *MYC2* and *ORA59* TF gene expression (Fig. 2). Furthermore, our results provide detailed biological evidence that the previously described antagonism between MYC2 and ERF-type TFs (Lorenzo et al., 2003; Lorenzo et al., 2004) is important for the differential regulation of the JA response as observed during plant-insect interactions. Using a two-choice assay, we show that *P. rapae* larvae preferred to feed on plants that express the ERF-branch, while expression of the MYC2-branch had no effect on the preference of *P. rapae* (Fig. 4). We thus conclude that by prioritizing the MYC2-branch of the JA pathway over the ERF-branch, Arabidopsis plants rewire the JA signaling pathway away from the *P. rapae*-preferred ERF-branch of the JA pathway, possibly to minimize attractiveness of the leaf tissue for insect feeding. We found no evidence for a role of glucosinolates in the preference of *P. rapae* for the ERF-branch of the JA pathway (Fig. 6; Table 1). The induced responses leading to feeding preference by the insects thus still remain elusive. Interestingly, application of oral secretion of *P. rapae* into mechanically damaged leaf tissue induced the ERF-branch of the JA pathway (Fig. 5), suggesting that elicitors in the oral secretion are potentially capable of steering the JA response towards expression of the *P. rapae*-preferred ERF-branch. During the interaction of *P. rapae* larvae with wild-type Col-0 plants, the MYC2/ERF balance shifts, however, towards the MYC2 branch, indicating that the arms race between plant and attacker during this interaction is decided in favor of the plant.

P. rapae larvae prefer to feed on plants that express the ERF-branch of the JA pathway

It is generally accepted that the JA signaling pathway is important in plant defense against herbivorous insects (Howe and Jander, 2008). Growth and development of *P. rapae* larvae was indeed significantly enhanced on JA-insensitive Arabidopsis *coi1-1* plants (Fig. 3; Reymond et al., 2004; Bodenhausen and Reymond, 2007; Van Oosten et al., 2008). However, in comparison to the generalist herbivores *Spodoptera exigua* (Beet armyworm) and *Spodoptera littoralis* (Egyptian cotton worm), the effect on the performance of *P. rapae* larvae was relatively small (Bodenhausen and Reymond, 2007; Van Oosten et al., 2008), probably because this specialist herbivore is well-adapted to the defense responses that are triggered in the wild-type plants

(Wittstock et al., 2004). Also in a two-choice assay, *P. rapae* larvae had a significant preference for the JA-insensitive *coi1-1* plants (Fig. 4D), again highlighting the role of JA in defense against insect feeding. In wild tobacco (*Nicotiana attenuata*) plants, silencing of *COI1* resulted in greater damage levels inflicted by the local herbivore community in a field experiment (Paschold et al., 2007). Laboratory choice assays confirmed that the *COI1*-silenced wild tobacco plants were preferred by herbivores (Paschold et al., 2007), which is in similarity to what we observed with Arabidopsis.

In mutant Arabidopsis *coi1-1* plants, the JA signaling pathway is completely blocked. This is in clear contrast to JA-response mutants such as *jin1* and *jar1*, which still display a strong but altered JA response in response to insect herbivory. JA-Ile production is compromised in *jar1-1* (Staswick and Tiryaki, 2004), and was found to be responsible for activating COI1-mediated degradation of JAZ repressors proteins that suppress MYC2 action (Chini et al., 2007; Thines et al., 2007; Chung et al., 2008). Previously, it was demonstrated that *jar1-1* plants do not show significant differences in JA-responsive gene expression in response to wounding, probably because the reduced, albeit not nil, level of bioactive JA-Ile in wounded *jar1-1* leaves exceeds the level needed to saturate the JA-Ile-induced transcriptional response (Koo et al., 2009). Indeed, the MYC2-marker gene *VSP2* was not significantly affected in *P. rapae*-infested *jar1-1* plants (Fig. 1). However, in contrast to *P. rapae*-infested Col-0 plants, the expression of *PDF1.2* was not suppressed in *jar1-1*, indicating that the *jar1-1* mutation significantly affects the MYC2-mediated suppression of the ERF-branch during insect feeding. The observation that *jin1* and *jar1* plants were significantly more preferred by *P. rapae* larvae than wild-type Col-0 plants, and were similarly preferred when given a choice between both mutants (Fig. 4), suggests that the phenotype that the mutants share, namely strong activation of the ERF-branch upon insect feeding, is crucial for the preference of *P. rapae.* These molecular and ecological phenotypes of *jin1* and *jar1* plants are shared with *ORA59*-overexpressing *35S:ORA59* plants, which also express the ERF-branch of the JA pathway and are also significantly more preferred by *P. rapae* larvae (Fig. 2 and 4). Activation of the MYC2-branch in Col-0 did not make the plant less attractive than *jin1-1/* RNAi-*ORA59* plants that were unable to activate either the MYC2- or the ERF-branch (Fig. 4C). We thus conclude that *P. rapae* larvae were stimulated to feed from plants that express the ERF-branch rather then that they were being deterred by plants that express the MYC2-branch of the JA pathway.

Effect of the differential JA response on insect feeding

One of the ways by which caterpillars can discriminate for food quality is by taste. As measured by electrophysiological activity, chemoreceptors in taste sensilae of the larvae of *P. rapae* respond to several secondary plant substances, such as naringenin and strychnine. These 'bitter' compounds were classified as deterrents to *P. rapae* larvae, because in choice tests there was consumed less of cabbage leaf discs covered with these compounds (Zhou et al., 2009). Besides taste cells specialized in deterrents, also cells responding to stimulatory feeding compounds have been discovered. Caterpillar species respond differentially to specific sugars and amino acids as a way to determine plant quality. As a specialist on glucosinolate-containing plants, *P. rapae* contains two glucosinolate-sensitive taste cells that aid the larvae to discriminate between glucosinolate-containing hosts and glucosinolate-lacking non-hosts (Schoonhoven and van Loon, 2002). It has been proposed that wound-induced responses are important in determining the pattern of feeding by insect herbivoress, in which the insect moves away from a site that is heavily induced for defense (Edwards and Wratten, 1983). In line with this, larvae should settle longer on plants containing a less detrimental induced defense, making it possible to study effectiveness of defense in choice tests. Our results suggest that *P. rapae* caterpillars stay longer on Arabidopsis plants in which the ERF-branch of the JA pathway is activated, suggesting that the ERF-branch regulates the production of metabolites that are feeding stimulants for the *P. rapae* larvae.

Glucosinolates are secondary metabolites consisting out of a β-thioglucose moiety, a sulfonated oxime moiety, and a variable side chain which greatly determines level of toxicity (Hopkins et al., 2009). Indole glucosinolates are derived from tryptophan, whereas aliphatic glucosinolates are derived from methionine. Glucosinolates are implicated in stimulating oviposition of *P. rapae* butterflies (De Vos et al., 2008). We tested whether differences in glucosinolate composition between different genotypes might account for the observed preference of the *P. rapae* larvae for plants expressing the ERF-branch of the JA pathway. However, although amounts of several induced individual glucosinolates differed in the tested mutants from wild type, no evidence was found for a role for glucosinolates in the enhanced preference of caterpillars for leaf tissue expressing the ERF-branch of the JA pathway.

Rewiring of the JA signaling pathway during the Arabidopsis-*P. rapae* interaction

Application of oral secretion of *P. rapae* into mechanically inflicted wounds triggered the ERF-branch of the JA pathway (Fig. 5). This suggests that *P. rapae*

oral secretion contains elicitors that trigger the herbivore-preferred ERF-branch of the JA pathway in wounded leaf tissue, which may be a mechanism by which these specialist herbivores attempt to manipulate the JA response to their own benefit. Nevertheless, feeding by *P. rapae* larvae on wild-type Col-0 plants finally results in suppression of the ERF-branch because the MYC2-branch of the JA pathway becomes dominantly activated during the Arabidopsis-*P. rapae* interaction. Apparently, crosstalk between the MYC2 and ERF-type TFs leads to rewiring of JA signaling in favor of the MYC2-branch that strongly antagonizes the herbivore-preferred ERF-branch of the JA pathway. In pharmacological experiments, this antagonistic effect has previously been demonstrated to involve ABA signaling, which in combination with JA signaling results in the prioritization of the MYC2 branch over the ERF-branch (Anderson et al., 2004; Lorenzo et al., 2004). Herbivory by *P. rapae* has previously been shown to be associated with ABA-responsive gene expression (Bodenhausen and Reymond, 2007). Hence, it is tempting to speculate that activation of the ABA pathway during *P. rapae* feeding is responsible for redirecting the JA pathway towards the MYC2-branch and away from the herbivore-preferred ERF-branch. Natural variation of the MYC2/ERF balance exists between *A. thaliana* accessions (Ahmad et al., 2011), but to what extent this correlates with the level of resistance to insect herbivores or their feeding preference remains to be elucidated.

More examples of insect herbivores that manipulate the host's defense response have been described, often with a favorable outcome for the attacker. For instance, components in the oral secretion of the generalist herbivore *Helicoverpa zea* was shown to reduce the JA-dependent synthesis of nicotine, a toxin that is produced by tobacco plants to defend themselves against herbivory (Musser et al., 2002). In Arabidopsis, Zarate et al. (2007) showed that phloem feeding silverleaf whiteflies (*Bemisia tabaci*) switch on the SA defense pathway that antagonizes the JA defense pathway that hinders whitefly nymph development. A similar phenomenon was observed with caterpillars from *S. exigua*. Elicitors from salivary excretions of this herbivore suppressed effectual JA-dependent defenses through the activation of the SA pathway (Weech et al., 2008; Diezel et al., 2009). In Lima bean, infestation with the whitefly *B. tabaci* and the spider mite *Tetranychus urticae* was shown to suppress indirect JA-dependent defenses that were induced by the spider mites, resulting in reduced attraction of carnivorous enemies of the spider mites (Zhang et al., 2009). Recently, elicitors from insect eggs were found to activate the SA pathway in Arabidopsis at the site of oviposition. As a result, JA-dependent defenses were suppressed, resulting in an advantage for the newly hatched offspring that fed from the undefended tissue (Bruessow et al., 2010). These examples pinpoint hormone-regulated signaling pathways as important targets for plant attackers to manipulate the host's defense response. In addition, in the present study, we show that the plant

evolved to recognize the invader so that the hormone signaling route that favors the invader is suppressed.

MATERIALS AND METHODS

Plant growth conditions

Seeds of *Arabidopsis thaliana* wild-type Col-0, glabrous Col-5(*gl1*), mutants *jin1-1*(*gl1*), *jin1-1, jin1-2*, *jin1*-7 (SALK_040500), *jin1-10* (SALK_017005)(Lorenzo et al., 2004), and *jar1-1* (Staswick et al., 1992), and transgenics RNAi-*ORA59* and *35S:ORA59* (Pré et al., 2008) were sown on quartz sand and, after two weeks of growth, seedlings were transplanted into 60ml pots containing a sand–potting soil mixture (5:12 v/v) that had been autoclaved twice for 20 min with a 24-h interval (Pieterse et al., 1996). Mutant *coi1-1*(*gl1*) (Feys et al., 1994) and *coi1-1* plants were selected by size on vermiculite supplied with 50 μM MeJA, after which the genotype was confirmed as described (Xie et al., 1998). Non-glabrous *jin1-1* and *coi1-1* were produced by back-crossing *jin1-1*(*gl1*) and *coi1-1*(*gl1*) with Col-0. For genotyping of the *jin1-1* and *coi1-1* mutation the following primers were used: *jin1-1*-Fw: 5'-AAG CCA GCA AAC GGT AGA GA-3'; *jin1-1*-Rv 5'-ACG CGA GAA GAG CTG AAG AA-3'; Fw-*coi1-1* 5'-GAA AGG ATT ACA GAT CTG CC-3'; Rv-*coi1-1* 5'- CAT ATT GGC TCC TTC AGG AC-3'. Wild-type was distinguished from *coi1-1* by digesting the 530-bp PCR product with *XcmI,* which leaves the mutant PCR product intact (Xie et al., 1998). Plants were cultivated in a growth chamber with an 8-h day (200 μE m^{-2} s^{-1} at 24°C) and 16-h night (20°C) cycle at 70% relative humidity for another 4 weeks. Plants were watered every other day and received half-strength Hoagland solution (Hoagland and Arnon, 1938) containing 10 μM Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Pieris rapae assays

Pieris rapae (small cabbage white butterfly) was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) as described previously (Van Poecke et al., 2001). In all experiments, first-instar (L1) larvae were used. For gene expression analysis, three larvae were placed separately on fully expanded leaves of 6-week-old Arabidopsis plants using a fine paint-brush. In all cases, larvae were removed 24 hours after the start of the experiment. Leaves of damaged and undamaged plants were harvested at different time points after the start of caterpillar infestation.

P. rapae growth was monitored during a 10-day period after transfer of single larvae to individual 6-week-old Arabidopsis plant genotype (n=40-45) that were placed inside a Magenta GA-7 vessel (Sigma-Aldrich, Zwijndrecht, The Netherlands) with

a modified lid that contained an insect-proof mesh. The caterpillars were weighed to the nearest 0.1 mg on a microbalance (Sartorius AC211P) at 4, 7 and 10 days after the start of the experiment.

For *P. rapae* two-choice assays, two L1 larvae were released per plant in an arena (n=20) that consisted of two 6-week-old Arabidopsis plants of each tested genotype. The plants in the two-choice arena were in physical contact, which allowed the caterpillars to freely move through the arena. After 4 days the number of caterpillars present on each genotype was monitored. The frequency distribution of the caterpillars over the different genotypes was calculated and tested for statistical difference from a 50/50 choice proportion using Student's *t*-test.

Wounding assays

The effect of wounding was assessed by mechanically damaging the leaf tissue of 6-week-old plants. Three spots were punctured in each leaf using a sterile needle (1 mm diameter). Oral secretion was collected from L4/L5 larvae that were allowed to feed on uninduced Col-0 plants for 24 h as described (Mattiacci et al., 1995). In each of the wounds, a 1-µl droplet of freshly collected, undiluted or 5x diluted oral secretion was applied. A similar volume of tap water was applied as a control.

RNA extraction and northern blot analysis

Total RNA was isolated as described (Van Wees et al., 1999). For northern blot analysis, 10 or 15 µg of RNA were denatured using glyoxal and dimethyl sulfoxide (Sambrook et al., 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N^+ membrane (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. To check for equal loading, rRNA bands were stained with ethidium bromide. Northern blots were hybridized with gene-specific probes for *PDF1.2* and *VSP2* and *ORA59* as described previously (Leon-Reyes et al., 2010a). A probe for *MYC2* was made by PCR amplification of cDNA of MeJA-treated plants and the gene-specific primers *MYC2*-Fw 5'GGTCA CCGGT TTATG GAATG-3' and *MYC2*-Rv 5'CGTAT CCGTC ACCTC CTCAT3'. After hybridization with $\alpha[^{32}P]$-dCTP-labeled probes, blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad, Veenendaal, the Netherlands). The AGI numbers for the genes studied are At5g44420 (*PDF1.2*), At5g24770 (*VSP2*), At1g06160 (*ORA59*), and At1g32640 (*MYC2*). All gene expression analyses have been repeated with similar results.

RT-qPCR

SuperScript™ III Reverse Transcriptase was used to convert DNA-free total RNA into cDNA. PCR reactions were performed in optical 96-well plates (Applied Biosystems) with an ABI PRISM® 7900 HT sequence detection system, using SYBR® Green to monitor the synthesis of double-stranded DNA. A standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60°C to 95°C with a ramp speed of 1.9°C min^{-1}. *ORA59* transcript levels (primer pair from (Czechowski et al., 2004)) were calculated relative to the reference gene At1g13320 (Czechowski et al., 2005) using the $2^{-\Delta\Delta Ct}$ method described previously (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Glucosinolate measurements

Caterpillar-damaged leaves (infested with two L1 larvae for 4 days) were harvested from 6-week-old plants, frozen in liquid nitrogen and subsequently freeze-dried. Plant material was homogenized and a 100-mg aliquot was weighed in a 2-ml tube for glucosinolate extraction, as described previously (Kabouw et al., 2010). For each treatment, five replicas of a pool of 3 plants were used.

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CHAPTER 3

Abscisic acid is required for rewiring of the jasmonate signaling pathway during insect herbivory

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Abstract

Jasmonic acid (JA) and its oxylipin derivatives are important regulators of the defense response of plants to herbivorous insects and microbial pathogens. In *Arabidopsis thaliana*, insect herbivores and necrotrophic pathogens activate distinct, antagonistic branches of the JA signaling pathway: the MYC2-branch, which is regulated by the basic helix-loop-helix leucine zipper transcription factor MYC2, and the ERF-branch, which is controlled by the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain transcription factors ORA59 and ERF1. Arabidopsis plants infested by larvae of *Pieris rapae* activate the MYC2-branch, possibly to suppress the herbivore-preferred ERF-branch of the JA pathway, which was shown to be stimulated by oral secretion of the larvae (Chapter 2). Here, we investigated the role of the plant hormones abscisic acid (ABA) and ethylene (ET) in the rewiring of the JA response during *P. rapae* feeding. Analysis of the ABA biosynthesis mutant *aba2-1* revealed that ABA is required for repression of the ERF-branch in *P. rapae*-infested plants. Pharmacological experiments confirmed that ABA could suppress the ERF-branch in *P. rapae*-infested plants. Also in the MYC2-impaired *jin1-7* mutant ABA antagonized the ERF-branch, suggesting that ABA acts independently of MYC2. Conversely, exogenous application of ET stimulated the ERF-branch and repressed the MYC2-branch in *P. rapae*-infested plants. Analysis of the hormone signal signature of *P. rapae*-infested Arabidopsis plants revealed that besides the jasmonates JA, 12-oxo-phytodienoic acid (OPDA), and (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), also ABA accumulated in herbivore-damaged tissues, whereas ET levels remained unaltered. Collectively, these results indicate that during the Arabidopsis-*P. rapae* interaction, the MYC2-branch of the JA pathway is prioritized over the herbivore-preferred ERF-branch through ABA-mediated suppression of the ERF-branch, and that ET plays no role in this interaction. These results highlight the important modulating role of ABA in the hormone-regulated defense signaling network in plants.

Introduction

Plants are able to convert gaseous carbon dioxide into sugars to provide themselves with the energy needed to complete their lifecycle. As a result, plants are of special interest to insect herbivores and microbial pathogens that are dependent on external resources for their energy demand. To protect themselves, plants possess a highly sophisticated defense system that, like the animal innate immune system, recognizes non-self molecules or signals from injured cells, and responds by activating an effective defense response against the invader encountered. Active defense against biotrophic pathogens, which require a living host to fulfill their life cycle, is often largely dependent on programmed cell death, whereas defense against necrotrophic pathogens, which first kill host cells and live on the content, require a different defense strategy that often involves the production of antimicrobial compounds (Glazebrook, 2005; Laluk and Mengiste, 2010). Defense against insect herbivores is based on two strategies: direct and indirect defense. Upon herbivore attack, plants accumulate defense compounds, that can be toxic or repellent, which target the attacker directly. Indirect defenses are activated by decreasing the nutritional value of tissue, which affects the feeding behavior of the insect (Howe and Jander, 2008) and by emitting a volatile odor blend that attracts carnivorous enemies of the herbivores that feed on the plant (Dicke et al., 2009).

Because plant defenses are costly, they are often inducible so that they are only recruited in case of pathogen or insect attack (Walters and Heil, 2007; Ahmad et al., 2010). Plant hormones ermerged as important regulators of induced defense responses. The importance of the hormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) as primary signals in the regulation of plant defense is well established (Bari and Jones, 2009; Pieterse et al., 2009). Abscisic acid (ABA) emerged as an important regulator of induced defenses against pathogens and insects as well (Thaler and Bostock, 2004; Bodenhausen and Reymond, 2007; Asselbergh et al., 2008; Ton et al., 2009), although its role is less well characterized. JA and its oxylipin derivatives (collectively called here jasmonates (JAs)) are key players in the regulation of induced defenses against insect herbivores (Howe and Jander, 2008) and necrotrophic pathogens (Glazebrook, 2005; Laluk and Mengiste, 2010). Upon pathogen or insect attack, JAs are rapidly synthesized via the oxylipin biosynthesis pathway (Wasternack, 2007; Gfeller et al., 2010). Via the activity of the JA conjugate synthase JAR1, JA can be readily conjugated to amino acids such as isoleucine (Staswick and Tiryaki, 2004), resulting in biologically highly active (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca et al., 2009). The F-box protein COI1 (CORONATINE INSENSITIVE 1) functions as a JA-Ile receptor in the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI1} (Yan et al., 2009). Binding of JA-Ile to COI1 leads to degradation of JASMONATE ZIM-domain (JAZ)

transcriptional repressor proteins via the proteasome (Chini et al., 2007;Thines et al., 2007). In resting cells, JAZ proteins act as transcriptional repressors of JA signaling by physically interacting with transcriptional activators, such as the transcription factor MYC2 (Chini et al., 2007). JAZ protein degradation results in de-repression of the JA signaling pathway and the activation of a large number of JA-responsive genes (Memelink, 2009). Interestingly, transcriptional changes in response to diverse JA-inducing insect herbivores and pathogens show limited overlap, suggesting that the context in which the JA signal is perceived is crucial in tuning the JA-regulated response (De Vos et al., 2005; Pauwels et al., 2009).

Changes in hormone concentration or sensitivity, such as triggered upon interactions with herbivores and pathogens, activates a bouquet of hormone signaling events that steers adaptive plant responses. The final outcome of the activated defense response is greatly influenced by the composition and kinetics of the hormonal blend produced (De Vos et al., 2005; Mur et al., 2006; Koornneef et al., 2008; Leon-Reyes et al., 2010a). The plant hormones ET, ABA, and SA, which in various combinations and concentrations can be part of the signal signature that is produced upon pathogen or insect attack, emerged as important differential regulators of the JA response (Pieterse et al., 2009). While SA generally acts antagonistically on JA signaling (Verhage et al., 2010), ET and ABA each co-regulate a different branch of the JA pathway (Anderson et al., 2004; Lorenzo et al., 2004; Adie et al., 2007a; Kazan and Manners, 2008).

JA and ET are often simultaneously produced upon infection by necrotrophic pathogens (Penninckx et al., 1998; De Vos et al., 2005). In *Arabidopsis thaliana* (Arabidopsis), JA and ET signaling act synergistically on the expression of defense-related genes, such as *PLANT DEFENSIN1.2* (*PDF1.2*) (Penninckx et al., 1998). Two members of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of transcription factors (TFs), ERF1 and ORA59, emerged as principal integrators of JA and ET signaling (Lorenzo et al., 2003; Pré et al., 2008). Both TFs activate the ERF-branch of the JA pathway and confer resistance to necrotrophic pathogens (Berrocal-Lobo et al., 2002; Pré et al., 2008). RNAi-*ORA59* plants were demonstrated to be completely blocked in JA/ET-induced *PDF1.2* expression, indicating that ORA59 is required for induction of *PDF1.2*. Another important master regulator of JA-responsive gene expression is the TF MYC2 (originally called JIN1 for JASMONATE INSENSITIVE1)(Lorenzo et al., 2004). On the one hand, MYC2 acts in concert with ABA signaling in negatively regulating the ERF-branch of the JA pathway (Anderson et al., 2004; Lorenzo et al., 2004). On the other hand, MYC2 functions as a transcriptional activator of genes in the MYC2-branch of the JA pathway, including the JA marker gene *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Dombrecht et al., 2007). The MYC2-branch of

the JA pathway is associated with the wound-response and is thought to contribute to defense against insect herbivores (Lorenzo et al., 2004; Dombrecht et al., 2007), although it has also been reported to function in priming for enhanced pathogen defense (Pozo et al., 2008).

