Unwiring jasmonic acid defense signaling

Molecular regulation and ecological costs

Irene Vos

Irene A. Vos (2015)
Unwiring jasmonic acid defense signaling – molecular regulation and ecological costs
PhD thesis, Utrecht University

Cover & layout: Roy Marsman
Published by: Uitgeverij BOXPress, 's-Hertogenbosch
ISBN: 978-94-6295-271-3

The research described in this thesis was financed by VIDI grant no. 11281 of the Dutch Technology Foundation STW, which is part of the Netherlands Organization of Scientific Research, and ERC Advanced Investigator Grant no. 269072 of the European Research Council.

Unwiring jasmonic acid defense signaling

Molecular regulation and ecological costs

Ontrafeling van de jasmonzuur gereguleerde afweersignalering

Moleculaire regulatie en ecologische kosten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op vrijdag 28 augustus 2015 des middags te 2.30 uur

door

Irene Anneke Vos

geboren op 7 februari 1987 te Renkum

Promotor: Prof. dr. ir. C.M.J. Pieterse

Copromotor: Dr. S.C.M. van Wees

CONTENTS

CHAPTER 1

General introduction: Costs and benefits of hormone-regulated plant defenses

Adapted from:
Vos IA, Pieterse CMJ & Van Wees SCM, 2013
Costs and benefits of hormone-regulated plant defences
Plant Pathology **62,** 43-55.

ABSTRACT

Plants activate defense responses to protect themselves against microbial pathogens and herbivorous insects. However, induction of defenses comes at a price, as the associated allocation costs, autotoxicity costs and ecological costs form fitness penalties. Upon pathogen or insect attack, resources are allocated to defenses instead of to plant growth and reproduction, while above- and below-ground interactions with beneficial organisms may also be disturbed. The plant hormones salicylic acid, jasmonic acid, ethylene and abscisic acid are major players in the regulation of induced defenses and their associated fitness costs. Hormone-controlled signaling pathways cross-communicate, providing the plant with a finely-tuned defense regulatory system that can contribute to a reduction of fitness costs by repressing ineffective defenses. However, this sophisticated regulatory system causes ecological costs, because activated resistance to one organism can suppress resistance to another. Moreover, the system can be hijacked by invading organisms that manipulate it for their own benefit. Priming for enhanced defense emerged as a defense mechanism with limited fitness costs. Since priming results in a faster and stronger activation of defense only after pathogen or insect attack, the limited costs of the primed state are often outweighed by the benefits in environments with pathogen or herbivore pressure. The balance between protection and fitness is crucial for a plant's success and is therefore of great interest for plant breeders and farmers. By combining molecular knowledge and ecological relevance of defense mechanisms, one can gain fundamental insight into how and why plants integrate different immune signals to cope with their natural multitrophic environment in a cost-effective manner.

INTRODUCTION

During their lifetime, plants encounter innumerable attackers, including microbial pathogens and herbivorous insects that try to retrieve nutrients from the plant. Plants can ward off the majority of these attackers for which they rely on preformed defenses and activation of their innate immune system. Preformed plant defenses include physical barriers such as thick cuticles, rigid cell walls, thorns, needles and trichomes, and chemical weapons such as toxic or repellent compounds (Osbourn, 1996). In a second line of defense, inducible defenses can be activated when pattern recognition receptors of plants recognize general features of microbial pathogens, such as flagellin, lipopolysaccharides, peptidoglycan, β-glucans and chitin, referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs; Jones & Dangl, 2006; Pel & Pieterse, 2013). Similarly, recognition of so called damage-associated molecular patterns (DAMPs), such as galacturonides, systemins and AtPep1, which are endogenous elicitors that accumulate as a result of enzymatic degradation of plant cell walls or proteins upon attack by pathogens or insects, leads to activation of defenses (Figure 1; Lotze *et al.*, 2007; Boller & Felix, 2009; Heil, 2009; Ferrari *et al.*, 2013). Other defense-inducing compounds are the herbivore-associated molecular patterns (HAMPs), such as fatty acid-amino acid conjugates (FACs) from oral secretions (Felton & Tumlinson, 2008; Mithöfer & Boland, 2008) and effectors of pathogens that are produced to suppress immune responses but that the plants, under evolutionary pressure, have learned to recognize (Jones & Dangl, 2006).

The immune response that is activated upon pathogen or insect attack is modulated by the induced production of a hormonal blend in the plant. The plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) are important regulators of induced defense mechanisms (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). Whereas SA and JA are the main players, ET and ABA have more modulating roles (Van Loon *et al.*, 2006b; Ton *et al.*, 2009). The SA pathway is primarily induced by and effective against biotrophic pathogens, whereas the JA pathway in combination with ET is primarily induced by and effective against necrotrophic pathogens and the JA pathway together with ABA is primarily induced by and effective against herbivorous insects (Figure 1 & Figure 2; Penninckx *et al.*, 1998; Glazebrook, 2005; Howe & Jander, 2008; Vos *et al.*, 2013b). The quantity, composition and timing of the hormonal signal signature tailors the defense response specifically to the attacker at hand, thereby prioritizing effective over ineffective defenses, which minimizes fitness costs (De Vos *et al.*, 2005; Pieterse & Dicke, 2007).