Recent advances in plant immunity research underpin the pivotal role of cross-communicating hormones in the regulation of the plant's defense signaling network (Spoel and Dong, 2008; Pieterse et al., 2009; Verhage et al., 2010). Their powerful regulatory potential allows the plant to quickly adapt to its hostile environment and to utilize its resources in a cost-efficient manner. Plant enemies on the other hand, can rewire the plant's defense signaling network for their own benefit by affecting hormone homeostasis to antagonize the host immune response (Walling, 2008; Grant and Jones, 2009). Since JA-dependent defenses are predominantly regulated via the antagonistic MYC2- and ERF-branch of the JA signaling pathway, we previously set out to investigate the JA-dependent defense response of Arabidopsis that is activated upon feeding by larvae of the specialist insect herbivore *Pieris rapae* (small cabbage white butterfly). Feeding by *P. rapae* activated the expression of the MYC2-branch of the JA pathway (exemplified by the activation of the TF gene *MYC2* and the marker gene *VSP2*), while the ERF-branch, which is typically activated in response to necrotrophic pathogens, was repressed (exemplified by the suppression of the ERF TF gene *ORA59* and the marker gene *PDF1.2*). We demonstrated that although oral secretion of *P. rapae* stimulated the ERF-branch, feeding by *P. rapae* caterpillars activated the MYC2-branch of the JA pathway, resulting in active suppression of the ERF-branch of the JA pathway (Chapter 2). In a two-choice setup with various mutant and transgenic Arabidopsis genotypes in which the MYC2- or the ERF-branch of the JA pathway was positively or negatively affected, *P. rapae* consistently preferred plant genotypes in which the ERF-branch was activated (Chapter 2). This suggests that the herbivores were stimulated to feed from plants that expressed the ERF-branch, rather than that they were deterred by plants expressing the MYC2-branch. The MYC2-mediated suppression of the herbivore-preferred ERF-branch of the JA pathway in *P. rapae*-infested Arabidopsis plants may therefore be part of the defense strategy of the plant against this specialist herbivore. In the present study we investigated the role of ABA and ET in the rewiring of the JA pathway during the Arabidopsis-*P. rapae* interaction and provide evidence for a role of ABA in steering the plant response towards activation of the MYC-branch.

Results

ABA signaling is required for rewiring of the JA pathway during insect herbivory

Upon herbivory by tissue-chewing insects, Arabidopsis plants activate a plethora of JA-responsive genes (Reymond et al., 2004; De Vos et al., 2005). The JA-dependent transcriptional response of Arabidopsis to *P. rapae* feeding is predominantly regulated via the MYC2-branch of the JA pathway, while the ERF-branch is suppressed (Chapter 2). Since ABA and ET have been demonstrated to play a modulating role in the differential JA response (Lorenzo et al., 2003; Anderson et al., 2004), we investigated the significance of these hormones in the JA-regulated response that is induced upon *P. rapae* feeding. To this end, caterpillars of larval stage L1 were allowed to feed for 24 h on leaves of Arabidopsis wild-type Col-0, MYC2-impaired mutant *jin1-7,* ABA biosynthesis mutant *aba2-1,* and ET response mutant *ein2-1,* after which the caterpillars were removed. In Col-0 plants, *P. rapae* feeding strongly stimulated the accumulation of *VSP2* transcripts, while *PDF1.2* mRNA levels were hardly detectable (Fig. 1A). In *MYC2*-defective *jin1-7* plants, the transcriptional pattern of *PDF1.2* and *VSP2* was opposite to that observed in Col-0, confirming previous findings that in wild-type plants, *P. rapae* feeding activates the MYC2-branch of the JA response, while suppressing the ERF-branch in a MYC2-dependent manner (Chapter 2). In Col-0 plants, the *VSP2* transcript levels had dropped back to non-induced basal levels at 48 h after introduction of the caterpillars, which is 24 h after removal of the caterpillars, while at the same time *PDF1.2* mRNA levels started to increase, suggesting that stimulation of the MYC2-branch and concomitant suppression of the ERF-branch was effective until at least 12 h after removal of the insect herbivores. In mutant *ein2-1* plants, the expression patterns of *PDF1.2* and *VSP2* were similar as those observed in Col-0 plants, suggesting that ET signaling does not play a major role in the regulation of the differential JA response that is activated upon *P. rapae* feeding. Interestingly, *aba2-1* plants displayed the same expression pattern of the marker genes *PDF1.2* and *VSP2* as *jin1-7* plants, indicating that in wild-type plants ABA signaling is required for rewiring the JA response towards the MYC2-branch and away from the ERF-branch.

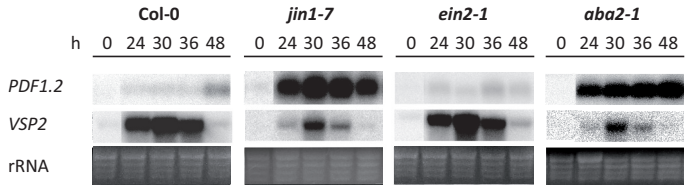

Figure 1: Differential JA-responsive gene expression in *P. rapae*-infested Arabidopsis plants. Northern blot analysis of JA-responsive *PDF1.2* (marker for ERF-branch) and *VSP2* (marker for MYC2-branch) gene expression in wild-type Col-0 plants, MYC2-impaired *jin1-7* mutant, ET response mutant *ein2-1,* and ABA biosynthesis mutant *aba2-1.* First-instar larvae of *P. rapae* were allowed to feed for 24 h on 5-week-old plants after which the caterpillars were removed. Infested leaves were harvested for RNA extraction at the time points indicated. The experiment was repeated with similar results.

ABA antagonizes the herbivore-induced ERF-branch of the JA pathway

To further investigate the role of ABA in the regulation of the differential JA response during insect herbivory, we determined the effect of exogenously applied ABA to herbivore-infested Col-0 and *jin1*-7 plants. As shown in Figure 2, application of 10 or 100 μM ABA by itself did not induce the expression of *PDF1.2* or *VSP2* in Col-0 or *jin1*-7. However, ABA had a strong negative effect in a dose-dependent manner on both the relatively low *P. rapae*-induced expression levels of *PDF1.2* in Col-0, and the relatively high levels of *PDF1.2* expression in *jin1*-7. These results indicate that ABA is able to antagonize the herbivore-induced ERF-branch of the JA pathway and that this suppressive effect functions independently of MYC2. Conversely, exogenous application of ABA to *P. rapae*-infested plants enhanced the herbivore-induced transcription of *VSP2* in Col-0 and *jin1*-7 (quantified expression levels shown in the bottom panel of Fig. 2). These results indicate that ABA positively affects the herbivore-induced MYC2-branch of the JA pathway in a MYC2-independent manner, resulting in enhanced *VSP2* and reduced *PDF1.2* expression levels.

ET antagonizes the herbivore-induced MYC2-branch of the JA pathway

To investigate the potential effect of ET in the regulation of the differential JA response during insect herbivory, we exogenously applied ET to herbivore-infested Col-0 and *ein2-1* plants and monitored the expression of *PDF1.2* and *VSP2*. As shown in Figure 3, application of 1 ppm of gaseous ET induced the expression of *PDF1.2* in Col-0, but not in the ET signaling mutant *ein2-1.*

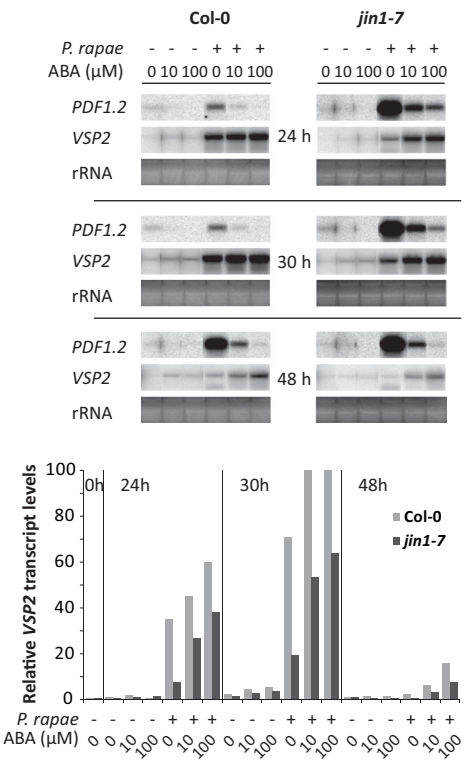

Figure 2: Effect of ABA on herbivore-induced *PDF1.2* and *VSP2* expression.
Northern blot analysis of JA-responsive *PDF1.2* and VSP2 gene expression in Col-0 and *jin1-7* plants that were infested or not with *P. rapae*, and treated or not with ABA. First-instar larvae of *P. rapae* were allowed to feed for 24 h on 5-week-old plants after which the caterpillars were removed. ABA was added to the plants one day before application of the larvae and one day after removal of the larvae by watering the plants with a solution containing 0, 10, or 100 µM ABA. Leaves were harvested for RNA extraction at the time points indicated. To check for loading, rRNA bands were stained with ethidium bromide. Signal intensities of *VSP2* mRNA on the northern blots were quantified using a Phosphor imager (bottom panel) and plotted relative to the highest level (set to 100%). Please note that the quantitative data displayed in the bottom panel are quantifications of the corresponding signal intensities in the top panel and thus do not contain error bars. The northern blot analyses have been repeated with similar results.

P. rapae feeding further enhanced the ET-induced expression of *PDF1.2* in Col-0 plants, confirming previous findings that ET and JA signaling act synergistically on *PDF1.2* transcription (Penninckx et al., 1998). Furthermore, ET treatment strongly antagonized the herbivore-induced expression of *VSP2*, suggesting that ET antagonized the MYC2-branch of the JA signaling pathway. In *ein2-1* plants, both the stimulating effect of ET on *PDF1.2* and the suppressive effect of ET on *VSP2* in *P. rapae*-infested plants were blocked, indicating that both ET-mediated processes are dependent on EIN2 and thus regulated via the ET signaling pathway.

Previously, the ERF-type TF ORA59 was demonstrated to play a dominant regulatory role in the JA/ET-dependent expression of *PDF1.2* (Pré et al., 2008). To investigate whether ORA59 is involved in the ET-mediated suppression of the MYC2-branch of the JA pathway, we monitored the expression of *PDF1.2* and *VSP2* in the *ORA59*-silenced line RNAi-*ORA59* and the control line RNAi-*GUS* (Pré et al., 2008). In RNAi-*ORA59* plants, *PDF1.2* was expressed at very low levels, demonstrating that the RNAi-*ORA59* plants were indeed impaired in ORA59 functioning. However, the suppressive effect of ET on herbivore-induced *VSP2* transcript levels was not significantly affected in RNAi-*ORA59,* as the level of *VSP2* suppression was similar as that observed in RNAi-*GUS* plants. Together, these results suggest that ET signaling suppresses the herbivore-induced MYC2-branch of the JA pathway and that this antagonistic effect functions independently of ORA59.

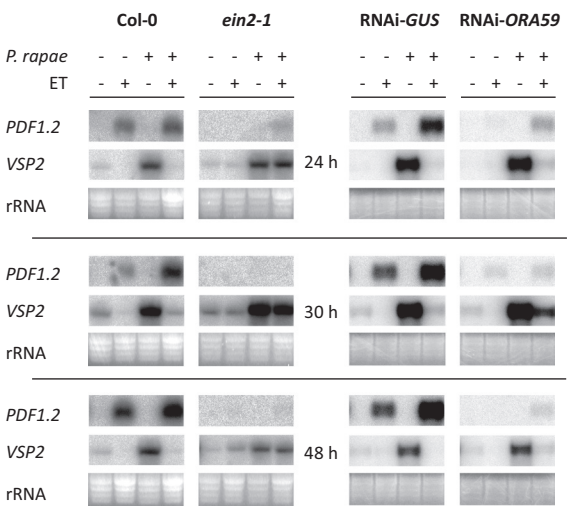

Figure 3: Effect of ET on herbivore-induced *PDF1.2* and *VSP2* expression.
Northern blot analysis of JA-responsive *PDF1.2* and *VSP2* gene expression in Col-0, *ein2-1,* RNAi-*GUS,* and RNAi-*ORA59* plants that were infested or not with *P. rapae*, and treated with a flow of air with or without gaseous ET (1 ppm, starting 24 h prior to infestation). First-instar larvae of *P. rapae* were allowed to feed for 24 h on 5-week-old plants after which the caterpillars were removed (t=24 h). Leaves were harvested for RNA extraction at the time points indicated. To check for equal loading, rRNA bands were stained with ethidium bromide. The experiment was repeated with similar results.

Signal signature of *P. rapae*-infested plants

To match the differential JA response observed in *P. rapae*-infested plants with the signal signature produced during insect herbivory, we monitored the accumulation of JA, its precursor OPDA, and the biologically highly active conjugate JA-Ile. In addition, we measured the levels of the hormones that have been demonstrated to modulate the JA response: ABA, ET, and SA. Again, first-instar larvae were allowed to feed for 24 h after which they were removed from the leaves. Subsequently, hormone levels were measured at different time points after removal of the caterpillars. Figure 4A shows that *P. rapae* feeding induced the production of OPDA, JA, and JA-Ile in Col-0 at most of the time points tested. In the *jin1*-7 and *aba2-1* mutants, the levels of OPDA, JA, and JA-Ile raised similarly (Table 1), indicating that the biosynthesis of JAs is not significantly affected by the mutations.

In Col-0 plants, ABA levels increased significantly within 24 h after herbivory, after which a drop to basal levels was observed (Fig. 4B; Table 1). As expected, ABA levels were low in the ABA biosynthesis mutant *aba2-1*. In contrast to that observed in Col-0 plants, *P. rapae* feeding did not induce the accumulation of ABA in *jin1*-7 plants, suggesting that herbivore-induced ABA biosynthesis is regulated by MYC2.

Free SA levels were determined in the same samples in which OPDA, JA, JA-Ile and ABA were measured. In none of the samples a significant change in the level of SA was detected (Table 1), which confirms previous findings that *P. rapae* feeding has no effect on SA biosynthesis in Arabidopsis (De Vos et al., 2005).

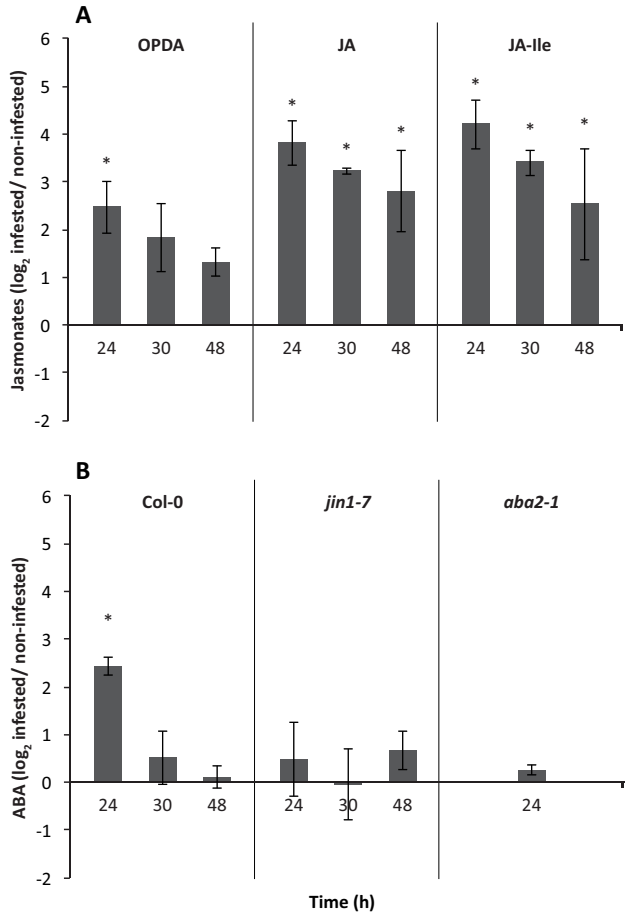

Figure 4: Changes in the production of OPDA, JA, JA-Ile and ABA in Col-0, *jin1-7,* and *aba2-1* plants upon herbivory by *P. rapae*.
OPDA, JA, JA-Ile (A), and ABA (B) levels were measured by Triple Quad LC/MS/MS in 5-week-old Col-0, *jin1-7,* and *aba2-1* plants on which *P. rapae* larvae were allowed to feed for 24 h. Hormone levels were determined in caterpillar-damaged leaves at different time points after removal of the insects. Depicted are the log_2-transformed fold induction values in hormone levels in *P. rapae*-infested leaves as compared to the levels in non-infested leaves of control plants that were harvested at the same time point. The corresponding absolute values are given in Table 1. Asterisks indicate statistically significant differences in comparison to non-infested plants (Tukey post-hoc tests; $P<0.05$).

Table 1: Production of OPDA, JA, JA-Ile, ABA, and SA in P. rapae-infested Col-0, *jin1-7*, and *aba2-1* plants[1].

			24 hours			30 hours		48 hours	
hormone	*P.r.*	Col-0	*jin1*	*aba2-1*	Col-0	*jin1*	Col-0	*jin1*
OPDA	-	255 ± 66	214 ± 38	206 ± 29	215 ± 62	214 ± 37	249 ± 73	186 ± 25
	+	1353 ± 224	1001 ± 120	1789 ± 371	1226 ± 740	509 ± 182	661 ± 244	666 ± 138
JA	-	36 ± 5	40 ± 11	61 ± 20	41 ± 17	31 ± 8	44 ± 18	38 ± 6
	+	538 ± 143	395 ± 64	325 ± 91	466 ± 148	298 ± 102	256 ± 31	251 ± 116
JA-Ile	-	0,8 ± 0,1	1,2 ± 0,6	2,0 ± 0,9	1,3 ± 0,8	1,1 ± 0,6	1,3 ± 0,7	0,9 ± 0,4
	+	16,3 ± 5,5	20,7 ± 5,6	13,9 ± 1,3	16,1 ± 7,8	17,7 ± 9,7	6,1 ± 1,1	10,0 ± 0,1
ABA	-	2,5 ± 0,3	3,1 ± 1,0	0,8 ± 0,2	3,4 ± 0,7	4,3 ± 1,6	2,7 ± 0,5	2,8 ± 0,8
	+	12,1 ± 2,1	5,5 ± 1,4	1,2 ± 0,2	4,9 ± 0,5	3,5 ± 0,2	3,1 ± 0,6	4,7 ± 1,9
SA	-	9,8 ± 1,3	19,7 ± 10,9	9,8 ± 3,7	26,9 ± 6,8	22,4 ± 4,1	18,4 ± 9,1	8,3 ± 2,3
	+	28,8 ± 1,8	25,5 ± 7,5	20,8 ± 7,3	54,8 ± 22,6	24,7 ± 12,1	14,8 ± 3,4	22,5 ± 4,5

ng g^{-1} (FW)

1 Absolute values (ng/g FW) of the levels of OPDA, JA, JA-Ile, ABA, and SA in 5-week-old Col-0, *jin1-7*, and *aba2-1* plants at different time points after herbivory by *P. rapae (P.r.)*. The hormone levels correspond with the fold-change induction levels depicted in Figure 4. Green highlights indicate statistically significant differences in comparison to non-infested plants (Tukey post-hoc tests; $P<0.05$).

To monitor the emission of ET during herbivory, *P. rapae*-infested Col-0 plants were placed in 2-L air-tight cuvettes. Subsequently, the production of ET was

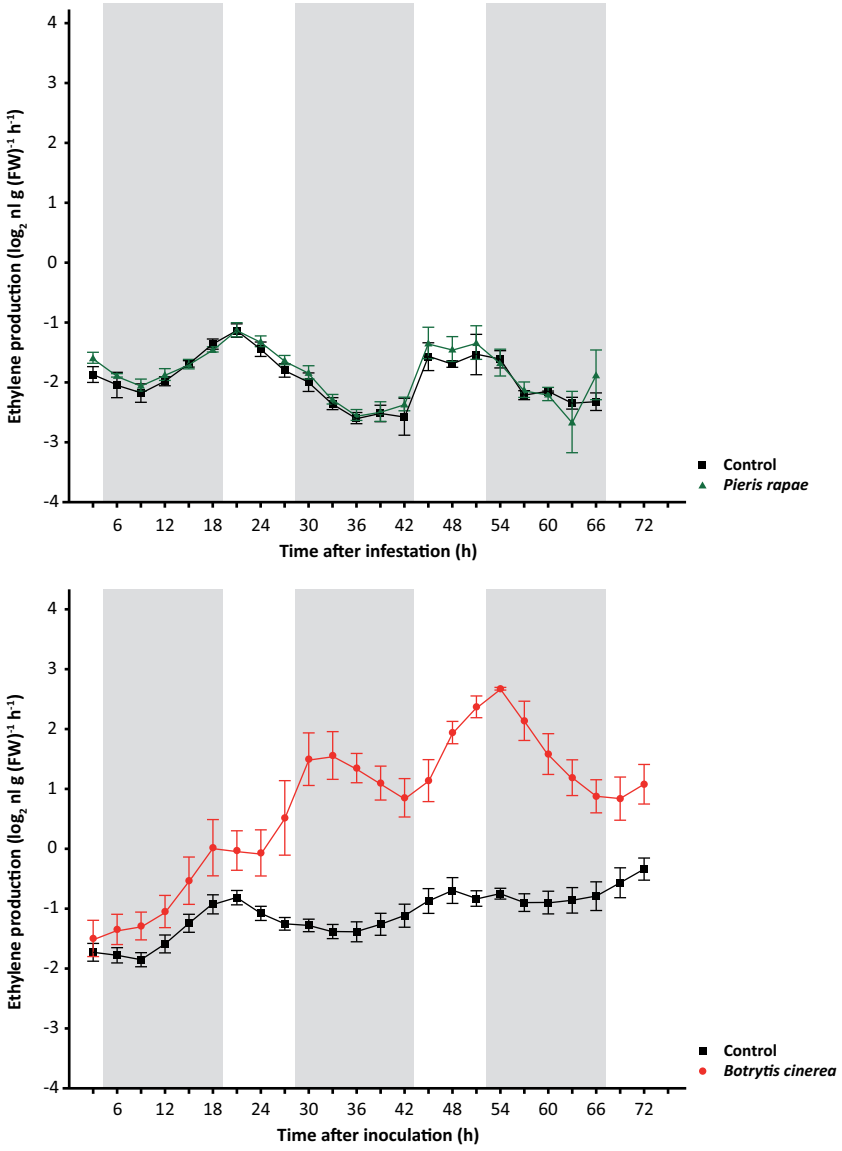

Figure 5: ET production of Col-0 plants during herbivory by *P. rapae* and infection by *B. cinerea*.
ET production was monitored in consecutive 3-h time intervals. Five-week-old Col-0 plants were infested with first-instar larvae of *P. rapae* (larvae fed on the leaves for the duration of the experiment) or inoculated with *B. cinerea* after which they were placed in 2-L air-tight cuvettes that were connected to a photoacoustic detection system, which allowed continuous detection of ET levels in the flush-through airflow. Error bars depict ± SE (*n*=6). The white area indicates the light period, and the shaded area indicates the dark period.

monitored over consecutive 3-h time intervals in a flow-through, highly sensitive photoacoustic detection system (Voesenek et al., 1990), which allows for continuous ET measurements during climate chamber growth conditions. Col-0 plants that were non-infested or inoculated with the ET-inducing necrotrophic fungus *Botrytis*

cinerea were used as negative and positive controls, respectively. Figure 5 shows that the production of ET was strongly enhanced in *B. cinerea*-infected plants. *P. rapae*-infested plants produced similar amounts of ET as did non-infested plants, indicating that *P. rapae* herbivory had no effect on the level of ET production.

Discussion

The plant immune system is based on recognition of attackers and subsequent activation of complex signaling cascades that result in effective direct and indirect defense strategies. The plant hormones JA, ET and SA play decisive regulatory roles in this process (Pieterse et al., 2009; Verhage et al., 2010). Defenses against insect herbivores have been demonstrated to be largely regulated by the hormone JA (Howe and Jander, 2008). A large number of defense-related genes is controlled by JA, in which MYC2 and ERF-type TFs play crucial regulatory roles (Anderson et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Pré et al., 2008; Memelink, 2009). The MYC2- and ERF-branch of the JA response are mutually antagonistic, hence the balance between both branches determines the set of JA-responsive genes that is activated upon stimulation of JA signaling. Previously, we showed that *P. rapae* oral secretion activates the ERF-branch of the JA response, and that *P. rapae* caterpillars prefer plants that express this branch of the JA pathway (Chapter 2). However, the final outcome of the interaction between Arabidopsis and feeding *P. rapae* larvae leads to activation of the MYC2-branch of the JA pathway, while the herbivore-preferred ERF-branch is strongly suppressed (Chapter 2). Previously, pharmacological evidence indicated that ABA co-regulates the JA-dependent activation of the MYC2-branch of the JA pathway, whereas ET co-regulates activation of the JA-dependent ERF-branch (Anderson et al., 2004; Lorenzo et al., 2004). Therefore, we hypothesized that ABA and ET play an important role in the differential JA response as observed during the Arabidopsis-*P. rapae* interaction.

ABA and the balance between the MYC2- and the ERF-branch during herbivory

In this study, we show that *aba2-1* plants failed to fully activate the MYC2-branch of the JA pathway and to concomitantly suppress the ERF-branch of the JA pathway in response to *P. rapae* feeding (Fig. 1), indicating that in wild-type plants ABA is essential for shifting the MYC2/ERF balance towards the MYC2-branch upon herbivory. A role for ABA in fine-tuning of the JA response was confirmed by

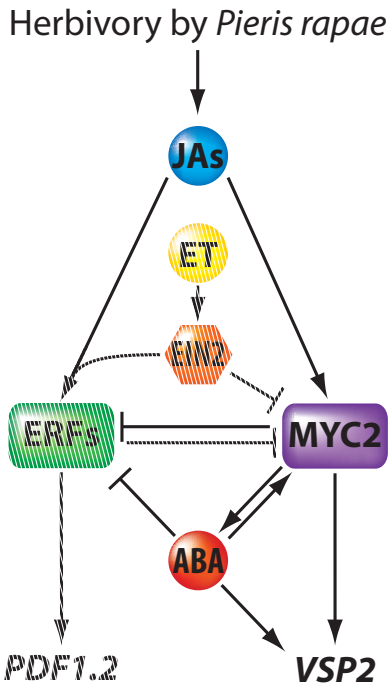

Figure 6: Model of differential regulation of JA responses of Arabidopsis during herbivory by *P. rapae*.
Feeding by *P. rapae* stimulates the production of JAs and ABA, resulting in activation of the MYC2-branch *(VSP2)* and concomitant suppression of the ERF-branch *(PDF1.2)* of the JA pathway. *P. rapae*-induced ABA production was found to be affected in *jin1-7* and it is thus placed downstream of MYC2 in the model. Although ET has the capacity to suppress the *P. rapae*-induced MYC2-branch (as indicated by the dashed components), it is not produced during the *P. rapae*-Arabidopsis interaction, and thus does not modulate the differential JA response in this *P. rapae*-Arabidopsis interaction model. The figure is based on combined data from Chapters 2 and 3. Arrows indicate a stimulating effect, whereas the blocked line indicates a suppressive effect.

the observation that exogenous application of ABA strongly suppressed the ERF-branch of the JA pathway in *P. rapae*-infested plants, while it stimulated the MYC2-branch (Fig. 2). In wild-type Col-0 plants, *P. rapae* feeding enhanced the production of the jasmonates OPDA, JA and JA-Ile, as well as that of ABA (Fig. 4; Table 1). ABA by itself did not alter the expression of the marker genes *PDF1.2* and *VSP2* (Fig. 2), which points to a model in which ABA plays an important modulating role in prioritizing the MYC2-branch of the JA pathway over the ERF-branch during insect feeding (Fig. 6). Previously, Reymond and coworkers (Reymond et al., 2004; Bodenhausen and Reymond, 2007) demonstrated that *P. rapae* feeding induced significant changes in the expression of a set of ABA-responsive genes. Moreover, they demonstrated that the generalist herbivore *Spodoptera littoralis* performed significantly better on the biosynthesis mutant *aba2-1*. Also in maize plants, increased ABA biosynthesis has been demonstrated upon herbivory of the leaves by *S. littoralis* and herbivory of the roots by the western corn rootworm *Diabrotica virgifera virgifera* (Erb et al., 2009). Together, these findings suggest that insect herbivory can lead to enhanced ABA biosynthesis. Our data pinpoint ABA as an important modulator of the MYC2/ERF-controlled differential JA response that shapes the final outcome of the *P. rapae*-induced JA-dependent defense response.

Interestingly, not only Col-0 plants showed suppression of the ERF-branch by exogenous application of ABA to *P. rapae*-infested plants, so did MYC2-impaired *jin1*-7 plants. This suggests that ABA can down-regulate ERF-dependent JA responses in a MYC2-independent manner. In wild-type plants *P. rapae*-induced ABA biosynthesis was largely dependent on MYC2 action, because ABA levels in *P. rapae*-infested *jin1*-7 plants were not significantly increased (Fig. 4; Table 1). Hence, the antagonistic effect of MYC2-dependent responses on the ERF-branch of the JA pathway may be partly caused by MYC2-mediated production of ABA.

ET and the balance between the MYC2- and ERF-branch during herbivory

We showed that ET can shift the MYC2/ERF balance towards the ERF-branch of the JA pathway during insect herbivory. Exogenous application of gaseous ET suppressed MYC2-dependent *VSP2* transcription in hervivore-infested plants, while it stimulated the ERF-branch marker gene *PDF1.2* (Fig. 3). Both effects were absent in the ET-insensitive mutant *ein2-1,* indicating that the modulating effect of ET was mediated via the ET signaling pathway. By contrast, *ORA59*-silenced RNAi-*ORA59* plants showed the same level of suppression of the MYC2-branch upon treatment with gaseous ET as did control RNAi-*GUS* plants, suggesting that the antagonistic effect of ET on MYC2-dependent gene expression can function independently of the ERF-type TF ORA59. Continuous monitoring of the production of ET in *P. rapae*-infested Arabidopsis plants using a highly sensitive photoacoustic detection system revealed that *P. rapae* feeding did not induce changes in the emission of ET (Fig. 5). Hence, although ET has the potential to modulate the balance between the MYC2- and the ERF-branch of the JA pathway, this hormone is unlikely to play a role in the outcome of the Arabidopsis-*P. rapae* interaction. These results also explain the observation of Bodenhausen and Reymond (2007), who found virtually no changes in the transcript profile of *P. rapae*-infested *ein2-1* plants in comparison to Col-0.

The plants used for our ET application experiments were grown in cuvettes under a continuous flush of fresh air which removes all endogenously produced ET. Under this condition, regular air-treated RNAi-*ORA59* plants showed the same *VSP2* expression pattern as Col-0 upon herbivory. However, when plants were grown under growth chamber conditions, RNAi-*ORA59* plants showed enhanced *VSP2* and *MYC2* expression levels compared to Col-0 plants, and *35S:ORA59* plants exhibited repressed *VSP2* expression upon *P. rapae* infestation (Chapter 2; Fig. 2). This suggests that under normal growth conditions, ORA59 can at least be partly responsible for ET-mediated suppression of the MYC2-branch of the JA pathway.

In conclusion, our data pinpoint ABA as an important modulator of the JA response that is induced upon herbivory. Because the signal signature produced in *P. rapae*-damaged tissue consists of JAs and ABA, whereas ET is lacking, the MYC2-branch of the JA pathway becomes stimulated resulting in the concomitant suppression of the ERF-branch (Fig. 6). As demonstrated in Chapter 2, suppression of the ERF-branch makes the leaf tissue less attractive for *P. rapae.* Although we found no evidence for a role for ET in the interaction between Arabidopsis and *P. rapae,* this hormone has been implicated in the response of Arabidopsis to other herbivores. For instance, *ein2-1* plants exhibited an enhanced level of resistance against the generalist herbivore *S. littoralis*, which was accompanied with significant EIN2-dependent changes in gene expression upon feeding of Col-0 plants (Stotz et al., 2000; Bodenhausen and Reymond, 2007). Apparently, the ET signaling pathway stimulates susceptibility to this generalist insect herbivore. A role for ET in stimulating caterpillar feeding corroborates our previous finding in Chapter 2 that expression of the ERF-branch enhances the feeding preference of *P. rapae* for these tissues. This study highlights that the hormonal context in which JAs are produced upon insect attack plays a decisive role in the final outcome of the plant's defense response.

MATERIALS AND METHODS

Plant material

Seeds of *Arabidopsis thaliana* accession Col-0, the mutants *jin1-7* (Lorenzo et al., 2004), *aba2-1* (Koornneef et al., 1982), and *ein2-1* (Alonso et al., 1999), and the transgenic lines RNAi-*ORA59* and RNAi-*GUS* (Pré et al., 2008) were sown in quartz sand. After two weeks, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with an 8-h day (24°C) and 16-h night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10 μM Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Pieris rapae infestations

Pieris rapae (small cabbage white butterfly) was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) as described previously (Van Oosten et al., 2008). *P. rapae* infestation of Arabidopsis plants was performed with freshly hatched first-instar (L1) larvae. On each plant 3 larvae were placed separately on fully expanded leaves of 5-week-old plants. After 24 h of feeding the larvae were removed after which herbivore-damaged leaves were harvested at different time points.

Botrytis cinerea inoculations

Botrytis cinerea inoculations were performed with strain B05.10 (Van Kan et al., 1997) as described previously (Van der Ent et al., 2008). Briefly, *B. cinerea* was grown on Potato Dextrose Agar (PDA; Difco Laboratories) for 5-7 days at 20°C under a short day-light regime (8-h day, 16-h night). Spores were collected and resuspended in half-strength Potato Dextrose Broth (PDB) to a final density of $5 \cdot 10^5$ spores per ml. After a 3-h incubation period, 5-µL droplets of the spores were applied as described (Van der Ent et al., 2008) .

Chemical treatments

Plants were treated with ABA (Sigma, Steinheim, Germany) by pouring 5 mL of a solution containing 0, 10, or 100 µM ABA onto the soil 1 day prior to transfer of *P. rapae* to the plants and 1 day after the 24-h feeding period. ABA solutions were diluted from a 1,000-fold concentrated stock in 96% ethanol. The control solution contained 0.1% ethanol.

Application of gaseous ET to the plants was performed as described (Millenaar et al., 2005; Leon-Reyes et al., 2009). In brief, gaseous ET (100 $\mu L \cdot L^{-1}$; Hoek Loos, Amsterdam, the Netherlands) and air (70% relative humidity) were mixed using flow meters (Brooks Instruments, Veenendaal, the Netherlands) to generate an output concentration of 1 $\mu L \cdot L^{-1}$ ethylene, which was flushed continuously through glass cuvettes (13.5 x 16.0 x 29.0 cm) at a flow rate of 75 $L \cdot h^{-1}$ and then vented to the outside of the building. The concentration of ET in the airflow was verified using gas chromatography as described (Millenaar et al., 2005). Five-week-old plants were placed in the cuvettes and remained there for the duration of the experiment. Control cuvettes containing plants were flushed with air (70% relative humidity) at the same flow rate. ET and air treatments started 1 day prior to transfer of *P. rapae* to the plants in the cuvettes and continued for the duration of the experiment. Light and temperature conditions were the same as described above.

RNA extraction and northern blot analysis

For RNA extraction, at least 5 plants per treatment were harvested at the time points indicated. RNA isolation was performed as described previously (Van Wees et al., 1999). For northern blot analysis, 15 µg of RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook et al., 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N^+ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The buffers used for electrophoresis and blotting were 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA blots were hybridized with probes for *PDF1.2* and *VSP2* as described (Leon-Reyes et al., 2010b). To check for equal loading, rRNA bands were stained with ethidium bromide. The AGI numbers for the genes studied are

At5g44420 (*PDF1.2*) and At5g24770 (*VSP2*). After hybridization with α-^{32}P-dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of *VSP2* mRNA on the northern blots were quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad, Veenendaal, the Netherlands).

Hormone analysis

For OPDA, JA, JA-Ile, ABA, and SA analysis, 0.5 g of *P. rapae*-infested damaged leaves as well as undamaged leaves from non-infested control plants were ground in a mortar with liquid nitrogen. The samples were extracted as described by López-Ráez et al. (2010b); 2 mL of cold ethyl acetate containing [2H6]-ABA was added to the samples at the start of the extraction as an internal standard (0.25 nmol) in order to calculate the recovery of the hormones measured. Hormone levels were analyzed by LC-MS on a Varian 320 Triple Quad LC/MS/MS. Ten µL of each sample was injected onto a Pursuit column (C18; 5 µm, 50 x 2.0 mm; Varian) that was connected to a precolumn (Pursuit Metaguard C18; 5 µm; 2.0 mm). Multiple reaction monitoring was performed for parent-ions and selected daughter-ions after negative ionization: OPDA 291/165 (fragmented under 18V collision energy), JA 209/59 (fragmented under 12V collision energy), JA-Ile 322/130 (fragmented under 19V collision energy), ABA 263/153, ABA-D_6 269/159 (both isoforms of ABA fragmented under 9V collision energy), and SA 137/93 (fragmented under 15V collision energy). The mobile phase comprised solvent A (0.05% formic acid) and solvent B (0.05% formic acid in MeOH) with settings as described (Diezel et al., 2009). The retention time of each compound was confirmed with pure compounds (ChemIm Ltd, Olomouc, Czech Republic). The surface area for each daughter-ion peak was recorded for the detected analytes. The analytes were quantified with standard curves made for each individual compound.

Ethylene measurements

ET production was measured in a laser-driven photoacoustic detection system (ETD-300, Sensor Sense, Nijmegen, the Netherlands) connected to a 6-channel valve control box in line with a flow-through system essentially as described (Voesenek et al., 1990). In brief, 5-week-old plants were placed in 2-L air-tight cuvettes (four plants per cuvette) which were incubated under growth chamber conditions (see above). After an acclimation time of 2 h, the cuvettes were continuously flushed with air (flow rate: 0.9 $L.h^{-1}$), directing the flow-through air from the cuvettes into a photoacoustic cell for ET measurements. ET levels were measured over consecutive 0.5-h time intervals, after which the machine switched to the next cuvette (n=6).

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CHAPTER 4

Systemic effects of insect herbivory on differential jasmonic acid signaling responses in Arabidopsis

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ABSTRACT

In Arabidopsis, the basic helix-loop-helix leucine zipper transcription factor MYC2 and APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain transcription factors antagonistically regulate distinct branches of the jasmonic acid (JA) signaling pathway. Feeding by larvae of the insect herbivore *Pieris rapae* (small cabbage white butterfly), results in activation of the MYC2-branch of the JA pathway and concomitant suppression of the herbivore-preferred ERF-branch of the JA pathway in insect-damaged leaves. Abscisic acid (ABA) was shown to play a decisive role in activation of the MYC2-branch and suppression of the ERF-branch upon herbivory. Here we investigated whether this differential JA response is expressed also in undamaged leaves of *P. rapae*-infested plants. In such systemic leaves, the *MYC2* transcription factor gene was induced to the same level as in the local leaves that were damaged by the insect. However, the systemic induction of the MYC2-branch marker gene *VSP2* was milder than the local induction. Also the de-repressed activation of the ERF-branch marker gene *PDF1.2,* as observed in herbivore-damaged leaves of MYC2-impaired *jin1*-7 plants, was absent in systemic leaves of herbivore-damaged *jin1*-7 plants. Comparison of the systemic and local hormone signal signature revealed that the levels of JA and (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) raised to a similar extent, but the enhanced production of the JA precursor 12-oxo-phytodienoic acid (OPDA) and ABA, as observed in local herbivore-damaged leaves, was absent in distant undamaged leaves. Together, these data point to a model in which herbivory leads to priming of the MYC2-branch of the JA pathway in systemic undamaged leaves, without fully activating costly JA-dependent defenses for which also ABA is required. This primed state may lead to accelerated activation of MYC2-dependent defenses when systemic tissue is attacked by insect herbivores. Whether activation of the full or the primed JA response as observed in local and systemic leaves, respectively, is caused by differences in the hormonal signal signature will need further study.

INTRODUCTION

Plants have evolved sophisticated mechanisms to defend themselves against attack by pathogens or insect herbivores. Once the immune system of a plant detects activity of a harmful organism, a local and systemic signaling cascade is activated involving numerous signaling compounds, defense-related hormones, transcriptional activators, and repressor-targeting degradation complexes. Important defense-related hormones are jasmonic acid (JA) and salicylic acid (SA). Their signaling cascades can be modified by ethylene (ET) and abscisic acid (ABA), two hormones that can play a decisive role in plant defense (Van Loon et al., 2006b; Ton et al., 2009). The nature of the hormone signal signature produced upon attack depends on the stimuli perceived, which enables the plant to activate attacker-specific defense responses (Pieterse et al., 2009; Verhage et al., 2010).

JA is an important hormone in the defense response of plants to insect herbivores and necrotrophic pathogens. Disruption of plant tissue by mechanical damage is sufficient to start production of JAs (Peña-Cortés et al., 1995; Howe et al., 1996; McConn et al., 1997), and wounding therefore to some extent reflects the response triggered by insect herbivory (Mithöfer et al., 2005). The initial phase of JA formation is localized in the chloroplasts, in which fatty acids derived from the organelles' membrane are modified by lipoxygenases (LOXs) into oxylipins. Desaturation and subsequent cyclization results in the formation of 12-oxo-phytodienoic acid (OPDA), the end-stage of oxylipin modification in the chloroplast. Subsequently, OPDA is translocated to the peroxisome, in which it is reduced by OPDA-REDUCTASE3 (OPR3) and undergoes three steps of ß-oxydation leading to formation of JA. Several modified JA isoforms have been found in plants, including hydroxylated-, methylated-, and amino-acid conjugated JAs, such as biologically highly active (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) (reviewed in Gfeller et al., 2010).

Recent evidence shows that the F-box protein COI1 (CORONATINE INSENSITIVE 1) functions as a JA-Ile receptor in the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI1} (Yan et al., 2009; Sheard et al., 2010). Binding of JA-Ile to COI1 leads to degradation of JASMONATE ZIM-domain (JAZ) transcriptional repressor proteins via the proteasome (Chini et al., 2007; Thines et al., 2007). JAZ proteins act as repressors of transcription factors (TFs), as has been shown for the basic helix-loop-helix leucine zipper transcription factor MYC2 (Chini et al., 2007), resulting in transcription or repression of downstream target genes. Although JA-Ile has been determined as the most potent initiator of JAZ degradation (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008; Yan et al., 2009), the role of other oxylipin isoforms remains largely unknown. Work on

OPDA-REDUCTASE-impaired *opr3* mutants revealed the JA-precursor OPDA as a direct regulator of a distinct set of JA-responsive genes (Stintzi and Browse, 2000; Taki et al., 2005; Bottcher and Pollmann, 2009), suggesting that the oxylipin signature may also influence the final outcome of the JA response.

Insect herbivory not only leads to induction of local JA-dependent defenses, it also activates defense responses in systemic undamaged tissues (Green and Ryan, 1972; Howe and Jander, 2008). Previously, we showed that prior infestation of *Arabidopsis thaliana* (Arabidopsis) with *P. rapae* induces a systemic defense response that inhibits *P. rapae* performance (De Vos et al., 2006a). This indicates that a systemic signal is spread throughout the plant, protecting the tissue for future damage (Howe and Jander, 2008). The systemic signal produced upon herbivory or wounding is still unknown, and may simply be JA itself or one of its isoforms (Schilmiller and Howe, 2005; Glauser et al., 2008). Grafting experiments with tomato revealed that JA biosynthesis is required at the site of tissue damage, whereas JA recognition is needed in distal plant tissue, to induce a JA response (Li et al., 2002a). Additional grafting experiments demonstrated that the *acx1* mutation, which impairs JA biosynthesis at the β–oxidation stage, disables the transmission of a systemic signal upon wounding, whereas perception of the signal is still intact (Li et al., 2005). This indicates that *de novo* JA biosynthesis in systemic tissue is not needed for the induction of a systemic JA response. However, elegant experiments in Arabidopsis by Koo et al. (2009), using dexametasone-inducible *OPR3* plants, showed that OPR3 is needed in systemic leaves to exhibit an increase in JA-Ile in this tissue upon wounding in distal leaves, which indicates that JA-Ile is synthesized *de novo* in this tissue. In *Nicotiana attenuata* plants, labeled JA-$^{13}C_6$-Ile was not retrieved in systemic leaves after $^{13}C_6$-Ile feeding in damaged local tissue (Wang et al., 2008a). These reports suggest that the molecular basis for the onset of a systemic JA response can vary between species.

In Arabidopsis, insect herbivores and necrotrophic pathogens activate distinct, antagonistic branches of the JA signaling pathway: the MYC2-branch that is regulated by the transcription factor MYC2, and the ERF-branch that is controlled by the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain transcription factors ORA59 and ERF1 (Anderson et al., 2004; Lorenzo et al., 2004; Pré et al., 2008). Previously, we showed that herbivory by larvae of the specialist herbivore *Pieris rapae* (small cabbage white butterfly) results in prioritization of activation of the MYC2-branch over the ERF-branch of the JA pathway (Chapter 2). Two-choice assays showed that *P. rapae* larvae prefer to feed from plants in which the ERF-branch of the JA pathway is activated (Chapter 2), suggesting that MYC2-mediated suppression of the ERF-branch during the *P. rapae*-Arabidopsis interaction may be part of the plant's defense strategy. Analysis of the hormone

signal signature revealed that the biosynthesis of OPDA, JA, JA-Ile and ABA is significantly upregulated in *P. rapae*-damaged leaves, whereas ET and SA levels remain unchanged (Chapter 3). ABA emerged as an important regulator in the stimulation of the MYC2-branch and the concomitant suppression of the ERF-branch in *P. rapae*-damaged leaves (Chapter 3), highlighting the important modulating role of ABA in the hormone-regulated defense signaling network in plants.

To investigate the spatial scale on which the differential JA response is activated during the Arabidopsis-*P. rapae* interaction, we monitored changes in transcript levels of the MYC2-branch marker gene *VSP2* and the ERF-branch marker gene *PDF1.2,* and compared hormone levels in damaged (local) and non-damaged (systemic) plant tissue of *P. rapae*-infested plants. We provide evidence that in contrast to herbivore-damaged leaves, distant undamaged leaves do not display the full differential JA response but may be primed for enhanced MYC2-dependent defenses.

RESULTS

Systemic effects of *P. rapae* feeding on differential JA-regulated responses

To investigate whether differential JA responses as observed in insect-damaged leaves are also expressed systemically, we monitored the gene expression of the key transcription factors regulating this process, *MYC2* and *ORA59,* and their respective target marker genes *VSP2* and *PDF1.2. P. rapae* caterpillars were allowed to feed for 24 h on wild-type Col-0 plants, after which the caterpillars were removed and both the damaged (local) and undamaged (systemic) leaf tissues analyzed for gene expression. Figure 1A shows that *MYC2* transcription was induced to high levels in both local and systemic leaves until 6 h after removal of the caterpillars (t=30 h). At later time points *MYC2* transcript levels decreased but remained elevated in comparison to non-infested plants. The level of *P. rapae*-induced *MYC2* expression was strikingly similar in damaged and undamaged leaves. In local infested leaves of wild-type Col-0 plants, *MYC2* transcription coincided with the activation of the MYC2-branch marker gene *VSP2,* which peaked also at 6 h after removal of the caterpillars (Fig. 1B). However, in systemic tissues *VSP2* transcription was remarkably lower. These results indicate that despite similar levels of induced *MYC2* gene expression in local and systemic tissues, activation of MYC2 downstream target genes is severely lower in systemic tissues.

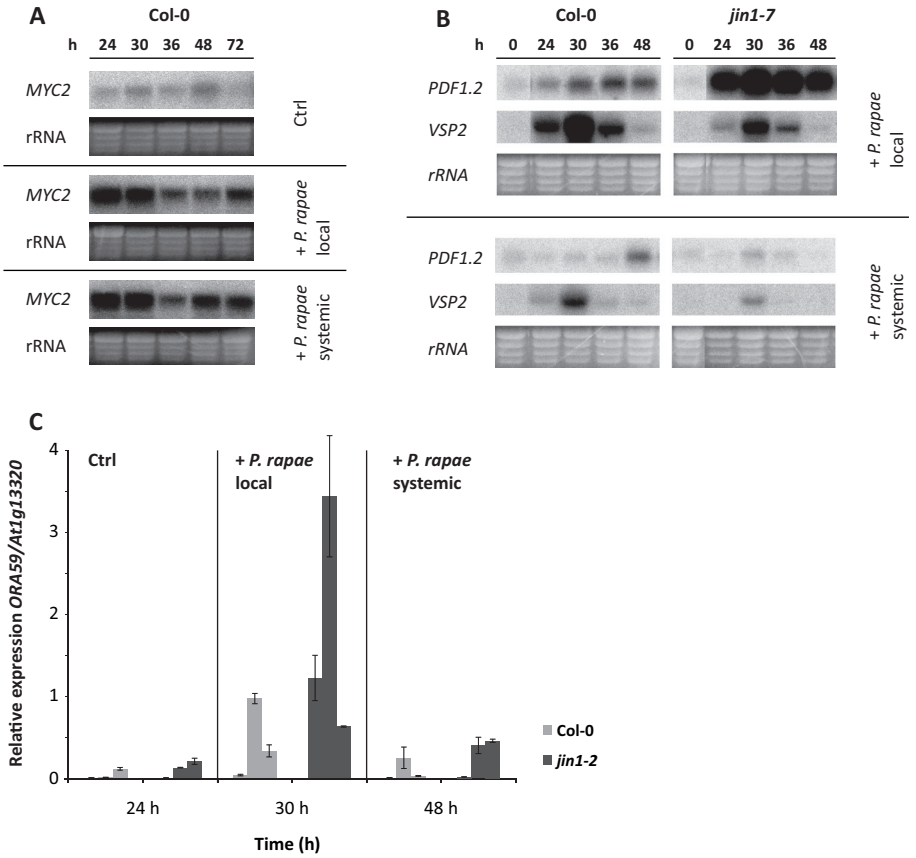

Figure 1: Local and systemic effects of P. rapae feeding on differential JA responses in Col-0 and *jin1-7* plants.

(A) Northern blot analysis of *MYC2* transcript levels in non-infested (Ctrl) and *P. rapae*-infested Col-0 plants. First-instar larvae were allowed to feed for 24 h after which they were removed (*t*= 24 h). Subsequently, damaged (local) and undamaged (systemic) leaf tissue was harvested for gene expression analysis. (B) Northern blot analysis of JA-responsive *PDF1.2* (marker for ERF-branch) and *VSP2* (marker for MYC2-branch) gene expression in damaged (local) and undamaged (systemic) tissue of *P. rapae*-infested Col-0 and MYC2-impaired *jin1-7* plants. (C) RT-qPCR analysis of ORA59 transcript levels (relative to *At1g13320*) in damaged (local) and undamaged (systemic) tissue of non-treated and *P. rapae*-infested Arabidopsis Col-0 and *jin1-7* plants.

To investigate whether, in analogy to responses in local infested tissues (Chapter 2), systemic *MYC2* expression leads to MYC2-dependent suppression of the ERF-branch of the JA pathway in systemic tissues, we monitored the expression of *PDF1.2* in undamaged leaves of *P. rapae*-infested *jin1*-7 plants. Figure 1B shows that, in contrast to herbivore-damaged *jin1*-7 leaves, systemic tissues did not show induced expression of *PDF1.2*. Similarly, expression of *ORA59,* a major transcriptional regulator of the ERF-branch of the JA signaling pathway whose expression typically coincides with the expression of *PDF1.2* (Pré et al., 2008), was strongly induced locally in herbivore-damaged *jin1*-7 leaves, but not systemically in undamaged tissues (Fig. 1C). In fact, the *PDF1.2* and *ORA59* expression levels were

similarly as in systemic tissue of *P. rapae*-infested Col-0 wild-type plants. Apparently, the ERF-branch of the JA pathway did not become activated in systemic leaves, neither in wild-type Col-0, nor in MYC2-impaired *jin1*-7.

Signal signature in systemic leaves of *P. rapae*-infested plants

The hormonal context in which bioactive JAs are produced is often decisive for the nature of the JA response that is triggered. To investigate whether the differences in JA-responsive gene expression in damaged and undamaged leaves of *P. rapae*-infested plants may be related to differences in the hormonal signal signature, we monitored the accumulation of JA, its precursor OPDA, and the biologically highly active amino acid conjugate JA-Ile. In addition, we measured the level of ABA production, which was previously shown to be important for the differential regulation of the JA response during *P. rapae* feeding (Chapter 3). Again, first-instar larvae were allowed to feed for 24 h after which they were removed from the leaves. Subsequently, hormone levels were measured at different time points after removal of the caterpillars. Previously, we demonstrated that OPDA, JA, JA-Ile and ABA levels significantly increased in *P. rapae*-damaged leaves (green shading in Fig. 2; data

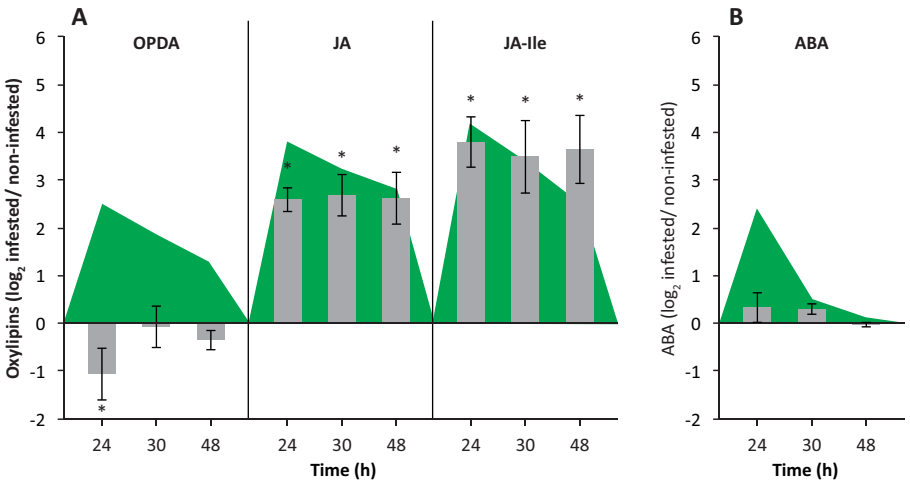

Figure 2: Systemic changes in the production of OPDA, JA, JA-Ile and ABA in Col-0 plants upon herbivory by *P. rapae*.
OPDA, JA, JA-Ile (A), and ABA (B) levels were measured by Triple Quad LC/MS/MS in 5-week-old Col-0 plants on which *P. rapae* larvae were allowed to feed for 24 h. Depicted are the log2-transformed fold induction values in hormone levels in undamaged leaves of *P. rapae*-infested plants as compared to the levels in leaves of non-infested plants that were harvested at the same time point. Green shading reflects the fold induction levels of the hormones in damaged leaves of *P. rapae*-infested plants (data from Chapter 3). Statistics were performed on a normalized dataset, which was acquired through ln-transformation of raw data. Asterisks indicate statistically significant differences in hormone levels between undamaged leaves of *P. rapae*-infested and non-infested control plants (ANOVA, Tukey post-hoc tests; *P*<0.05). Error bars represent ± SD.

from Chapter 3). Figure 2A shows that JA and JA-Ile levels also rose in systemic undamaged tissues and reached similar levels as in herbivore-damaged leaves. In contrast, OPDA and ABA levels did not rise in systemic tissues (Fig. 2A and 2B). OPDA levels were even significantly decreased after 24 h of *P. rapae* feeding. These results demonstrate that the systemic signature of OPDA, JA, JA-Ile and ABA production is different from that observed locally in herbivore-damaged leaves.

DISCUSSION

Previously, we demonstrated that herbivory by *P. rapae* on Arabidopsis leads to activation of the MYC2-branch of the JA pathway and concomitant suppression of the ERF-branch of the JA pathway in herbivore-damaged leaves (Chapter 2). ABA was demonstrated to play an important role in the regulation of the balance between the MYC2- and the ERF-branch of the JA pathway during *P. rapae* feeding (Chapter 3). Because *P. rapae* larvae were shown to have a preference for leaf tissue that expresses the ERF-branch of the JA pathway (Chapter 2), it is thought that MYC2-mediated suppression of the ERF-branch is part of the plant's defense strategy to limit herbivore damage. Here we investigated whether this differential JA response is extended to systemic undamaged tissues. We demonstrated that *P. rapae* feeding induces similar levels of *MYC2* gene expression in damaged and undamaged leaves (Fig. 1). However, in systemic undamaged leaves this did not lead to full induction

of the MYC2-branch of the JA pathway, as evidenced by low *VSP2* induction levels and lack of MYC2-mediated suppression of the ERF-branch marker genes *ORA59* and *PDF1.2* (Fig. 1B). This observation resembles a phenomenon that is called priming for enhanced defense (Conrath et al., 2006), which is also observed during rhizobacteria-induced systemic resistance (ISR). Upon root colonization by beneficial ISR-inducing rhizobacteria, aboveground plant tissues acquire an enhanced level of resistance that is effective against a broad spectrum of pathogens and even insect herbivores (Van Loon et al., 1998; Van Wees et al., 2008; Pineda et al., 2010). This type of induced resistance is not associated with direct activation of

defense-related genes, but a large set of predominantly JA-responsive genes become primed for accelerated expression after pathogen or insect attack (Van Wees et al., 1999; Hase et al., 2003; Pozo et al., 2008; Van Oosten et al., 2008). Previously, Pozo et al. (2008) demonstrated that induction of ISR by *Pseudomonas fluorescens* WCS417r was associated with enhanced expression of *MYC2* in systemic leaf tissue,

without directly activating the downstream JA-responsive target genes, resembling our observations in undamaged leaves of *P. rapae*-infested plants. Moreover, MYC2-impaired *jin1* mutants were blocked in ISR, highlighting the importance of MYC2 in this type of induced resistance and in priming for enhanced defense. De Vos et al. (2006a) demonstrated that local infestation by *P. rapae* larvae reduces the performance of this insect in systemic tissue, suggesting that indeed the observed systemic expression of *MYC2* can play a role in systemically induced resistance.

Previously, Van der Ent et al. (2009c) demonstrated that priming for enhanced defense by ISR-inducing rhizobacteria and the chemical agent β-aminobutyric acid (BABA) is associated with enhanced expression of a large set of transcription factor genes, including *MYC2,* without affecting the expression of downstream defense-related genes. After pathogen infection, the primed leaf tissues displayed accelerated expression of a large number of pathogen-responsive genes. As most defense-related regulatory transcription factors act as amplifiers in defense signaling cascades, even a modest induction during priming can be sufficient to enhance the defense signaling capacity, thereby giving the primed plant tissue a 'head start' during the early stages of pathogen or insect attack. It is therefore tempting to speculate that systemic induction of *MYC2* gene expression in response to insect herbivory primes the undamaged plant parts for enhanced expression of MYC2-dependent defenses to protect these plant parts against a secondary attack by insect herbivores. Priming has been demonstrated to entail limited fitness costs, especially in comparison to direct activation of defense. Moreover, these fitness costs were shown to be outweighed by the enhanced resistance benefits under pathogen pressure, which suggests that priming functions as an ecological adaptation of the plant to respond faster to its hostile environment (Van Hulten et al., 2006; Walters et al., 2008).

Analysis of the production of OPDA, JA, JA-Ile and ABA in systemic tissues revealed that the signature of these hormonal signals differs from that observed in herbivore-damaged leaves. While all four hormones significantly increased in herbivore-infested leaves (Chapter 3), in undamaged leaves only JA and JA-Ile levels were induced (Fig. 2). The levels of JA and JA-Ile were similar as those observed in undamaged leaves, which correlates with the comparable levels of *MYC2* gene expression in local and systemic leaves. The fact that *MYC2* expression in systemic leaves was not translated into activation of MYC2-dependent target genes may be caused by differences in the hormonal context in which JA and JA-Ile were produced. In undamaged tissue, neither OPDA nor ABA levels were upregulated (Fig. 2), which may be causally related to the strongly reduced activation of downstream MYC2-dependent target genes, such as *VSP2*. In support of this, we found that exogenous application of ABA has a synergistic effect on *P. rapae*-induced *VSP2* expression and that the ABA biosynthesis mutant *aba2-1* shows reduced *VSP2* levels upon *P. rapae* infestation (Chapter 3).

The lack of an increase in systemic ABA levels may be caused by an insufficient amount of locally produced ABA to translocate to undamaged leaves. In herbivore-damaged leaves, ABA production increased in a MYC2-dependent manner (Chapter 3). Despite the observed increase in MYC2 transcript levels in systemic tissue (Fig. 1A), ABA levels did not increase (Fig. 2B). This indicates that, apart from MYC2, additional signals are needed for ABA production to increase. Most likely, these signals consist of herbivore- or plant-derived damage-associated molecular patterns (HAMPs or DAMPs) (Lotze et al., 2007; Heil, 2009), but this remains to be elucidated.

In contrast to herbivore-damaged leaves in which OPDA production was significantly upregulated, undamaged leaves displayed a significant decrease in the level of this JA precursor. Previously, decreasing OPDA levels in systemic tissues of Arabidopsis were also observed upon mechanical damage (Koo et al., 2009). The drop in OPDA suggests that turnover of the existing pool of this JA precursor is increased, which points to de novo production of JA in systemic leaf tissue. However, it cannot be ruled out that the observed systemic increase in JA and JA-Ile is partly caused by systemic transport from herbivore-damaged leaf tissue. Nevertheless, the difference in OPDA levels in damaged (OPDA up) and undamaged (OPDA down) leaves may be causally related to the differential activation of the MYC2-branch of the JA pathway in local and systemic leaves. OPDA was previously found to be a potent nuclear Ca^{2+} inducer, causing a concentration-dependent increase in nuclear Ca^{2+} levels (Walter et al., 2007). Ca^{2+} is an important second messenger in plants and a rise in free nuclear Ca^{2+} concentration activates kinases and phosphatases involved in regulating transcriptional activity (Mazars et al., 2009). In addition, OPDA is able to add a nucleophilic compound to enzymes, transcription factors or tripeptide glutathione (GSH), thereby changing the activity of these proteins (Bottcher and Pollmann, 2009). Therefore, OPDA and its effect on protein activity might well be involved in the differential regulation of the MYC2 target genes in damaged and undamaged leaves of *P. rapae*-infested plants. However, the molecular basis of this phenomenon clearly needs further investigation.

MATERIALS AND METHODS

Plant growth conditions

Seeds of *Arabidopsis thaliana* wild-type Col-0, and mutants *jin1-2* and *jin1-7* (Lorenzo et al., 2004) were sown on quartz sand and, after two weeks of growth, seedlings were transplanted into 60mL pots containing a sand-potting soil mixture

(5:12 v/v) that had been autoclaved twice for 20 min with a 24-h interval. Plants were cultivated in a growth chamber with an 8-h day (200 μE m^{-2} s^{-1} at 24°C) and 16-h night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland solution (Hoagland and Arnon, 1938) containing 10 μM Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Pieris rapae assays

Pieris rapae (small cabbage white butterfly) was reared on Brussels sprouts plants (*Brassica oleracea gemmifera* cv. Icarus) as described previously (Van Poecke et al., 2001). In all experiments, first-instar (L1) larvae were used. For gene expression analysis, three larvae were placed separately on fully expanded leaves of 5-week-old Arabidopsis plants using a fine paint-brush. In all cases, larvae were removed 24 h after the start of the experiment. Leaves were harvested at different time points after caterpillar infestation. Leaves damaged by caterpillar feeding were harvested separately from undamaged leaves of infested and uninfested control plants.

RNA extraction and northern blot analysis

Total RNA was isolated as described (Van Wees et al., 1999). For northern blot analysis, 10 μg of RNA were denatured using glyoxal and dimethyl sulfoxide (Sambrook et al., 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N^+ membrane (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. To check for equal loading, rRNA bands were stained with ethidium bromide. Northern blots were hybridized with gene-specific probes for *PDF1.2* and *VSP2* as described previously (Leon-Reyes et al., 2010a). A probe for *MYC2* was made by PCR amplification of cDNA of MeJA-treated plants and the gene-specific primers *MYC2*-Fw 5'GGTCA CCGGT TTATG GAATG-3' and *MYC2*-Rv 5'CGTAT CCGTC ACCTC CTCAT3'. After hybridization with $\alpha[^{32}P]$-dCTP-labeled probes, blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad, Veenendaal, the Netherlands). The AGI numbers for the genes studied are At5g44420 (*PDF1.2*), At5g24770 (*VSP2*), and At1g32640 (*MYC2*). All gene expression analyses have been repeated with similar results.

RT-qPCR

SuperScript™ III Reverse Transcriptase was used to convert DNA-free total RNA into cDNA. PCR reactions were performed in optical 96-well plates (Applied Biosystems) with an ABI PRISM® 7900 HT sequence detection system, using SYBR® Green to monitor the synthesis of double-stranded DNA. A standard

thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60°C to 95°C with a ramp speed of 1.9°C min^{-1}. The following primers were used to detect *ORA59* (At1g06160): Fw 5'-TTCCC CGGAG AACTC TTCTT-3', Rv 5'-GCCTG ATCAT AAGCG AGAGC-3'. Transcript levels were calculated relative to the reference gene At1g13320 (Czechowski et al., 2005) using the $2^{-\Delta\Delta Ct}$ method described previously by (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Hormone analysis

For OPDA, JA, JA-Ile, and ABA analysis, 0.5 g of undamaged leaves of *P. rapae*-infested and non-infested control plants were ground in a mortar with liquid nitrogen. The leaf samples were harvested from the same plants as those used for hormone analysis of local, *P. rapae*-damaged leaves. The extraction and hormone analysis was performed as described by López-Ráez et al. (2010b) (see also Chapter 3).

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CHAPTER 5

Priming for enhanced defense in Arabidopsis: a field study on fitness benefits

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ABSTRACT

Plants possess an inducible defense system that regulates the activation of defenses against pathogens. Plants that are primed for disease resistance have acquired the capacity to respond faster and/or stronger to pathogen attack. Under controlled growth conditions, primed plants were previously found to have a fitness advantage compared to nonprimed plants when infected by pathogens. Here, we tested whether priming also benefits plants when cultivated under natural growth conditions in the field. *Arabidopsis thaliana* mutants *edr1* and *jin1* were tested, since these mutants are primed for enhanced activation of diverse hormone-regulated defense signaling routes. Mutant *edr1* is constitutively primed for salicylic acid (SA)-dependent defenses and cell wall defenses, leading to enhanced resistance to biotrophic pathogens like *Hyaloperonospora arabidopsidis*. Mutant *jin1* is primed for defenses regulated by the ERF-branch of the jasmonic acid (JA) signaling pathway, which is correlated with enhanced resistance to necrotrophic pathogens like *Plectosphaerella cucumerina*. Challenge of field-grown Arabidopsis plants with *H. arabidopsidis* and *P. cucumerina* induced the expression of marker genes for SA- and JA-dependent defenses to similar extents in both the priming mutants *edr1* and *jin1* and in the wild-type Col-0. In agreement with this, field-grown Col-0 displayed similar resistance levels as mutants *edr1* and *jin1* to *H. arabidopsidis* and *P. cucumerina*. Moreover, fitness of the plants, as measured by radial rosette growth, was affected to similar extents by pathogen infection in the different plant genotypes tested. This suggests that under field conditions the wild-type Col-0 plants may have acquired a primed capacity to express defenses to a level that is similar to that observed in the constitutively primed mutants, which hints at a role for priming in the adaptation of plants to their environment. Strikingly, we also detected that after an initial reduction of rosette growth by pathogen infection, all plants subsequently fully compensated for this growth loss. Infection by *H. arabidopsidis* was even found to nearly double the seed set, indicating a fitness benefit acquired by infection with this biotrophic pathogen.

INTRODUCTION

The efficiency of induced plant defenses to combat pathogen attack greatly contributes to the success of a plant to resist the infection. When defenses are expressed too late or at too low levels, the plant will become diseased. Plants that are primed for disease resistance possess an enhanced defensive capacity to express defenses faster and/or stronger upon pathogen perception, leading to improved protection to future environmental stresses (Conrath et al., 2002; Conrath et al., 2006).

Priming for disease resistance has been described for several plant species and can be induced by diverse stimuli. Local infection by a necrotizing pathogen triggers systemic acquired resistance (SAR) in distant plant tissue that is associated with direct activation of SAR marker genes like *PR-1* (*PATHOGENESIS-RELATED 1*)(Ryals et al., 1996; Sticher et al., 1997; Durrant and Dong, 2004; Van Loon et al., 2006a), but also with priming of defense responses in systemic tissue (Mur et al., 1996; Cameron et al., 1999; Van Wees et al., 1999). SAR is dependent on endogenous accumulation of the plant hormone salicylic acid (SA; Gaffney et al., 1993) and requires the regulatory protein NPR1 (NONEXPRESSOR OF PR GENES 1; Cao et al., 1994; Dong, 2004). Besides pathogen infection, also insect herbivory can prime plants for a faster and stronger response to subsequent stresses in both systemic tissue (De Vos et al., 2006a) and in neighboring plants by means of the production of volatile organic compounds (Engelberth et al., 2004; Ton et al., 2007). These responses are also inducible by the plant hormone jasmonic acid (JA) that is produced in the plant after infestation by insects (Howe and Jander, 2008). In addition, beneficial micro-organisms, such as plant growth-promoting rhizobacteria and mycorrhizal fungi, can enhance the defense capacity of plants (Van Wees et al., 2008). Induced systemic resistance (ISR) triggered by non-pathogenic rhizobacteria like *Pseudomonas fluorescens* WCS417r functions independently of SA, but requires intact JA and ethylene (ET) signaling pathways (Pieterse et al., 1998). The enhanced resistance in leaves of WCS417r-mediated ISR-expressing Arabidopsis may be entirely attributed to priming, since expression of JA/ET-dependent defenses is not directly induced in ISR-expressing tissue, but an augmented induction is observed upon subsequent pathogen or insect attack (Van Wees et al., 1999; Hase et al., 2003; Verhagen et al., 2004; Van Oosten et al., 2008; Van der Ent et al., 2009a). Recently, the basic helix-loop-helix leucine zipper transcription factor MYC2 that regulates a part of the JA signaling pathway was identified as an important regulator of ISR. Mutant *jin1* (*jasmonate insensitive1*) that is impaired in MYC2 was affected in WCS417r-induced resistance and priming of defense responses (Pozo et al., 2008). Also ISR triggered by the beneficial fungus *Piriformospora indica* was shown to be dependent on MYC2 (Stein et al., 2008).

Besides MYC2, an additional set of 120 transcription factors was demonstrated to be induced or repressed during WCS417r-mediated ISR (Van der Ent et al., 2009c). It is thought that these signaling intermediates remain inactive until a subsequent stress stimulus is perceived, after which they contribute to the enhanced defensive capacity of primed plants (Conrath et al., 2006; Van der Ent et al., 2009c). Recently, the MAP kinases MPK3 and MPK6 were shown to accumulate in response to priming concentrations of the SA mimic BTH. Only upon subsequent exposure to pathogens the accumulated MPKs were activated, leading to augmented defense gene expression (Beckers et al., 2009). Hence, priming of defense responses can be established by enhanced accumulation of inactive signaling intermediates such as transcription factors (Van der Ent et al., 2009c) and MAP kinases (Beckers et al., 2009).

In general, priming of SA-dependent SAR responses is mostly effective against pathogens with a (hemi-)biotrophic lifestyle (Delaney et al., 1994), while priming of JA-dependent ISR responses is predominantly successful to ward off necrotrophic pathogens (Ton et al., 2002; Van der Ent et al., 2008) and generalist herbivores (Van Oosten et al., 2008). Pathogen-induced SAR, rhizobacteria-induced ISR and resistance induced by the chemical β-aminobutyric acid (BABA) commonly are associated with priming of callose-containing cell wall appositions, that are induced in the plant at the sites of pathogen attack, which is an effective defense response against a variety of both biotrophic and necrotrophic pathogens (Ton and Mauch-Mani, 2004). Priming of callose deposition is regulated likely through different mechanisms by the different inducing stimuli (Ton and Mauch-Mani, 2004; Pozo et al., 2008; Van der Ent et al., 2009c).

Expressing resistance responses is costly for the plant, as is demonstrated with Arabidopsis *cpr* (*constitutive expression of PR genes*) mutants that constitutively display inducible defenses, which is associated with reduced growth rate and seed set compared to wild-type plants under both growth chamber and field conditions (Heidel et al., 2004; Van Hulten et al., 2006). When under pathogen pressure, *cpr* mutants expose a fitness level that equals that of wild type (Van Hulten et al., 2006) or is in some cases lower (Heidel and Dong, 2006), demonstrating that the fitness costs associated with constitutively induced defenses are similar as or higher than the resistance benefits. In contrast, priming of Arabidopsis plants imposes only a minimal reduction in fitness on the plant, which is outweighed by the resistance benefit of priming when plants are attacked by pathogens (Van Hulten et al., 2006). Priming may thus represent an important ecological adaptation to resist environmental stress.

Priming has predominantly been studied under tightly controlled growth conditions with stabile temperature, light intensity, humidity, and nutrient availability for plant growth. In natural environments the above mentioned parameters are different and fluctuate continuously. Furthermore, in nature plants interact with plants and numerous other naturally-occurring organisms, such as herbivores and micro-organisms that can be beneficial or detrimental to the plant (Pieterse and Dicke, 2007). All of these factors may affect priming responses and likely influence the plant's energy balance, so that the effect of priming and the costs associated with it can be different under natural conditions compared to controlled conditions. Studies on the fitness consequences of primed plants under natural conditions will be instrumental to better understand the ecological impact of priming of defense responses. In earlier studies, priming-inducing chemicals and micro-organisms were shown to protect plants against diseases in the field (Zehnder et al., 2001; Beckers and Conrath, 2007; Goellner and Conrath, 2008; Walters, 2009). However, priming of cellular defense responses was not determined in these studies. Since most chemical priming agents can activate defenses directly when applied at high doses (Kohler et al., 2002; Van Hulten et al., 2006) and many protective micro-organisms can directly inhibit pathogens, the protection observed in the field can not automatically be attributed to priming of defenses.

In this study we investigated the fitness effects of priming for defense responses under field conditions using the Arabidopsis constitutive priming mutants *edr1* and *jin1*. Previously, we demonstrated that under controlled growth chamber conditions *edr1* (*enhanced disease resistance1*), which is mutated in a MAPKKK (Frye et al., 2001), displayed augmented SA-dependent gene expression and callose deposition compared to wild-type Col-0 when attacked by the biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis* (Frye and Innes, 1998; Van Hulten et al., 2006). This constitutive priming phenotype of *edr1* was associated with enhanced resistance to *H. arabidopsidis* and a fitness benefit in the presence of this pathogen compared to infected Col-0 plants (Van Hulten et al., 2006). In mutant *jin1*, repression of the ERF-branch of the JA signaling pathway by MYC2 is released, resulting in primed expression of a set of JA/ET-dependent genes like *PDF1.2* (*PLANT DEFENSIN 1.2*) (Anderson et al., 2004; Chapter 2 and 3). As a consequence, the priming mutant *jin1* displayed enhanced resistance to the necrotrophic pathogen *Plectosphaerella cucumerina* (Lorenzo et al., 2004). In our field study, we monitored Col-0 and the two priming mutants with regard to activation of defense responses, and costs and fitness of infection with *H. arabidopsidis* and *P. cucumerina*.

RESULTS

Choice of constitutive priming mutants *edr1* and *jin1* to study priming in the field

Constitutive priming mutants *edr1* and *jin1* harbor a single ethylmethane sulfonate (EMS)-induced point mutation (Frye and Innes, 1998; Lorenzo et al., 2004), and could thus be used as primed plants in the field without the need to apply to strict GMO regulations, which would be needed if transgenic plants were used in the field. Moreover, by using different genotypes, primed (mutant) and non-primed (wild-type) plants could be randomized in a plot which would be impossible if a priming stimulus like a chemical were to be applied.

First, we verified the constitutive priming phenotypes of *edr1* and *jin1* under controlled growth conditions. To this end, we determined the expression of the SA-responsive marker gene *PR-1* and the JA-responsive marker gene *PDF1.2* after treatment with concentration ranges of SA and methyl-JA (MeJA). As expected, *edr1* mutants showed an augmented *PR-1* gene expression after SA treatment compared to wild-type Col-0 plants (Fig. 1A and C). Also *jin1* mutants showed enhanced levels of SA-induced *PR-1* expression albeit to a lesser extent than *edr1* (Fig. 1A and C). These results are in line with previous observations of higher *PR-1* gene expression after pathogen attack in *edr1* and *jin1* compared to Col-0 (Frye and Innes, 1998; Lorenzo et al., 2004; Laurie-Berry et al., 2006; Van Hulten et al., 2006). *PDF1.2* gene induction by MeJA treatment was clearly enhanced in *jin1* compared to Col-0 (Fig. 1B and D), as was described previously (Anderson et al., 2004; Lorenzo et al., 2004). In *edr1*, augmented *PDF1.2* gene expression was observed only after treatment with the highest dose of MeJA (100 μM). This indicates that *edr1* is slightly primed for JA-inducible responses, which is possibly due to a modestly elevated expression level of the transcription factor *ERF1* in *edr1* (Wawrzynska et al., 2008).

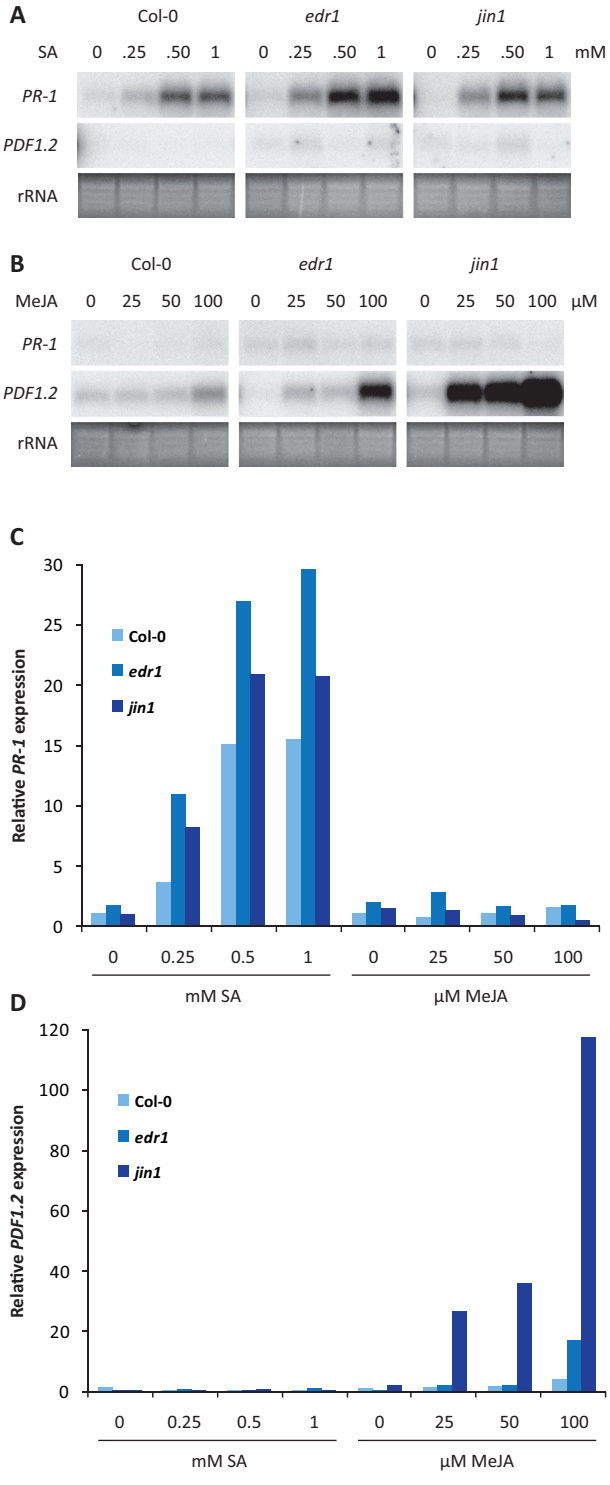

Figure 1: Responsiveness to exogenously applied SA and MeJA of Col-0, *edr1* and *jin1*, grown under controlled conditions.

(A) Northern blot analysis of *PR-1* and *PDF1.2* gene expression in 5-week-old plants 24 h after treatment with different concentrations of SA. (B) Northern blot analysis of *PR-1* and *PDF1.2* gene expression in 5-week-old plants 6 h after treatment with different concentrations of MeJA. (C) Signal intensities of *PR-1* signals on Northern blots as shown in (A) and (B). *PR-1* transcript level of mock-treated Col-0 was set at 1. (D) Signal intensities of *PDF1.2* signals on Northern blots as shown in (A) and (B). PDF1.2 transcript level of mock-treated Col-0 was set at 1.

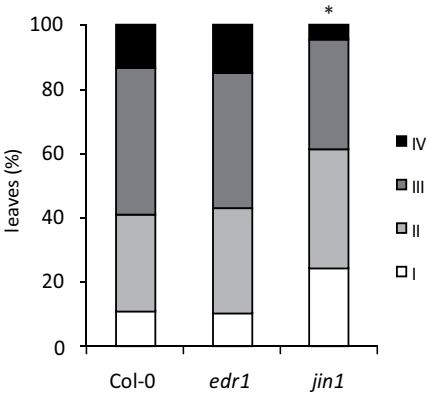

Figure 2: Resistance of Col-0, *edr1* and *jin1*, grown under controlled conditions, to *P. cucumerina*. Disease severity after drop inoculation with *P. cucumerina* spores of 5-week-old plants was quantified 7 days after inoculation. Disease rating is expressed as the percentage of leaves in disease classes I, no symptoms; II, moderate necrosis at inoculation site; III, full necrosis size of inoculation droplet, and IV, spreading lesion. Asterisk indicates statistically significant different distribution of the disease classes compared to Col-0 (χ2 test; P<0.05; n=240 leaves).

In the field experiment, we used the biotrophic pathogen *H. arabidopsidis,* which is sensitive to SA-dependent defenses, and the necrotrophic pathogen *P. cucumerina,* which is sensitive to JA-dependent defenses, as challenging pathogens to determine the effect of priming for defense. We previously demonstrated that under controlled growth conditions, mutant *edr1* is enhanced resistant to *H. arabidopsidis* (Van Hulten et al., 2006), while mutant *jin1* displays resistance levels comparable to Col-0 (Pozo et al., 2008). Here, we tested under controlled conditions the resistance of both mutants against *P. cucumerina*. Mutant *jin1* was enhanced resistant, confirming former findings (Fig. 2; Lorenzo et al., 2004). Mutant *edr1* showed the same level of resistance as Col-0 (Fig. 2). This indicates that the moderately enhanced responsiveness of *edr1* to MeJA (Fig. 1B and D) and *jin1* to SA (Fig. 1A and C) did not lead to increased resistance against *P. cucumerina* and *H. arabidopsidis*, respectively (Fig. 2; Pozo et al., 2008).

Figure 3: Impression of the setup of a plot in the field experiment. In the left picture the Arabidopsis plants are 4 weeks old and have been grown in the field for almost 2 weeks, which is time point of treatment with pathogens or mock. In the right picture the plants are 6 weeks old, which is in this particular plot 2 weeks after mock treatment; empty spots indicate that plants were harvested for analysis. White labels indicate Col-0 plants, green labels *edr1* mutants, and purple labels *jin1* mutants.

Setup of the field experiment

The field experiment of which the results are described here was preceded by two other common garden experiments in which only few plants survived. We had learned from the previous experiments that the survival of Arabidopsis plants under field conditions improved significantly by using plants that had been transplanted into the field as 2-week-old greenhouse-grown seedlings instead of growing plants from the seed stage in the field. In addition, the use of maze nettings to protect Arabidopsis against bright sunlight significantly contributed to plant survival.

For the field experiment described here, plants were grown for 2 weeks in a controlled greenhouse compartment and then transferred to an uncontrolled plastic semi-dome greenhouse to acclimatize to the low temperatures in the field (at that time frost was observed at night). Five days later, the plants were transplanted into a field site near Utrecht, The Netherlands (52°05'24.33" N; 5°10'21.81" E). In total there were 11 plots in a random block design, each containing 37 plants per genotype, which were randomly divided per plot. Fig. 3 shows an impression of a field plot. Plants in the plots were spaced 3.9 cm apart to prevent crowding and water was supplied when needed. With the exception of active protection from slugs, no measures were taken to exclude naturally occurring pathogens, insects, or rodents from the field plots. Of the 1227 transplanted plants all but 2 survived until harvest. Besides some degree of anthocyanin production induced after transfer of the seedlings from controlled greenhouse conditions to uncontrolled semi-dome greenhouse conditions, the plants seemed to develop normally and grew steadily. Few insects crawling in the plots were detected but hardly any symptoms caused by herbivory or spontaneous pathogen infections were spotted in this experiment. After growing for 10 days in the field, all plants of each plot received a pathogen or mock treatment.

Mutants *edr1* and *jin1* were not enhanced resistant to *H. arabidopsidis* or *P. cucumerina* in the field

Inoculation with *H. arabidopsidis* and *P. cucumerina* leads under controlled growth chamber conditions to clearly visual disease symptoms, but in the field experiment there were only few observations of necrosis in the pathogen-inoculated plants. However, RT-quantitative PCR (RT-qPCR) using pathogen-specific primers revealed that pathogen-specific mRNA was detected in the field-grown plants at increasing levels at 2, 4 and 7 days after inoculation, indicating an active infection by the pathogens (Fig. 4A and 4B). No statistically significant differences in pathogen mRNA accumulation were found between the different genotypes tested (Fig. 4A

and B). This indicates that the field-grown *edr1* and *jin1* plants were not enhanced resistant to *H. arabidopsidis* and *P. cucumerina*, respectively, which differs from the situation under controlled growth chamber conditions (Fig. 2; Van Hulten et al., 2006; Pozo et al., 2008). This suggests that, under field conditions, *edr1* and *jin1* either do not exhibit a priming phenotype, or their priming phenotype does not lead to enhanced resistance against *H. arabidopsidis* and *P. cucumerina* in comparison to Col-0 plants.

Although *in planta* growth of both pathogens in the field was detected, it was noted that the observed mRNA levels of *H. arabidopsidis Actin2* and *P. cucumerina β-Tubulin* in infected Col-0 plants grown under controlled growth chamber conditions is usually 10-fold higher (ΔCt of -3; data not shown). The lower infection rate in field-grown plants could possibly be explained by less favorable conditions for infection in the field. Alternatively, plants may have acquired a relatively high disease resistance level in the field.

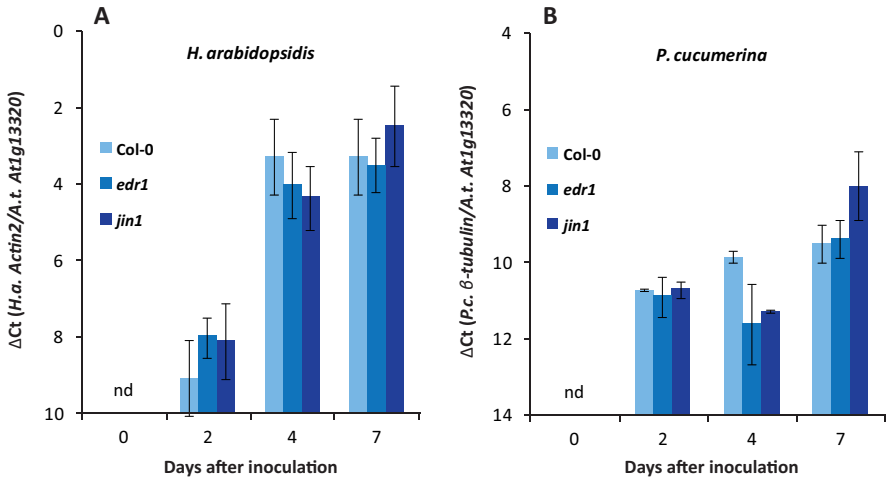

Figure 4: Growth of *H. arabidopsidis* and *P. cucumerina* in field-grown Col-0, *edr1* and *jin1* plants. (A) ***H. arabidopsidis Actin2*** **ΔCt levels and (B)** ***P. cucumerina*** *β-tubulin* **ΔCt levels relative to *At1g13320* in Col-0, *edr1* and *jin1* plants grown under field conditions at 2, 4 and 7 days after inoculation with *H. arabidopsidis* or *P. cucumerina*. Shown are means ±SE of 3 independent biological replicas, each consisting of 4 plants.**

Callose induction levels are similar in *edr1* and Col-0, but lower in *jin1* under field conditions

Deposition of callose in the plant cell wall is induced as a defense response upon infection by *H. arabidopsidis*. Previous results obtained from plants that were grown under controlled growth chamber conditions have shown that mutant *edr1* responds with augmented deposition of callose-containing papillae at the site of attempted pathogen entry (Van Hulten et al., 2006). Figure 5 shows that under field conditions

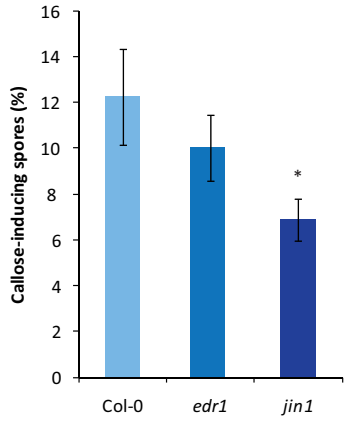

Figure 5: Quantification of callose deposition in field-grown *H. arabidopsidis*-inoculated Col-0, *edr1* and *jin1* plants.
Callose deposition was quantified at 2 days after inoculation with *H. arabidopsidis* by determining the percentage of spores on each individual leaf that induced callose-containing depositions in the epidermal cell layer at the site of attempted pathogen entry. Shown are means ±SE (*n*=30 leaves). Asterisk indicates statistically significant difference between the mutant and Col-0 (Student's *t*-test; *P*<0.05).

edr1 did not show enhanced callose formation in comparison to Col-0 at 2 days after inoculation with *H. arabidopsidis*. This suggests that when the plants were grown in the field, either *edr1* expressed a negligible priming potential for callose deposition, or Col-0 was primed for callose deposition to a level that was comparable to that of *edr1*. The lack of difference in callose deposition may contribute to the lack of enhanced resistance of *edr1* to *H. arabidopsidis* observed in the field (Fig. 4A).

Mutant *jin1* displayed significantly less callose deposition upon *H. arabidopsidis* infection compared to Col-0 in the field (Fig. 5), whereas under controlled growth chamber conditions no such difference was observed (Pozo et al., 2008). Rhizobacteria that trigger ISR had been shown to prime plants for callose deposition. In mutant *jin1*, both ISR and priming of callose deposition induced by the rhizobacteria was impaired (Pozo et al., 2008). In the field experiment, priming of callose induced by natural environmental factors may explain the greater callose deposition levels detected in Col-0 compared to *jin1*. Priming of callose in Col-0 may further contribute to the similar callose deposition levels observed in *edr1* and Col-0.

Induction of *PR-1* and *PDF1.2* expression by pathogens in field-grown plants is not enhanced in *edr1* and *jin1* compared to Col-0

Expression of defense responses was further investigated by monitoring activation of the SA-dependent marker genes *PR-1* and *PR-5*, and the JA-dependent marker gene *PDF1.2*. Using RT-qPCR, it was found that both *H. arabidopsidis* and *P. cucumerina* induced expression of *PR-1*, *PR-5*, and *PDF1.2* in Col-0 at 2, 4 and 7 days after inoculation with the pathogens, in most cases to a statistically significant higher level compared to the mock treatment (Fig. 6A and B; expression of *PR-5* followed the same pattern as *PR-1* in all treatments (data not shown)). Induction of these

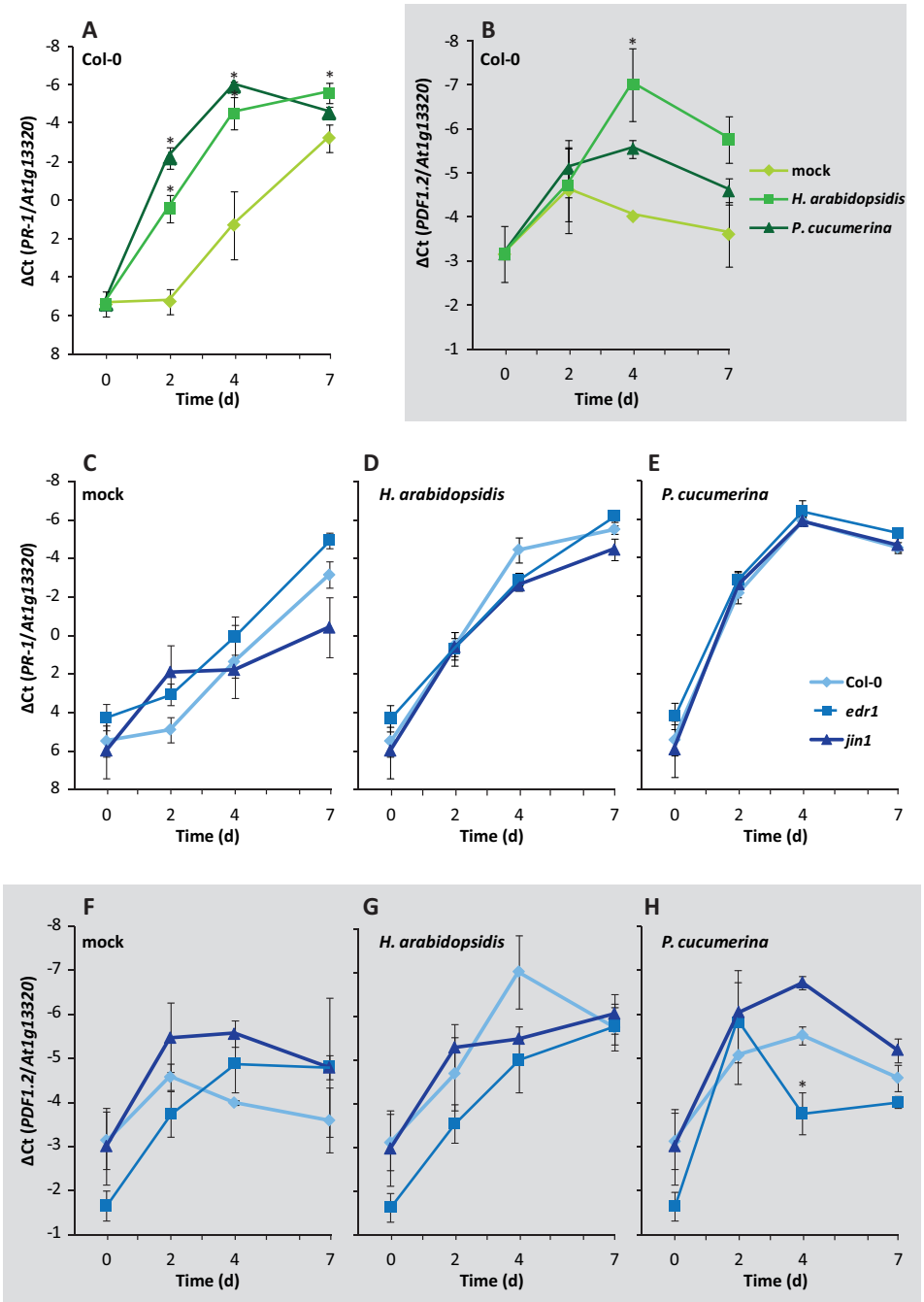

Figure 6: *PR-1* and *PDF1.2* expression in field-grown Col-0, *edr1* and *jin1* plants after pathogen challenge.

PR-1 (white background) and *PDF1.2* (grey background) expression was monitored in Col-0, *edr1* and *jin1* plants at 0, 2, 4 and 7 days after challenge with *H. arabidopsidis* or *P. cucumerina*. *PR-1* ΔCt levels and *PDF1.2* ΔCt levels (relative to *At1g13320*) are depicted for Col-0 after mock, *H. arabidopsidis* and *P. cucumerina* treatment **(A, B)**, and for Col-0, *edr1* and *jin1* after mock **(C, F)**, *H. arabidopsidis* **(D, G)**, and *P. cucumerina* **(E, H)** treatment. Shown are means ±SE of 3 independent biological replicas, each consisting of 4 plants. Asterisks indicate statistically significant differences compared to mock **(A, B)** or Col-0 **(C-H)** (LSD test; $P<0.05$).

genes by the two tested pathogens has also been reported for plants grown under controlled growth chamber conditions (Thomma et al., 2001; Van Hulten et al., 2006; Huibers et al., 2009).

Comparison of the *PR-1* and *PDF1.2* gene expression levels in the *edr1* and *jin1* mutants with Col-0 plants revealed in general no statistically significant differences (Fig. 6C-H). *PR-1* levels tended to be higher in *edr1* than in Col-0 after mock treatment at all time points tested, whereas no differential augmented *PR-1* expression was observed after infection by the pathogens (Fig. 6C-E). Also under controlled growth chamber conditions *edr1* occasionally showed enhanced basal expression of *PR-1*, which was nonetheless, like in the field experiment, very low compared to pathogen-induced expression (Fig. 1A and 1C; Van Hulten et al., 2006). *PDF1.2* transcript levels tended to be enhanced in *jin1* after both mock and *P. cucumerina* treatment (Fig. 6F and H). Enhanced basal levels of *PDF1.2* in *jin1* have not been observed before under controlled growth chamber conditions (Chapters 2 and 3) and may be related to field conditions. The significantly lower *PDF1.2* expression level observed in *edr1* at one time point after *P. cucumerina* infection (Fig. 6H) seems an outlier and may be due to the generally high variability in *PDF1.2* expression levels between the different biological replicates of the field experiment. Despite the great variance in gene expression levels, their general similarity that was overall observed between infected *edr1*, *jin1* and Col-0 plants correlates with similar levels of pathogen growth in all genotypes in the field (Fig. 4A and B). Lack of difference in defense gene induction supports the idea that Col-0 plants in the field experiment may have become primed for disease resistance.

Mutants *edr1* and *jin1* do not have a fitness benefit under pathogen pressure in the field

Plant fitness under field conditions was determined by measuring rosette diameter and seed set. The data on seed set will be described in a following section. At the day of inoculation, when the plants were 4 weeks old, *edr1* mutants had the smallest rosette diameter in all field plots, whereas rosettes of *jin1* mutants had the largest size (Fig. 7A). Seven and 13 days later, *edr1* mutants were still behind in growth to the same extent (Fig. 7B-E), which may have been caused by drought stress that is known to reduce the size of *edr1* (Tang et al., 2005). When grown under controlled growth chamber conditions no or only minor growth defects were found for *edr1* (Frye and Innes, 1998; Tang et al., 2005; Van Hulten et al., 2006). More importantly, the similar growth rates of *edr1* and Col-0 observed after infection by a pathogen (Fig. 7D and E) indicates that the effect on fitness by pathogen infection was not

different in *edr1* from Col-0. The temporal effects of pathogen infection on rosette growth in all genotypes will be described in the following section.

Mutant *jin1* plants were larger than Col-0 plants when 4 weeks old, but this difference had ceased to nil for both mock- and pathogen-treated plants 2 weeks later (Fig. 7C), which was due to a reduced relative rosette growth rate in *jin1* compared to Col-0 in the first and second week after treatment (Fig. 7D and F). The growth rate of *jin1* was affected to similar extents by pathogen infection as Col-0, implying that there was no fitness (dis)advantage of *jin1* under pathogen

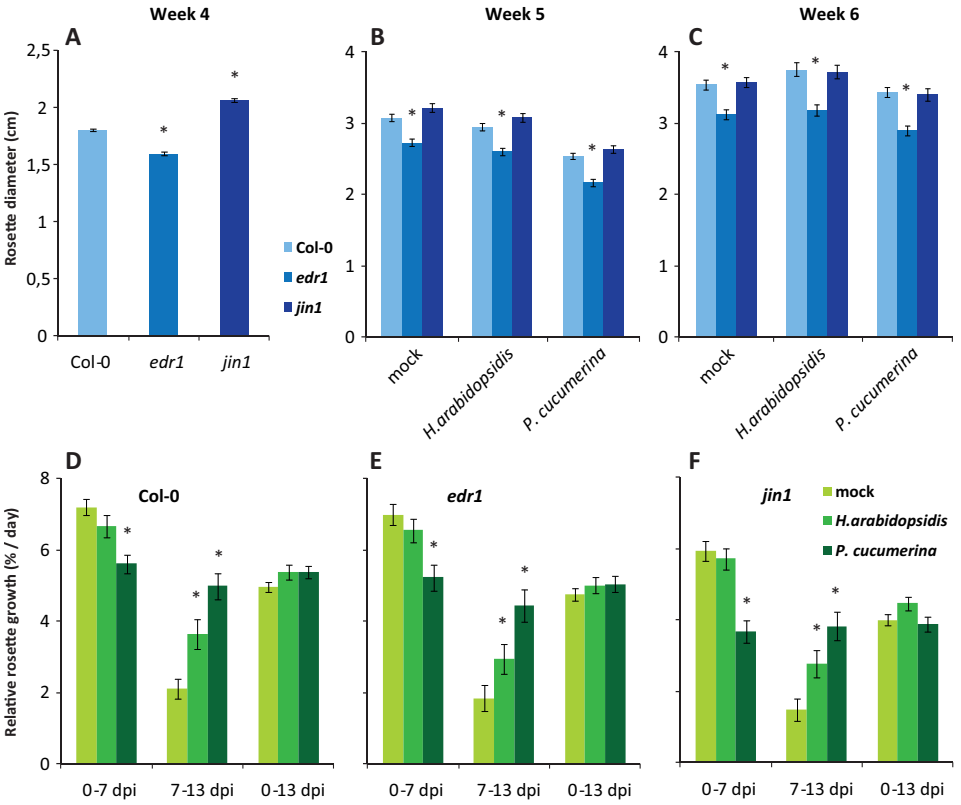

Figure 7: Rosette growth as a measure of fitness of field-grown Col-0, *edr1* and *jin1* plants. **(A)** Rosette diameters of 4-week-old Col-0, *edr1* and *jin1* plants. Asterisks indicate statistically significant differences between mutants and wild-type Col-0 (Student's *t*-test; P<0.05, n=407 plants). **(B)** Rosette diameters of 5-week-old Col-0, *edr1* and *jin1* plants, 1 week after mock treatment or inoculation with *H. arabidopsidis* or *P. cucumerina*. Asterisks indicate statistically significant differences within each treatment between mutants and wild-type Col-0 (Student's *t*-test; *P*<0.05, n=100-140 plants). **(C)** Rosette diameters of 6-week-old Col-0, *edr1* and *jin1* plants, 13 days after treatment. For details, see legend to Figure 7B. **(D-F)** Relative rosette growth per day of Col-0, *edr1*, and *jin1* plants after mock treatment or inoculation with *H. arabidopsidis* or *P. cucumerina*. Shown are the means ±SE of the relative increase in rosette diameter per day as compared with the rosette diameter 1 week earlier, calculated for all individual plants. Week 1 covers 0-7 days past inoculation (dpi) and week 2 covers 7-13 dpi. Asterisks indicate statistically significant differences between mock- and pathogen-treated plants (Fischer's LSD test; *P*<0.05, *n*=100-140 plants).

pressure. The faster development of *jin1* at an early age correlated with 5 days earlier onset of bolting of *jin1*, commencing when the plants were 5 weeks old, which was also found under controlled growth chamber conditions for this particular mutant allele *jin1-2* (data not shown).

Pathogen-induced growth reduction is followed by growth compensation

Analysis of the relative rosette growth per day monitored over 0-7 days past inoculation (dpi; week 1) revealed a significantly reduced rate of rosette growth of all genotypes in the first week after challenge with *P. cucumerina* and a similar trend was visible for *H. arabidopsidis*-inoculated plants (Fig. 7D-F). Interestingly, both *H. arabidopsidis*- and *P. cucumerina*-treated plants compensated for this growth depression by accomplishing higher rosette growth rates than mock-treated plants at 7-13 dpi (week 2; Fig. 7D-F). When relative rosette growth per day was calculated over the total 2-week-period after inoculation, hardly any differences were observed between pathogen- and mock-treated plants. This indicates that the pathogen-challenged plants had fully compensated the initial growth retardation following pathogen attack within two weeks.

H. arabidopsidis infection enhances seed production in field-grown Arabidopsis

Seed production was determined in inflorescences that were harvested at five weeks after treatment with mock or pathogens, when most plants had finished producing new flowers and had started to senesce. Upon mock treatment, mutants *edr1* and *jin1* produced similar seed quantities as Col-0 (Fig. 8A), indicating that the mutations did not significantly affect this fitness trait. However, a trend towards lower seed production of *edr1* and higher seed set of *jin1* was observed, correlating with smaller and greater rosette sizes, respectively (Fig. 7A-C). Unexpectedly, infection of Col-0 by *H. arabidopsidis* caused a significant increase in seed production with 70% (Fig. 8B). The average weight of 100 randomly selected seeds did not significantly between treatments (data not shown), indicating that differences in seed weight reflect differences in seed number. Also in mutant *jin1* a statistically significant increase in seed production was observed after *H. arabidopsidis* infection, while the increase in mutant *edr1* was not statistically significant (Fig. 8B). The differential effect of *H. arabidopsidis* on seed production of the different genotypes can not be explained by

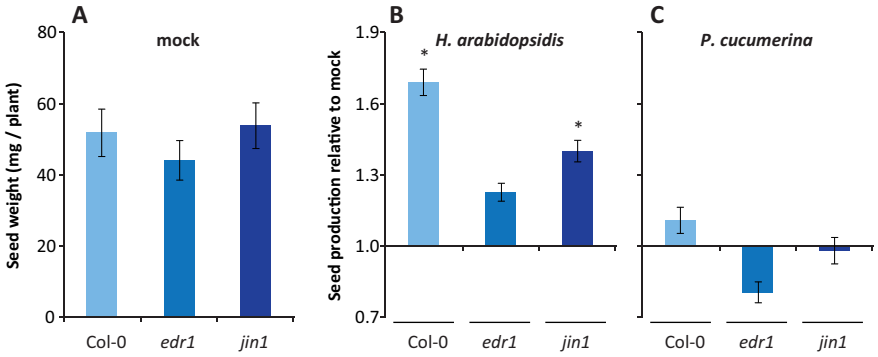

Figure 8: Seed production of field-grown Col-0, *edr1* and *jin1* plants after inoculation with *H. arabidopsidis* or *P. cucumerina*.
(A) Total weight of the seeds produced per plant by 10-week-old, mock-treated Col-0, *edr1* and *jin1* plants that were grown in the field. No statistically significant differences were revealed by Student's *t*-testing ($P<0.05$, $n=119$ plants). (B) Seed production in *H. arabidopsidis*-inoculated plants relative to mock-treated plants. Asterisks indicate statistically significant differences in seed production between pathogen- and mock-treated plants per plant genotype (Fisher's LSD test; $P<0.05$, $n=239$ plants). (C) Seed production in *P. cucumerina*-inoculated plants relative to mock-treated plants (Fisher's LSD test; $P<0.05$, $n=207$ plants). For details see the legend to Figure 8B. For performing statitistics on normally distributed data, the data used for statistics were ln-transformed. In all figures, data shown are means ±SE.

differential priming of defenses, since we showed that the level of resistance and defense gene expression was comparable in all genotypes. These results indicate that infection by *H. arabidopsidis* increased seed production in Arabidopsis and that the *edr1* and *jin1* mutations did not provide a fitness benefit in the field.

In contrast to *H. arabidopsidis*, *P. cucumerina* caused no significant alterations in seed set compared to mock treatment for all plant genotypes, although pathogen-treated *edr1* and *jin1* mutants showed a trend toward lower seed production (Fig. 8C).

Growth compensation and enhanced seed production after pathogen infection under controlled growth chamber conditions

The phenomena of (i) growth compensation after growth reduction due to pathogen infection and (ii) enhanced seed production after infection with *H. arabidopsidis* that were observed under non-controlled field conditions were verified in Col-0 plants that were grown under controlled growth chamber conditions. Like in the field experiment, inoculation with *H. arabidopsidis* and *P. cucumerina* caused a significant reduction in rosette growth compared to mock treatment in the first week after inoculation (Fig. 9A). In the second week, pathogen-treated plants exhibited enhanced growth rates, which led in *P. cucumerina*-treated plants to full recovery from the growth reduction and in the *H. arabidopsidis*-treated plants to partial recovery growth compensation had occurred partially, which is

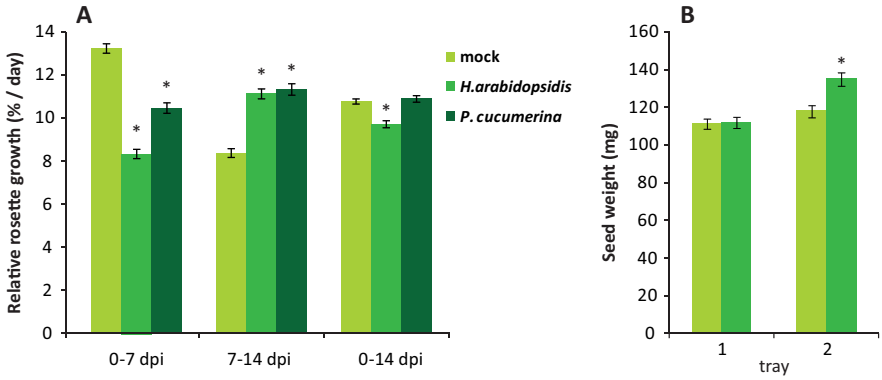

Figure 9: Relative rosette growth and seed set in infected Col-0 plants under controlled growth chamber conditions.
(A) Relative rosette growth of greenhouse-grown wild-type Col-0 plants after treatment with mock, *H. arabidopsidis* or *P. cucumerina*. Shown are means ±SE of the relative increase in rosette diameter per day as compared with the rosette diameter 1 week earlier, calculated for all individual plants. Week 1 covers 0-7 days past inoculation (dpi) and week 2 covers 7-14 dpi. Relative growth from time of inoculation until 2 weeks after inoculation is indicated by 0-14 dpi. Absolute growth was greater in the first week than in the second. Asterisks indicate statistically significant differences between mock- and pathogen-inoculated plants (ANOVA; P< 0.05, n=40 plants). (B) Total seed weights (±SE) of mock- and *H. arabidopsidis*-treated Col-0 plants. In tray 1, watering was ceased upon observation of first ripe seeds, while tray 2 was watered for a subsequent two weeks. Asterisk indicates significant difference between mock- and pathogen-inoculated plants (Student's *t*-test; *P*<0.05, *n*=20 plants).

possibly related to great growth reduction that was inflicted in the first week. In general these results show that *H. arabidopsidis*- and *P. cucumerina*–inoculated plants compensate for pathogen-induced growth reduction, under both field and growth chamber conditions.

The effect of *H. arabidopsidis* inoculation on seed production was determined in two sets of plants that had been cultivated under different watering regimes. In tray 1 watering was stopped when the first seeds had ripened, whereas in tray 2 watering was continued for another two weeks, allowing the development of new flowers. In tray 1 no difference in seed production was observed between mock-*H. arabidopsidis*-treated plants (Fig. 9B). In contrast, in tray 2 *H. arabidopsidis*-inoculated plants produced significantly more seeds, confirming that under field and controlled growth chamber conditions, infection by *H. arabidopsidis* can lead to enhanced seed production if the plants are allowed to develop seeds to full extent.

DISCUSSION

To our knowledge all studies to date on priming for defenses have been executed with plants grown under controlled growth chamber conditions. Here, we aimed to study the priming phenomenon under field conditions, where plants are exposed to natural environmental challenges like nutrient shortages, temperature fluctuations, and simultaneous interactions with naturally present herbivores and microbes. Besides findings on priming in the field, this study uncovered novel effects of pathogen infection on fitness traits.

Constitutive priming mutants *edr1* and *jin1* displayed neither enhanced defenses nor fitness benefits when grown under pathogen pressure in the field

For the field study we used the Arabidopsis mutants *edr1* and *jin1* that are constitutively primed for enhanced SA- or JA-dependent defense responses, respectively. Under growth chamber conditions, *PR-1* and *PDF1.2* indeed displayed a potentiated expression pattern after exogenous application of SA or MeJA, respectively (Fig. 1). Priming of these SA- and JA-dependent defenses was associated with enhanced resistance to *H. arabidopsidis* (Van Hulten et al., 2006) and *P. cucumerina* (Lorenzo et al., 2004; Fig. 2) in *edr1* and *jin1* plants, respectively. However, the performance of the priming mutants in the field was comparable to that of wild-type Col-0. From our field study we conclude that the mutations did not provide a benefit or a disadvantage in the field when the plants were under pathogen pressure. Infection with *H. arabidopsidis* and *P. cucumerina* induced comparable levels of expression of the SA and JA defense marker genes *PR-1* and *PDF1.2* in all three tested genotypes (Fig. 6). Furthermore, deposition of callose at sites of attempted penetration of the cell wall by *H. arabidopsidis* was not enhanced in the mutants in comparison to Col-0 (Fig. 5). In accordance, the mutants were not enhanced resistant to the pathogens tested (Fig. 4). Correspondingly, no fitness benefits of the mutants under pathogen pressure were observed under field conditions, as determined by rosette growth and seed production (Fig. 7 and 8). Together, these results indicate that the priming phenomenon and associated fitness benefits under disease pressure in *edr1* and *jin1* as observed under controlled growth conditions, was not detectable in our field experiment.

Although upon pathogen challenge in the field *edr1* and *jin1* generally displayed no significantly augmented defense responses in comparison to Col-0, the mock-treated *edr1* and *jin1* plants consistently exhibited enhanced basal levels of *PR-1* and

PDF1.2, respectively (Fig. 6C and F). Slightly enhanced basal levels were observed occasionally under controlled climate chamber conditions for *edr1* (Van Hulten et al., 2006; Fig. 2), but not for *jin1* (Chapters 2 and 3). This differential effect on basal expression levels may be related to stress encountered under field conditions. The enhanced basal levels of *PR-1* and *PDF1.2* in the mutants under field conditions did not lead to enhanced resistance against pathogen infection, but this may be explained by the relatively low level of basal defense expression and the non-augmented expression after pathogen challenge.

Did Col-0 plants acquire a primed defensive capacity in the field?

Explanations for the lack of difference in disease resistance and fitness between wild-type Col-0 and the mutants *edr1* and *jin1* in our field experiment under pathogen pressure could be that either 1) the mutants did not express the constitutive priming phenotype, or 2) wild-type Col-0 plants gained a primed state when grown in the field. The first explanation, loss of constitutive priming in *edr1* and *jin1,* is unlikely because the molecular mechanisms behind these priming phenomena in the different mutants are very different and therefore unlikely to be both suppressed. A more plausible explanation may be that wild-type plants grown in the field also acquired priming of defenses, resulting in similar levels of pathogen-induced defense gene expression and deposition of callose as observed in the mutants (Fig. 5 and Fig. 6D-H). An indication for priming of Col-0 in the field comes from our finding that callose deposition in response to *H. arabidopsidis* was higher in Col-0 than in *jin1* (Fig. 5). We previously showed that *jin1* is impaired in *P. fluorescens* WCS417r-induced priming for enhanced callose deposition in response to *H. arabidopsidis* (Pozo et al., 2008). Therefore the higher level of callose deposition in field-grown Col-0 may result from priming by e.g. rhizobacteria that were naturally present at the field site. Priming for enhanced callose deposition in Col-0 may also explain the same level of callose in *edr1* and Col-0 in the field, while under controlled conditions this response is augmented in *edr1* due to priming (Van Hulten et al., 2006).

A direct enhanced activation of defense responses in all tested genotypes instead of priming of defenses could be an alternative explanation for the lack of difference in resistance of Col-0 versus the mutants observed in the field. However, at the time of inoculation the basal expression levels of *PR-1* and also of the wound/herbivory-marker genes *VSP2* (*VEGATIVE STORAGE PROTEIN 2*) and *LOX2* (*LIPOXYGENASE 2*) were remarkably similar in the field trial compared to controlled growth chamber experiments (Fig. 6; data not shown). This low level of

defense activation in mock-treated field-grown plants is in agreement with the low natural pathogen and herbivore pressure in the field experiment. However, basal *PDF1.2* gene expression was approximately 16 times higher in field-grown plants than in plants grown in controlled environments (data not shown). Why *PDF1.2* gene expression was enhanced in our field-grown plants is currently unknown, but it may point to some level of constitutive activation of JA/ET responses. This could have played a role in the relatively high resistance level of the field-grown plants to *P. cucumerina*, but not to *H. arabidopsidis*. Even though the *PR-1* transcript levels were very low at the day of pathogen treatment, over the course of the field experiment the *PR-1* levels increased, also in the mock-treated plants. This was likely caused by the cold temperatures (around freezing point) at night. It was recently reported that a cold treatment causes the transcription factor NTL6 to bind to promoters of cold-responsive *PR* genes, amongst which *PR-1*, *PR-2* and *PR-5*, which results in enhanced resistance against *Pseudomonas syringae* pv. *syringae* DC3000 bacteria (Seo et al., 2010).

Growth compensation following growth reduction induced by pathogen infection

At 2 weeks after treatment, pathogen-inoculated plants had the same rosette sizes as mock-treated plants (Fig. 7C). However, in the first week after pathogen inoculation the relative rosette growth was found to be reduced in all plants, especially after *P. cucumerina* challenge (Fig. 7D-F). Interestingly, in the second week the plants compensated for this growth depression (Fig. 7D-F), resulting in the same rosette size as mock-treated plants at the end of the second week. The same phenomenon of growth depression followed by compensation was observed when plants were cultivated under controlled growth chamber conditions (Fig. 9A). Pathogen-induced growth depression followed by compensation was demonstrated for both Col-0 and the mutants *edr1* and *jin1* (Fig. 7D-F), suggesting that the mutations do not affect these pathogen-induced growth effects. Previous fitness studies related to BABA-induced priming did not include time courses (Van Hulten et al., 2006), but it would be interesting to verify in BABA-treated plants whether priming interferes with the growth depression and compensation phenotype observed in pathogen-inoculated plants.

Non-treated, field-grown *edr1* and *jin1* displayed reduced and enhanced growth phenotypes, respectively, when 4 weeks old (Fig. 7A), which was also observed under controlled growth conditions (Frye and Innes, 1998; Van Hulten et al., 2006) (for *jin1-2* data not shown). Drought stress to which *edr1* is known to be extra

sensitive may have contributed to the reduced size of *edr1* in the field (Tang et al., 2005). The differential growth rate of the mutants seems not related to priming for disease resistance, because the induced defense responses and the resistance level were similar as in Col-0 in the field (Fig. 4-6).

Growth compensation for damage has been described extensively in the context of herbivory (Trumble et al., 1993). But also compensation for growth depression caused by treatment with the defense-inducing SA analogue BTH has been demonstrated (Dietrich et al., 2005). In our field experiment, the depression in growth in the first week after pathogen challenge is possibly caused by allocation of limited resources from the primary metabolism of the plant to the synthesis of defense-related compounds. Upon pathogen attack there is a large transcriptional and metabolic reprogramming in the plant (Hahlbrock et al., 2003); defense-related gene expression is upregulated, while simultaneously photosynthesis is downregulated (Berger et al., 2007). The growth compensation that we observed following the growth depression is likely associated with the transient nature of pathogen-induced defense responses, freeing up resources for plant growth when defense responses go down again. Compensatory growth in plants attacked by herbivores is in general achieved through increases in photosynthesis, nutrient uptake, utilization of stored resources and activation of dormant meristems (Tiffin, 2000). More detailed study is necessary to elucidate the mechanisms behind the compensatory growth observed in pathogen-treated plants.

The biotrophic pathogen *H. arabidopsidis* increases seed production in infected plants

An unexpected result that was retrieved from the field experiment was the increase in production of seed in Col-0 and *jin1* upon infection with *H. arabidopsidis* (Fig. 8). Attempts to uncover this phenomenon in controlled conditions revealed that the period in which Arabidopsis develops new flowers is extended in *H. arabidopsidis*-inoculated plants. As a biotroph, *H. arabidopsidis* relies on a living host and suppresses defense responses and cell death (Slusarenko and Schlaich, 2003; De Jong and Van den Ackerveken, 2009; Baxter et al., 2010). It is well possible that it is also able to suppress senescence and, by doing so, increases proliferation time in the plant, creating the ability to produce more spores. Remarkably, no statistical increase in seed weight upon *H. arabidopsidis* infection was observed in *edr1*, although a trend was visible (Fig. 8). Mutant *edr1* is known to display enhanced senescence upon ET application (Frye and Innes, 1998; Tang et al., 2005), which may affect the period of flower development and thus seed set. It can be that a delay of senescence can not be

achieved in the *edr1* mutant by the pathogen, since it is already enhanced sensitive to display senescence. It would be interesting to know how *H. arabidopsidis* is able to extend time untill plant senescence, something worthwhile to investigate in future research. The fact that the benificial effect of *H. arabidopsidis* on seed set was only detected when the plants had received an extended watering period suggests that *H. arabidopsidis* prolonged the seed set period and thus an enhancement in seed production could only ensue when the plants were allowed enough time to develop seeds, a situation that had occurred also in the field experiment. It is tempting to speculate that the biotrophic downy mildew pathogen *H. arabidopsidis* benefits from delaying plant senescence, causing the plant to live longer and consequently set more seeds.

Conclusions

It is conceivable that Arabidopsis plants may have acquired a primed defensive capacity to fend off pathogens under the conditions of this field experiment. This raises the question whether the priming phenomenon can only be observed under optimal laboratory conditions. However, the fact that the primed state is an inducible physiological state indicates that the default status of a plant is non-primed. It would be challenging to investigate whether or not plants grown in their natural habitat, in which they co-evolved with indigenous micro-organisms, acquire the primed state as well. The presumably acquired priming of Arabidopsis plants under our field conditions suggests that priming may be an important trait for plants to adapt to their environment. It is well possible that in certain Arabidopsis accessions that grow in environments with high disease pressure a primed capacity to respond to SA and/or JA has naturally been evolved. Recently, Ahmad and co-workers provided evidence for naturally occurring variation in primed responsiveness of distinctly regulated basal defence mechanisms (Ahmad et al., 2010; Ahmad et al., 2011). It would be interesting to test whether this natural variation correlates with the ability to deal with pathogens under field conditions.

MATERIALS AND METHODS

Cultivation of pathogens and plant genotypes

Hyaloperonospora arabidopsidis WACO9 was maintained on susceptible Ws-0 plants by weekly transfer to healthy 14-day-old seedlings as described by Koch and Slusarenko (1990). Sporangia were obtained by washing diseased leaves in demineralized water.

Debris was filtered out using Miracloth (Merck) and the spores were resuspended in demineralized water to a final density of 5 x 10^4 sporangia/mL.

Plectosphaerella cucumerina, isolated from infected Arabidopsis, was grown on 19.5 g/L potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) plates for two weeks at 22°C. Spores were harvested as described by Van Wees *et al.* (2003), and resuspended in half-strength Potato Dextrose Broth (PDB) to a final density of 5 x 10^6 spores/mL.

Arabidopsis thaliana accession Col-0 and the mutants *edr1* (Frye and Innes, 1998) and *jin1-2* (Lorenzo et al., 2004), which are both mutants in the Col-0 background, were cultivated as described below for the different types of experiments.

SA and MeJA treatments and Northern blot analysis

Arabidopsis seeds were sown on quartz sand and, after two weeks of growth, seedlings were transplanted into 60-mL pots containing a potting soil-sand mixture (12:5 v/v) that had been autoclaved twice for 1 h with a 24-h interval. Plants were cultivated in a growth chamber with an 8-h day (200 μE m^{-2} s^{-1} at 24°C) and 16-h night (20°C) cycle at 70% relative humidity (RH) for another 3 weeks. Five-week-old plants were treated with SA (Malinkrodt Baker, Deventer, the Netherlands) or MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands) by dipping the rosettes in a solution, supplemented with 0.02% Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands). The MeJA solution was prepared from a 100 mM MeJA stock solution in 96% ethanol. Mock treatment consisted of dipping the leaves in 0.02% Silwet L-77, amended with 0.1% ethanol, which equals the ethanol concentration in the 100 μM MeJA solution. Plants were placed at 100% RH after the chemical treatments. Leaves from 3-5 plants per sample were harvested at 6 h and 24 h after treatments. RNA extraction, Northern blotting, labeling of specific probes for *PR-1* and *PDF1.2*, and blot hybridization were performed as described previously (Leon-Reyes et al., 2010b). After hybridization with α[^{32}P]-dCTP-labeled probes, blots were exposed for autodiography and signals were quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, the Netherlands) with corresponding software.

P. cucumerina bioassays

Five-week-old plants were challenge inoculated with *P. cucumerina* by applying 5-μL droplets containing 5 x 10^6 spores/mL potato dextrose broth (PDB) to 5-8 fully expanded leaves and plants were maintained at 100% RH. Disease symptoms were evaluated at 7 days after inoculation.

Field experiment

Seeds (800 per genotype) were imbibed in 0.1% agar at 4°C for 3 days. Subsequently, they were sown on non-autoclaved potting soil-sand mixture (12:5) in trays consisting of 200 cells (Teku Seedling Tray cat. no. JP3050/230) so that each cell contained one seed. Plants were kept in a greenhouse compartment with an 8-h day (24°C) and 16-h night (20°C) cycle at 100% RH till germination. After germination, plants were cultivated in the same compartment at 70% RH. Two-week-old seedlings were placed in an uncontrolled plastic semi-dome greenhouse to acclimatize for 5 days. On 15 April 2008 the seedlings, that possessed 4 true leaves, were transplanted to a field site near Utrecht, the Netherlands (Latitude 52° 5'23.88"N, Longitude 5°10'21.70"E). The seedlings were transplanted with an intact root system surrounded by ~ 1 cm^3 of the original potting soil-sand mixture. A total of 37 plants/genotype/plot were randomly divided over 11 different plots, which were positioned in a random block design. Plants were spaced 3.9 cm apart within each plot and plots were spaced 30 cm apart in two rows which were spaced 150 cm apart. To protect the plants from slugs, Escar-Go® pellets (ecostyle) were scattered between the plots at the beginning of the trial. Shade was provided by maze nettings placed at 1.5 meter above the plants. Plants were watered regularly (three times per week on average). Plants were not protected by active management from insect herbivores or pathogens.

On 25 April 2008 all plants within each plot were sprayed with water, 5 x 10^4 *H. arabidopsidis* sporangia/mL (4 plots), or 5 x 10^6 *P. cucumerina* spores/mL (3 plots), using an artist paintbrush. To allow infection by the pathogens the plants were kept at 100% RH for 48 h by enclosing the plants with see-through lids. Duplicate or triplicate samples of plant material were harvested for gene expression analysis, each consisting of 4 plants per sample, at 0, 2, 4 and 7 days after treatment. Rosette diameters were measured from pictures that had been taken at 0, 7 and 13 days after inoculation. To this end, two opposing longitudinal measurements were taken of each rosette and averaged using Adobe® Photoshop 7.0. On-picture rulers were used to convert measured pixels to realistic centimeters. On 3 June 2008 inflorescences were harvested to determine seed set and left to dry in a paper bag for 3 months. Seeds were weighed on a microbalance (Sartorius AC211P) with a 0.0001 g resolution.

Deposition of callose

H. arabidopsidis-inoculated plants were harvested for callose staining at 2 days after inoculation (n=30). Callose staining was performed as described previously (Ton et al., 2005). Two days after inoculation, leaves were harvested and incubated overnight in 96% ethanol. Subsequently, the leaves were washed in 0.07 M phosphate buffer pH 9, incubated for 15 min in 0.07 M phosphate buffer containing 0.005% Calcofluor

(fluorescent brightner; Sigma-Aldrich Chemie BV. Zwijndrecht, the Netherlands) and 0.01% aniline-blue (water blue; Fluka, Buchs, Switzerland), and then incubated overnight in 0.07 M phosphate buffer containing 0.01% aniline-blue. Leaves were analyzed by epifluorescence microscopy using a UV filter. Callose depositions were quantified as the proportion of callose-inducing sporangia per infected leaf.

Reverse Transcription quantitative PCR (RT-qPCR)

Total RNA was obtained by phenol/chloroform extraction and LiCl precipitation, as described by (Sambrook et al., 1989). Five μg of RNA was digested with Turbo DNA-freeTM (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. RNA was checked for genomic DNA contamination by PCR. Subsequently, DNA-free total RNA was converted into cDNA using oligo-dT20 primers (Invitrogen, Breda, the Netherlands), dNTPs, and SuperScriptTM III Reverse Transcriptase (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Prior to RT-qPCR analysis of the genes of interest, all cDNA samples were normalized to a Ct value (threshold cycle) of 21±0.5 of the constitutively expressed reference gene *At1g13320* (Czechowski et al., 2005).

PCR reactions were performed in optical 384-well plates (Applied Biosystems) with an ABI PRISM® 7900 HT sequence detection system, using SYBR® Green to monitor the synthesis of double-stranded DNA. The qPCR analysis was performed by mixing 1 μl of cDNA with 5 μl 2x SYBR® Green Master Mix reagent (Applied Biosystems) and 200 nM of a gene-specific primer pair in a total volume of 10 μl. Gene-specific primers for *PR-1* (At2g14610), *PR-5* (At1g75040), *PDF1.2* (At5g44420), *VSP2* (At5g24770) and *LOX2* (At3g45140) were used (Van der Ent et al., 2009c). Relative abundance of *P. cucumerina* in plants was determined by RT-qPCR using *P. cucumerina* ß-*Tubulin*-specific primers *Pcßtub* Rv 5'-CAAGT ATGTT CCCCG AGCCG T-3' (Sanchez-Rodriguez et al., 2009) and *Pcßtub* Fw 5'-TGTCA GGACG GAAGA GCTG-3'. Relative abundance of *H. arabidopsidis* in plants was determined by RT-qPCR using *H. arabidopsidis Actin2*-specific primers *HpACT* Fw 5'-GTGTC GCACA CTGTA CCCAT TTAT-3' and Rv 5'-ATCTT CATCA TGTAG TCGGT CAAG T-3' (Huibers et al., 2009).

For each reaction two technical replicas were analyzed. A standard thermal profile was used for all RT-qPCR reactions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60°C to 95°C with a ramp speed of 1.9°C min-1. ΔCt values were calculated relative to the reference gene *At1g13320* (Czechowski et al., 2005).

Fitness studies under controlled growth chamber conditions

For determining consequences of pathogen infection on radial rosette growth, 3-week-old Col-0 plants were spray-inoculated with *H. arabidopsidis* or *P. cucumerina* in a similar way as in the field. Plants were kept at 15°C during the whole experiment, which approaches average day temperatures reached during the field trial. A 12-h day (200 μE m^{-2} s^{-1}) and 12-h night cycle at 70% relative humidity was used. After challenge inoculation, plants were maintained at 100% RH Rosette diameters were determined in a similar way as in the field trial.

To determine the consequence of *H. arabidopsidis* infection on seed production, Col-0 plants were sprayed weekly with a spore suspension (5 x 10^4 spores/mL) or water when the plants were between 3 and 5 weeks of age. After treatment, the plants were maintained at 100% RH for 48 h to allow the pathogen to infect the plants. When five weeks old, plants were moved to long-day conditions (16-h day, 8-h night cycle) to induce flowering. While in one of the trays watering was seized when first siliques yellowed and opened, the other tray was watered for a subsequent two weeks. After 12 weeks seeds were harvested and weighed on a on a microbalance (Sartorius AC211P) with a 0.0001 g resolution.

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CHAPTER 6

Summarizing discussion

Differential regulation of plant defense responses to health-threatening attackers

Like all living organisms, plants encounter numerous different stresses, which often come about unexpectedly, and therefore flexibility in responsiveness to stresses is necessary to maximize chances of survival. To achieve this flexibility, the plant has evolved receptors that can be activated by specific stimuli leading to initiation of stress signaling pathways, which subsequently results in activation of appropriate responses. In this way, the plant does not need to waste energy on constitutive activation of responses that are superfluous at certain situations. Instead, the plant responds properly to a given stress by inducing protective responses when it needs to. Plants cope with abiotic stresses like cold and drought, but as photoautotrophic organisms, plants also have to cope with numerous deleterious organisms that are depending on plant nutrients for their demand of energy. The overwhelming number of plants on our globe proves that plants can successfully deal with diverse stresses.

A vast number of plant species are exploited by men to provide food, feed and consumables. Plant quality is very important for this exploitation and can be spoiled by diseases and plaques. Plant health has therefore continuously received attention of farmers, breeders and plant scientists. Expanding fundamental knowledge on regulation of plant defense responses and the impact it has on resistance against different attackers may help to engineer more resistant crops in the future.

Plant defense responses are orchestrated by different plant hormones. Each hormone is capable of initiating several defense responses in the plant, but it is the signal signature of the combined hormonal blend that determines the final outcome of activated defenses (Pieterse et al., 2009; Verhage et al., 2010). In line with this, we previously demonstrated that in *Arabidopsis thaliana* (Arabidopsis), depending on the feeding lifestyle of the attacker, certain defense responses are activated, coinciding with induction of specific blends of phytohormones (De Vos et al., 2005). Because of this, differential sets of genes activated or repressed upon attack by herbivorous insects and necrotrophic fungi that are mostly defended by the plant through defenses regulated by jasmonic acid (JA), show only marginal overlap (De Vos et al., 2005). Also in other studies, the nature of the JA response was demonstrated to be highly dependent on the context in which it is induced (Pauwels et al., 2009). It is generally thought that in this way, defense responses can be specified to be most detrimental towards the attacker encountered. However, during the continuous arms-race between the plant and its attackers, the latter have also found ways to interfere with the activated defense response by diverting the response through e.g. interference with the hormonal blend produced (reviewed in Robert-Seilaniantz et al., 2007; López et al., 2008; Grant and Jones, 2009).

From our previous studies on the signal signature and transcriptome changes induced by different attackers of Arabidopsis, it became clear that the JA-dependent transcriptional changes induced upon feeding by caterpillars of small cabbage white (*Pieris rapae*) was significantly different from that observed upon infection by the JA-inducing necrotrophic fungus *Alternaria brassicicola,* even though both attackers induced the production of significant amounts of JA (De Vos et al., 2005). In both cases approximately 200 genes showed a significant change in gene expression, the majority of which in a JA-dependent manner. However, the overlap was only marginal. *P. rapae* induced a branch of the JA signaling pathway that is associated with expression of the JA-responsive marker gene *VSP2,* whereas *A. brassicicola* triggered a branch of the JA pathway that is associated with expression of the JA-responsive marker gene *PDF1.2.* We previously gained important insights into how Arabidopsis is able to deal with beneficial microbes, pathogens, and insect herbivores. Together with our collaborators at the Entomology laboratory of the Wageningen University, we used caterpillars of *P. rapae* served as important insect herbivores to study plant-insect interactions (De Vos et al., 2005; De Vos et al., 2006b; De Vos et al., 2006a; De Vos et al., 2007; Pieterse and Dicke, 2007; Van Oosten et al., 2008; Pineda et al., 2010). The PhD research described in this thesis aimed to investigate the biological and molecular basis of the differential regulation of the JA response as observed upon pathogen and insect attack. To this end, we further explored the interaction between Arabidopsis and *P. rapae* as a starting point and asked the following research questions:

- What is the molecular basis of the differential JA response as observed during the Arabidopsis-*P. rapae* interaction?

- What is the role of hormone crosstalk in the differential JA response?

- What is the biological relevance of differentially regulating the JA response upon insect herbivory? Is it a mechanism to enhance resistance? Or is it a trick of the herbivore to avoid effectual defenses?

- What is the spatial scale of the observed differential JA response? Is it also expressed systemically throughout the plant?

- How does interference with regulators of defense affect the performance of plants in the field?

MYC2 as a master regulator of the differential JA response

Previous research pinpointed the transcriptional regulator MYC2 as crucial in steering the JA-dependent response. This basic helix-loop-helix leucine zipper-type transcription factor (TF) controls one of two branches of the JA signaling pathway that have been implicated in defense to pathogen and insect attack (Anderson et al., 2004; Lorenzo et al., 2004; Lorenzo and Solano, 2005; Dombrecht et al., 2007; Kazan and Manners, 2008; Fernandez-Calvo et al., 2011). The MYC2-branch of the JA pathway is associated with the wound-response and is thought to contribute to defense against insect herbivores (Lorenzo et al., 2004; Dombrecht et al., 2007). The MYC2-branch regulates transcription of a large set of JA-responsive genes (Dombrecht et al., 2007), of which the insect resistance-related *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Liu et al., 2005) serves as a marker (Memelink, 2009). Activation of the MYC2-branch of the JA pathway is often associated with a concomitant suppression of the ERF-branch of JA defense, which is controlled by APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain transcription factors, such as ERF1 and ORA59 (Lorenzo et al., 2004; Pré et al., 2008). The ERF-branch is implicated in defense against pathogens with a necrotrophic lifestyle and is associated with transcription of pathogen resistance-related *PLANT DEFENSIN2.1* (*PDF1.2*) (Berrocal-Lobo et al., 2002; Lorenzo et al., 2004; Pré et al., 2008).

Because of the important role of TF MYC2 in steering the JA defense response, *jin1* mutants that are impaired in MYC2 function respond in a different way not only to exogenous application of JA than do wild-type plants (Lorenzo et al., 2004), but also to pathogen infection (Anderson et al., 2004; Lorenzo et al., 2004) and to insect herbivory by e.g. *P. rapae* (Chapter 2). As a result of the lack of a major repressor of the ERF-branch of the JA pathway in *jin1* mutants, this branch is activated faster and stronger upon pathogen attack, resulting in primed activation of ERF-regulated genes and enhanced resistance against necrotrophic pathogens such as *Botrytis cinerea* and *Plectosphaerella cucumerina* (Lorenzo et al., 2004). This phenomenon was also found in our lab and exploited to study the effect of primed defense responses on plant fitness in the field (Chapter 5), which will be discussed later.

In Chapter 2 we show that, while wild-type Col-0 plants respond to *P. rapae* feeding by activating the MYC2-branch of the JA pathway, mutant *jin1* plants strongly activate the ERF-branch. In wild-type plants, this may mean that the plant actively responds to herbivory by prioritizing the MYC2-branch of the JA pathway because this branch may be most detrimental for the herbivores. It may also mean that the specialist herbivore has found a way to interfere with the direction of the JA

response by activating and exploiting the negative regulatory function of MYC2 on the ERF-branch to avoid effectual defenses that could be regulated by the ERF-branch. Mutations in the *MYC2* gene did not translate into changes in the performance of *P. rapae* larvae when growing on either wild-type Col-0 or MYC2-mutant *jin1* plants. However, in two-choice assays, *P. rapae* larvae showed a significant preference for *jin1* plants and other Arabidopsis transgenic genotypes that activated the ERF-branch upon insect feeding. This strongly indicates that activation of the MYC2-branch in wild-type plants is beneficial for the plant, and not a tactic of the insect herbivore to suppress effectual defenses. Interestingly, we found that oral secretions of the caterpillars have the potential to activate the ERF-branch of the JA pathway when applied to mechanically inflicted wounds. This suggests that compounds in the oral secretion of *P. rapae* have the potential to manipulate the plant response toward the caterpillar-preferred ERF-regulated branch of the JA response. However, by activating the MYC2-branch of the JA pathway, plants prevent stimulation of the ERF-branch by the herbivore, thereby becoming less attractive to the attacker. We thus conclude that by prioritizing the MYC2-branch of the JA pathway over the ERF-branch, Arabidopsis plants rewire the JA signaling pathway away from the *P. rapae*-preferred ERF-branch of the JA pathway, possibly to minimize attractiveness of the leaf tissue for insect feeding.

Recent evidence that activation of the MYC2-branch is detrimental to grazing caterpillars, whereas the ERF-branch is not, comes from a study on MYC2-homologs MYC3 and MYC4 and the role of these three TFs in defenses activated by the generalist herbivore *Spodoptera littoralis*. The triple mutant *myc2 myc3 myc4* showed transcript levels of *PDF1.2* that largely exceeded the levels in wild-type plants, indicative of a strongly activated ERF-branch, which was associated with increased performance of the larvae of *S. littoralis* to a similar extent as was observed on JA-insensitive *coi1-1* mutant plants (Fernandez-Calvo et al., 2011). This observation underlines that the branch of the JA-pathway that is regulated by MYC-type TFs regulates defenses affect the performance of insect herbivores, whereas the ERF-branch of the JA pathway seems to have little effect. The fact that the performance of *P. rapae* was not affected on MYC2-impaired *jin1* plants in our study may be caused by the fact that *P. rapae* is a specialist herbivore and highly adapted to defenses of brassicaceous plant species. Our observation that activation of the ERF-branch of the JA pathway actually stimulates *P. rapae* larvae to feed from this tissue adds another layer of complexity to the molecular interplay between plants and insect herbivores. In Arabidopsis accession Col-0 the MYC2-branch of the JA pathway dominates over the ERF-branch upon insect feeding. However, we recently demonstrated that there is significant natural genetic variation in Arabidopsis in terms of shifting the balance between the MYC2- and the ERF-branch of the JA pathway (Ahmad et al., 2011). Hence, it would be interesting

to investigate whether this balance plays a role in insect resistance in nature by studying in detail the relation between activation of the MYC2- and ERF-branch and insect resistance in the large set of Arabidopsis accessions that is available to the Arabidopsis community.

The role of the hormonal signature during herbivory in the differential regulation of the JA response

To further understand how the plant switches on the MYC2-branch of the JA pathway in response to *P. rapae* feeding, while suppressing the ERF-branch, a closer look was taken into the hormonal signature that is produced in the plant upon caterpillar feeding. To this end, a selection of hormones that are known modulators of the JA response were chosen for examination (Chapter 3): The JA precursor 12-oxo-phytodienoic acid (OPDA), which is the first cyclic pre-form of JA and has been shown to have the capacity to autoregulate a specific set of genes (Stintzi and Browse, 2000; Taki et al., 2005; Bottcher and Pollmann, 2009); JA, which is the first formed bioactive jasmonate; (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), which has been shown to be the most bioactive isoform of JA (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008; Yan et al., 2009); ABA, which is a co-regulator of the MYC2-branch of the JA pathway (Anderson et al., 2004); salicylic acid (SA), which is known to antagonize the JA signaling pathway in general (Koornneef et al., 2008; Koornneef and Pieterse, 2008; Pieterse et al., 2009); and ET, which has been shown to be a co-regulator of the ERF-branch of the JA pathway (Lorenzo et al., 2003; Pré et al., 2008; Memelink, 2009). In response to herbivory by *P. rapae*, increased levels of OPDA, JA, JA-Ile and also ABA were observed, whereas SA and ET levels remained unaltered. Remarkably, ABA levels were not increased in damaged tissue of *jin1*-7 mutant plants, suggesting that MYC2 plays an important regulatory role in the production of ABA during *P. rapae* feeding.

Mutant and pharmacological analysis of the role of ABA in the differential regulation of the JA response during *P. rapae* feeding revealed that ABA is not only an essential co-activator of the MYC2-branch of the JA pathway, it is also required for repression of the ERF-branch in *P. rapae*-infested plants (Chapter 3). Conversely, exogenous application of ET stimulated the ERF-branch and repressed the MYC2-branch in *P. rapae*-infested plants, suggesting that ET potentially antagonizes the MYC2-branch of the JA pathway. However, ET is not produced by Arabidopsis Col-0 plants in response to feeding by *P. rapae*, hence this process is found not to affect the outcome of the defense response that is activated in response to *P. rapae* feeding. Together, our results indicate that during the Arabidopsis-*P. rapae* interaction, the

MYC2-branch of the JA pathway is prioritized over the herbivore-preferred ERF-branch through ABA-mediated suppression of the ERF-branch, and that ET plays no role in this interaction. These results highlight the important modulating role of ABA in the hormone-regulated defense signaling network in plants.

Previously, Erb and colleagues (2009) reported that feeding on maize roots by the Western corn rootworm *Diabrotica virgifera virgifera* causes profound increases in ABA levels in aboveground tissues, whereas mechanical removal of a comparable amount of root tissue did not cause a rise in ABA levels. Although less profound, a significant increase in ABA levels was also observed upon feeding by *S. littoralis* in the same study. Also Reymond and colleagues (Bodenhausen and Reymond, 2007) found evidence for a role of ABA in defense against *P. rapae* feeding. They found significantly induced changes in the expression of a set of ABA-responsive genes upon *P. rapae* feeding. Moreover, they demonstrated that the generalist herbivore *S. littoralis* performed significantly better on the ABA biosynthesis mutant *aba2-1*. Together, these findings suggest that insect herbivory can lead to enhanced ABA biosynthesis. Our data pinpoint ABA as an important modulator of the MYC2/ERF-controlled differential JA response that shapes the final outcome of the *P. rapae*-induced JA-dependent defense response.

Systemic effects on the differential JA response

Previous evidence from our lab revealed that herbivory by *P. rapae* can induce resistance in systemic tissues to protect undamaged tissues against future encounter with malicious organisms. Plants previously infested with *P. rapae* larvae displayed enhanced resistance, not only against secondary attack by *P. rapae*, but also against infection by *turnip crinkle virus* (TCV) (De Vos et al., 2006a). The observed increase in resistance can be due to direct induction of defense genes in non-damaged tissues of an infested plant, but systemic priming can also be part of the increased resistance observed. In Chapter 4 we approached this question by monitoring activation of the JA-dependent defense marker genes *VSP2* and *PDF1.2*. Both genes were marginally or not at all activated in systemic tissues, suggesting that direct activation of systemic defenses is unlikely to play a role in herbivory-induced systemic resistance. However, *MYC2* mRNA levels increased to similar levels in systemic leaves as it did in damaged leaves, apparently without activating downstream target genes such as *VSP2*. This points to a model in which herbivory leads to priming of the MYC2-branch of the JA pathway in systemic undamaged leaves, without fully activating costly JA-dependent defenses. This primed state may lead to accelerated activation of MYC2-dependent defenses when systemic tissue is attacked by insect herbivores.

Activation of MYC2 followed by downstream activation of genes such as *VSP2* is dependent on preceeding degradation of JAZ repressor proteins, which involves increase in levels of JA-Ile (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008; Yan et al., 2009). Therefore, the activation of downstream MYC2 targets might simply not have been observed because JA-Ile was not present in systemic tissues. However, this was not the case, since levels of JA and JA-Ile were remarkably similar in systemic leaves in comparison to *P. rapae*-damaged leaves. Nonetheless, the hormonal signature of local and systemic leaves differed at the level of ABA and OPDA. In contrast to damaged leaves, ABA levels did not increase in undamaged leaves of *P. rapae*-infested plants, and OPDA levels even significantly decreased (Chapter 4). The crucial role of ABA in unfolding the MYC2-branch of the JA pathway, which was supported by findings with the ABA biosynthesis mutant *aba2-1* (Chapter 3), may thus explain the observation that the MYC2-branch of the JA pathway was not activated, even though *MYC2* mRNA levels were similar to those in local, damaged leaves. In this scenario, JA and/or JA-Ile production are responsible for enhanced activation of *MYC2* in local and systemic leaves, most likely resulting in enhanced levels of MYC2 TFs in both tissues. The production of ABA in *P. rapae*-damaged plants triggers locally the expression of the MYC2-branch of the JA signaling pathway and the concomitant suppression of the ERF-branch; systemically, ABA levels do not increase and therefore no effect on activity of the MYC2- or ERF-branch can be observed. Nevertheless, the enhanced pool of MYC2 TFs may prime the systemic tissues for accelerated expression of genes in the MYC2-branch of the JA pathway once these tissues are attacked by *P. rapae* or another (ABA-inducing) attacker.

Alternatively, the decreased OPDA levels in systemic tissues may have had an effect on transcriptional activity of JA-responsive genes by affecting nuclear calcium levels (Walter et al., 2007) or nucleophiles (Bottcher and Pollmann, 2009), which are both phenomena demonstrated to be specifically associated with OPDA function. Nevertheless, other factors that were beyond the scope of our research may also play a role in the systemic response.

Fitness benefits of priming under field conditions

The tentative role of MYC2 in priming for enhanced herbivore-induced defenses in systemic tissues, resembles the role of MYC2 in priming for enhanced defenses during rhizobacteria-induced systemic resistance (ISR) (Pozo et al., 2008; Van Wees et al., 2008; Van der Ent et al., 2009a). That TFs can play an important role in priming for enhanced defense was also shown in a study on transcriptional changes

upon treatment of plants with the priming-inducing agents *Pseudomonas fluorescens* WCS417r and the nonprotein amino acid β-aminobutyric acid (BABA). Although primed plants did not display direct activation of defense-related genes, a large set of genes encoding TFs were shown to be significantly induced (Van der Ent et al., 2009c). It was hypothesized that an increased pool of latent TFs brings the plant tissue in an enhanced state of readiness, and allows an accelerated activation of cellular defense responses once the primed tissue is attacked by a pathogen or an insect herbivore. Although MYC2-impaired *jin1* plants are blocked in priming induced by rhizobacteria, the ERF-branch is de-repressed in this mutant resulting in a faster and stronger activation of JA-responsive genes that are regulated via the ERF-branch after attack by JA-inducing pathogens or insects. As a result, *jin1* plants are more resistant to pathogens with a necrotrophic life style (Lorenzo et al., 2004). The priming phenomenon is predominantly studied in plants that are grown under controlled laboratory or climate chamber conditions. Hence, an important question that arises in research on priming is whether the ability to achieve a primed state is also present in plants growing in nature. Controlled laboratory conditions may be merely free of stress, keeping levels of defense unnaturally low. The variable and more hostile conditions in the field may sensitize the defense potential of the plant, making field-grown plants constitutively primed.

To investigate the significance of priming in the field, we subjected wild-type Col-0 plants and *jin1* mutants that are primed for the ERF-branch of the JA pathway to field conditions. To investigate whether increase in disease pressure would influence costs and benefits of this primed response, a subset of the plots was treated with the necrotrophic fungus *Plectosphaerella cucumerina*. Apart from *jin1*, also mutant *edr1* was used for studying the costs and benefits of priming under natural conditions. Mutant *edr1* also shows a primed response for defense, but instead of JA-dependent defenses, SA-dependent defenses are primed in this mutant (Van Hulten et al., 2006; Chapter 5), resulting in increased resistance levels against biotrophic pathogens (Frye and Innes, 1998; Van Hulten et al., 2006). The costs and benefits of the primed response of *edr1* in the field was investigated by treating the plants with the biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis.*

Mutant e*dr1* is slightly more sensitive to abiotic stress (Wawrzynska et al., 2008). In our field trial, we observed smaller rosette diameters of *edr1* plants throughout the experiment, suggesting that a certain level of abiotic stress affected the plants. In our field trial, Col-0, *jin1* and *edr1* plants showed similar levels of resistance to *P. cucumerina* and *H. arabidopsidis,* which is in contrast to plants grown under controlled climate chamber conditions where mutants *jin1* and *edr1* showed enhanced resistance to *P. cucumerina* and *H. arabidopsidis,* respectively. Moreover, expression of the JA-marker gene *PDF1.2* and the SA-marker gene *PR-1* was not enhanced in the

mutants compared to wild type after pathogen infection. This suggests that wild-type plants were already primed for enhanced defense when growing in the field, resulting in little or no differences in the level of resistance in comparison to the constitutively primed mutants. On the other hand, disease pressure was rather low in the field and disease symptoms were hardly observed (Chapter 5). Whether this was due to a reduced ability of the pathogens to infect under the field conditions or whether increased plant resistance was induced by other factors, such as abiotic stresses, remains to be solved.

Natural outbreak of pathogens or pests during a field trial with wild-type and primed genotypes or plants treated with priming agents might shed more light on the existence of priming in the field. In line with this, chemical induction of SAR was shown to increase resistance of Arabidopsis plants in the field against natural-occurring bacterial infections (Traw et al., 2007). Also chemically-induced priming by BABA results in enhanced disease resistance in the field (reviewed in Beckers and Conrath, 2007). However, priming chemicals have been shown to induce direct defenses when too high levels are applied (Kohler et al., 2002; Van Hulten et al., 2006), which makes it hard to conclude whether the observed increase in protection can be attributed to priming or direct induced defenses. Priming is effective if disease occurrence is uncertain. If plants face constant threat by certain pathogens in their natural environment, the levels of transcriptional activators may become adapted in such a way that the primed state is incorporated into basal defense (reviewed in Ahmad et al., 2010). This existence of such a phenomenon may be uncovered in a suite of accessions obtained from different growing habitats with specific environmental conditions that favor specific pathogens.

An unexpected result that was extracted from the field trial was the flexibility of Arabidopsis plants to compensate for pathogen-induced growth depression in the weeks after the infection. Surprisingly, infection by biotrophic pathogen *H. arabidopsidis* had a positive effect on seed production in the last stages of plant growth, which is likely caused by a delay in senescence under suitable conditions. Both phenomena that we observed in the field were reproducible in the lab and provide a basis for further ecological studies on the dynamics and flexibility of the plant's immune system in nature.

Concluding remarks

In nature, plants have to cope with a wide variety of biotic and abiotic stress conditions that frequently occur simultaneously in different combinations for

varying duration, and greatly affect plant growth. This especially impacts current agriculture, where diverse ecosystems have been replaced with monocultures that are much more vulnerable to changing climatic conditions and evolving biotic stresses. Despite crop protection measures, global losses in agriculture due to pathogens and pests are estimated at 25-40% for the major food and cash crops, which represents a value of over €500 billion worldwide. In the context of climate change, it is highly likely that the frequency and complexity of (a)biotic stress conditions will increase and further threaten the ecological balance in ecosystems and the performance of crops in the field. This urgently demands the development of novel strategies for sustainable crop protection, which imposes a major challenge to the plant science research community.

The evolutionary arms race between plants and their enemies provided plants with a highly sophisticated defense system. Recent advances in plant immunity research underpin the pivotal role of cross-communicating hormones in the regulation of the plant's defense signaling network (Rojo et al., 1999; Robert-Seilaniantz et al., 2007; Koornneef and Pieterse, 2008; López et al., 2008; Navarro et al., 2008; Spoel and Dong, 2008; Bari and Jones, 2009; Grant and Jones, 2009; Pieterse et al., 2009; Verhage et al., 2010). Their powerful regulatory potential allows the plant to quickly adapt to its hostile environment and to utilize its resources in a cost-efficient manner. Specialized plant enemies on the other hand, developed ingenious mechanisms to promote susceptibility to pathogens and pests, e.g. by affecting hormone homeostasis to antagonize the host immune response, thereby imposing new threats to the plant (Grant and Jones, 2009). In this PhD research, we zoomed in on the differential regulation of the MYC2- and the ERF-branch of the JA signaling pathway that is activated in response to insect herbivory and infection by necrotrophic pathogens, respectively. We demonstrated that elicitors in the oral secretion of *P. rapae* potentially steer the JA response towards the insect-preferred ERF-branch of the JA pathway. However, herbivore-induced ABA plays a decisive role in rewiring the JA response towards the MYC2-branch of the JA signaling pathway, thereby suppressing the insect-preferred ERF-branch of the pathway. We also demonstrated that in systemic undamaged tissues of herbivore-infested plants, the MYC2-branch of the JA pathway becomes primed, which may lead to enhanced expression of effectual defenses when these tissues become infested by insect herbivores. Finally, we put priming for enhanced defenses to the test in the field. We demonstrated that field-grown wild-type plants may be constitutively primed, e.g. due to environmental stimulation. Moreover, we gained insight into the dynamics and flexibility of the plant's immune system under field conditions.

In nature, plants simultaneously or sequentially interact with multiple beneficial and antagonistic organisms with very different lifestyles. In many cases, the adaptive

responses that are triggered upon recognition of these alien organisms are regulated by plant hormones. However, knowledge on how the hormone-regulated plant immune signaling network functions during multi-species interactions is still in its infancy. Hence, a detailed analysis of the molecular mechanisms and biological significance of hormonal crosstalk in plant defense, such as performed in this PhD research, will foster important new insights into how plants selectively adapt to the (combined) effects of biotic stresses. Because of the high level of complexity of regulatory mechanisms in the hormone-regulated defense signaling network, the bioinformatics and systems biology tools that become increasingly available (Volodarsky et al., 2009; Pitzschke and Hirt, 2010; Breeze et al., 2011) will be extremely valuable in this respect. The combination of elucidating molecular regulatory mechanisms and the ecological impact they have provides a rational basis for developing innovative approaches to sustainably increase crop production, which is necessary to meet the demand of a growing human population in a changing climate, without adversely affecting our ecological footprint.

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SUMMARY

Plants are attacked by a plethora of potentially devastating pathogens and pests. To protect themselves, plants have evolved a sophisticated immune system in which phytohormones play pivotal regulatory roles. Jasmonic acid (JA) emerged as an important hormonal regulator of defense responses that are triggered by insect herbivores and microbial pathogens with a necrotrophic life style. Although JA accumulates in response to invasion by both types of attackers, JA-dependent defenses against herbivores and necrotrophs are antagonistically regulated by different branches of the JA signaling pathway. This PhD research aimed to gain detailed insight into the molecular mechanisms and biological significance of this differential JA response, with the ultimate goal to understand how plants are able to fine-tune their immune system to survive in continuously changing and hostile environments.

In model plant *Arabidopsis thaliana* (Arabidopsis), MYC2 and ERF-type transcription factors are major players in the differential control of the two distinct branches of the JA pathway. This is nicely exemplified in Arabidopsis plants being attacked by larvae of the insect herbivore *Pieris rapae* (small cabbage white butterfly). Herbivory activates the MYC2-branch of the JA pathway, leading to concomitant suppression of the ERF-branch. Using Arabidopsis genotypes affected in MYC2- or ERF-TF functioning, we demonstrated that the insect larvae prefer to feed from plant tissues that express the ERF-branch of the JA pathway. Interestingly, factors in oral secretion of the caterpillars were shown to steer the JA pathway towards the insect-preferred ERF-branch. However, during the interaction of the insect with Arabidopsis, the MYC2/ERF balance was rewired towards the MYC2 branch, thereby suppressing the insect-preferred ERF-branch of the JA pathway. This "hide-the-candy" strategy of the plant sheds new light on the mechanisms involved in the evolutionary arms race between plants and their herbivorous enemies.

A second hormone, abscisic acid (ABA), appeared to play an important modulating role in the differential JA response. Upon insect feeding, ABA production was significantly increased. Using mutant and pharmacological approaches we demonstrated that ABA is essential for rewiring the JA pathway towards expression of the MYC2-branch, thereby preventing stimulation of the insect-preferred ERF-branch.

We also demonstrated that herbivory primed undamaged, systemic tissues for enhanced MYC2-dependent defenses, which potentially protects healthy tissues against future insect attack. In a field study, we investigated

the performance of Arabidopsis mutants that are constitutively primed for enhanced defenses. From this study we learned that plants may be primed when grown under natural conditions. Moreover, putting molecular mechanisms to the test in a field setting contributes greatly to our understanding of both the complexity and flexibility of the plant's immune system.

The detailed analysis of molecular mechanisms and biological significance of hormonal regulation of the plant immune signaling network fostered important new insights into how plants selectively adapt to the diverse biotic stresses in nature. This provides a rational basis for developing innovative approaches to sustainably increase crop production in times of increased demand and ever-growing environmental challenges.

SAMENVATTING

Planten zijn in staat energie uit licht om te zetten in suikers. Dit maakt hen een primaire bron van energie en bouwstenen voor andere organismen. In de strijd om het voortbestaan hebben planten daarom talloze mechanismen ontwikkeld om zichzelf te verweren tegen een grote verscheidenheid aan belagers (hoofdstuk 1). Het afweerproces van de plant begint met herkenning door middel van geavanceerde receptoren en resulteert uiteindelijk in een breed scala aan afweermechanismen, zoals aanmaak van proteolytische enzymen en het inkapselen van ziekteverwerkers.

Onderzoek in onder meer de modelplant *Arabidopsis thaliana* (Arabidopsis) heeft aangetoond dat plantenhormonen van essentieel belang zijn in de activatie van het afweerproces. Zo speelt het hormoon salicylzuur (SA) een belangrijke rol in de afweer tegen pathogenen met een biotrofe (plantbehoudende) levensstijl. Het hormoon jasmonzuur (JA) speelt een rol bij de afweer tegen zowel pathogenen met een necrotrofe (plantdodende) levensstijl als tegen herbivore insecten. Naast SA en JA zijn de hormonen ethyleen (ET) en abscisinezuur (ABA) belangrijke regulatoren van de uiteindelijke afweerrespons. De combinatie van de geproduceerde hormonen en dientengevolge interacties tussen de door verschillende hormonen aangeschakelde signaleringsroutes zijn bepalend voor de uitkomst van de geactiveerde afweerrespons. Het is moeilijk om te bepalen of de waargenomen respons van de plant de meest effectieve afweerreactie weergeeft. Dit komt onder andere doordat aanvallers mechanismen hebben ontwikkeld om de afweer van de plant te manipuleren, bijvoorbeeld door moleculen die de werking van planthormonen nabootsen te produceren waardoor de immuunrespons van de aangevallen plant ontregeld wordt.

In voorgaand onderzoek werd al gevonden dat elke belager een unieke afweerreactie van de plant initieert. Ook al werd bij een aanval met verschillende belagers bijvoorbeeld primair JA geproduceerd, de overlap in op JA reagerende genen die werden geactiveerd was marginaal. Om de factoren die voor deze differentiële JA respons verantwoordelijk zijn verder te onderzoeken hebben wij gekeken wat er gebeurt in een Arabidopsisplant die wordt aangevallen door één insectensoort, namelijk de rups van het kleine koolwitje (*Pieris rapae*). Dit kan dan een basis vormen voor het begrijpen van verschillen in respons ten opzichte van andere aanvallers. De rupsen van het kleine koolwitje zijn berucht om de schade die zij aanbrengen aan planten uit de koolfamilie (*Brassicacae*). Het zijn zogenaamde specialisten die zich hebben aangepast aan de afweermechanismen van de plant en vormen daarmee een grote bedreiging.

De JA-gereguleerde afweerrespons kan worden onderverdeeld in twee delen (takken). De JA-afhankelijke afweer tegen necrotrofe pathogenen wordt gereguleerd door ERF/AP2-type transcriptiefactoren (ERFs) zoals ERF1 en ORA59 en wordt positief beïnvloed door ET. Een gen coderend voor een antimicrobieel eiwit (*PDF1.2*) is door ons gebruikt als merker voor de activatie van de ERF-tak van de JA respons. De andere tak van de JA respons is betrokken bij de afweer tegen insecten en wordt, onder invloed van ABA, gereguleerd door transcriptiefactor MYC2. Een gen coderend voor een eiwit met schadelijke eigenschappen voor insecten (*VSP2*) hebben wij gebruikt als merker voor de activiteit van de MYC2-tak van de JA route. Door de nauwe antagonistische balans tussen de twee takken van de JA respons zorgen defecten in één van de twee takken voor verhoogde activatie van de andere tak zodra de JA respons wordt geactiveerd.

In wildtype Arabidopsis leidt vraat door *P. rapae* tot inductie van de MYC2-tak van de JA respons en een sterke onderdrukking van de ERF-tak. Om te onderzoeken of dit een voordeel voor de plant of voor het insect oplevert, is uitgebreid onderzoek gedaan met behulp van Arabidopsis mutanten met defecten in de MYC2-tak, zoals *jin1*. Vraat door *P. rapae* in mutant *jin1* had een verhoogde activatie van de ERF-tak tot gevolg, wat resulteerde in een voorkeur van rupsen om van deze planten te eten (hoofdstuk 2). Ook andere Arabidopsis genotypes waarin de ERF-tak van de JA respons de overhand had, kregen de voorkeur van *P. rapae*. In wildtype planten is het aanschakelen van de MYC2-tak van de JA respons dus een strategie om vraatschade door insecten te beperken. Elicitoren in spuug van rupsen bleken wel de capaciteit te hebben om de reactie van de plant zodanig te manipuleren dat de ERF-tak wordt aangeschakeld. Echter, dit bleek niet afdoende in de onderzochte Arabidopsis-insect interactie om de activatie van de MYC2-tak te onderdrukken. Verder bleek, ondanks de capaciteit van *P. rapae* om met speciale smaakpapillen glucosinolaten waar te nemen, geen verband tussen samenstelling van deze afweerstoffen en de voorkeur van de rupsen.

Omdat de hormoonsamenstelling zo bepalend is voor de afweerreactie van de plant, is onderzocht hoe deze samenstelling in door vraat verwonde planten verschilt van onbeschadigde planten. Hiertoe zijn concentraties van de JA-precursor OPDA, JA, JA-isoleucine (JA-Ile; één van de meest bioactieve vormen van JA), ABA, SA en ET gemeten. Concentraties van SA en ET verschilden niet tussen onbeschadigde en aangevreten bladeren, terwijl OPDA, JA, JA-Ile en ABA concentraties waren verhoogd (hoofdstuk 3). Opmerkelijk hierbij was dat MYC2 de productie van ABA positief bleek te beïnvloeden. Wanneer ET of ABA exogeen wordt toegediend aan planten die worden aangevreten door *P. rapae* verschuift de geactiveerde respons naar respectievelijk de ERF-tak of de MYC2-tak. Deze omleiding van de geactiveerde respons bleek onafhankelijk van respectievelijk ORA59 en MYC2. Samenvattend

kan worden geconcludeerd dat het ontbreken van ET samen met verhoogde niveaus van de hormonen JA(-Ile) en ABA zorgt voor activatie van de MYC2-tak van de JA respons tijdens vraat door *P. rapae*.

Eerder onderzoek heeft aangetoond dat wanneer een plant eenmaal is aangevreten door een insect, deze plant een sterkere afweerreactie laat zien bij een volgend incident van vraat. Om de basis van deze verhoogde resistentie te achterhalen is gekeken naar genexpressie en hormoonsamenstelling in onbeschadigde (systemische) bladeren van een aangevreten plant. De genexpressie van *MYC2* was in systemische bladeren vergelijkbaar met het niveau dat werd gevonden in aangevreten bladeren (hoofdstuk 4). Deze verhoogde niveaus resulteerden echter niet in directe activatie van de MYC2-gereguleerde JA responsen. De hormoonsamenstelling in systemische bladeren was vergelijkbaar met aangevreten bladeren, maar verhoogde niveaus van OPDA en ABA ontbraken. Doordat ABA de potentie heeft om de MYC2-tak te stimuleren en OPDA om transcriptiefactoren te activeren kan de afwezigheid van deze metabolieten een mogelijke verklaring geven voor het uitblijven van een volledige afweerrespons. Daarbij kan de verhoogde aanmaak van MYC2 duiden op een capaciteitsverhoging van afweer, een fenomeen dat priming wordt genoemd, die wordt ingezet indien ook de systemische plantendelen worden aangevallen.

Afweer kost veel energie en een plant kan er bij gebaat zijn om alleen het vermogen van de afweer te verhogen zonder de afweer volledig aan te schakelen; in deze staat zijn planten geprimed. Het wegvallen van negatieve regulatoren kan ook leiden tot priming. Zo is MYC2 mutant *jin1* geprimed voor de afweer tegen necrotrofe pathogenen omdat de ERF-tak van de JA respons niet meer wordt onderdrukt. EDR1 is een negatieve regulator van de SA respons en de *edr1* mutant is daarom geprimed voor de SA-afhankelijke afweer die effectief is tegen biotrofe pathogenen. Dit fenomeen van verhoogde resistentie en daarmee gepaard gaande intensivering van geactiveerde genexpressie zoals wordt waargenomen onder klimaatcel condities werd echter niet terug gevonden in het veld (hoofdstuk 5). Zo lieten wildtype planten in het veld eenzelfde mate van resistentie zien tegen de necrotrofe schimmel *Plectosphaerella cucumerina* en de biotrofe oomyceet *Hyaloperonospora arabidopsidis* als respectievelijk de constitutief geprimede mutanten *jin1* en *edr1*, die in klimaatcellen verhoogd resistent zijn. Onder natuurlijke omstandigheden zijn mogelijk zoveel factoren van stress aanwezig dat alle geteste planten geprimed zijn. Hierbij kan worden gedacht aan abiotische factoren zoals kou of droogte, maar bijvoorbeeld ook aan biotische factoren, zoals de aanwezigheid van wortel-koloniserende bacteriën die ook in staat zijn een plant te primen. Dit werd ondersteund door een relatief hoge mate van resistentie van alle planten en de verhoogde mate van afzetting van de afweer merker callose in wiltype planten ten opzichte van de twee mutanten. Het veldexperiment toonde verder aan dat planten de capaciteit

hebben om groeiremming die gepaard gaat met de geactiveerde afweer na infectie te compenseren in een later stadium. Daarnaast bleek infectie met *H. arabidopsidis* de zaadproductie positief te beïnvloeden, mogelijk door uitstel van het afsterven van de plant.

Al met al kon worden aangetoond dat planten een grote mate van flexibiliteit vertonen in het omgaan met ziektes en plagen in het lab en het veld. Afweer tegen herbivoren is gereguleerd door JA, waarbij een nauwe balans tussen verschillende hormonen de richting van de afweerreactie bepaalt. Dit leidt in beschadigde bladeren tot directe activatie van de MYC2-tak, terwijl in systemische bladeren het potentieel tot het vertonen van deze afweerreactie wordt verhoogd door verhoogde transcriptie van *MYC2*.Activatie van de MYC2-tak gaat gepaard met onderdrukking van de ERF-tak, die nadelig is voor de plant onder herbivoordruk. Larven vertoonden een voorkeur voor vraat op planten waarin de ERF-tak geactiveerd is. De plant bleek echter in staat tot activatie van de MYC2-tak ondanks elicitoren in het spuug, dat de potentie vertoonde juist de ERF-tak te activeren. Dit toont de veerkrachtigheid van planten om zich steeds weer aan te passen aan hun omgeving, waarin indringers voortdurend op de loer liggen. Het onderzoek beschreven in dit proefschrift draagt bij aan het inzicht in hoe het signaleringsnetwerk van het immuunsysteem van planten is gereguleerd. Het begrijpen van deze processen en de daaraan gerelateerde ecologische consequenties is essentieel in de ontwikkeling van innovatieve aanpakken om de voedselproductie in een tijd van toenemende vraag te kunnen vergroten. Dit alles met de intentie om natuurlijke ecosystemen in stand te houden en zo mogelijk zelfs uit te breiden.

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Adriaan Verhage

CURRICULUM VITAE

Adriaan Verhage werd geboren op 16 februari 1982 te Delft. In 1999 slaagde hij voor zijn HAVO aan het Erfgooierscollege te Huizen, om vervolgens in 2001 zijn VWO diploma aan het Willem de Zwijgercollege te Bussum te behalen.

Adriaan startte in 2001 met zijn studie Biologie aan de Universiteit van Utrecht en begon na de bacherlorfase aan een master `Plant Biology` welke bestond uit twee onderzoeksstages. Allereerst binnen de leerstoelgroep Fytopathologie, waar in 2004 onder leiding van Dr. Annemart Koornneef onderzoek werd verricht, getiteld: 'Preparation for a mutant screen to elucidate the mechanism underlying cross-talk between salicylic acid- and jasmonic acid-dependent defense pathways in *Arabidopsis thaliana*'. Zijn tweede onderzoeksstage werd uitgevoerd binnen het onderzoeksinstituut behorende tot het 'Consejo Superior de Investigaciones Científicas' (CSIC) aan het 'Estación Experimental del Zaidín' (EEZ) te Granada (Spanje). Dit alles onder leiding van Dr. María José Pozo Jiménez en onder externe supervisie van de Universiteit van Granada. Gedurende zes maanden werd onderzoek verricht met als titel: 'Plant defense reactions and signalling pathways involved in the arbuscular mycorrhizal symbiosis and associated induced resistance to plant pathogens'. Het genoemde werk werd gepresenteerd in 2006 op het '5th International Conference on Mycorrhiza' (ICOM5) te Granada. In augustus 2006 behaalde Adriaan zijn titel master of science (MSc.) waarna in september van dat jaar gestart werd met promotieonderzoek bij de leerstoelgroep plant-microbe interacties (voormalige leerstoelgroep fytopathologie) onder begeleiding van Prof. dr. ir. C.M.J. Pieterse en Dr. S.C.M. van Wees. Het aan dit proefschrift ten grondslag liggende onderzoek werd uitgevoerd tot september 2010. Sinds december 2010 is Adriaan werkzaam als fytopathologisch onderzoeker (specialisme nematologie) binnen het zaadbedrijf Rijk Zwaan te De Lier.

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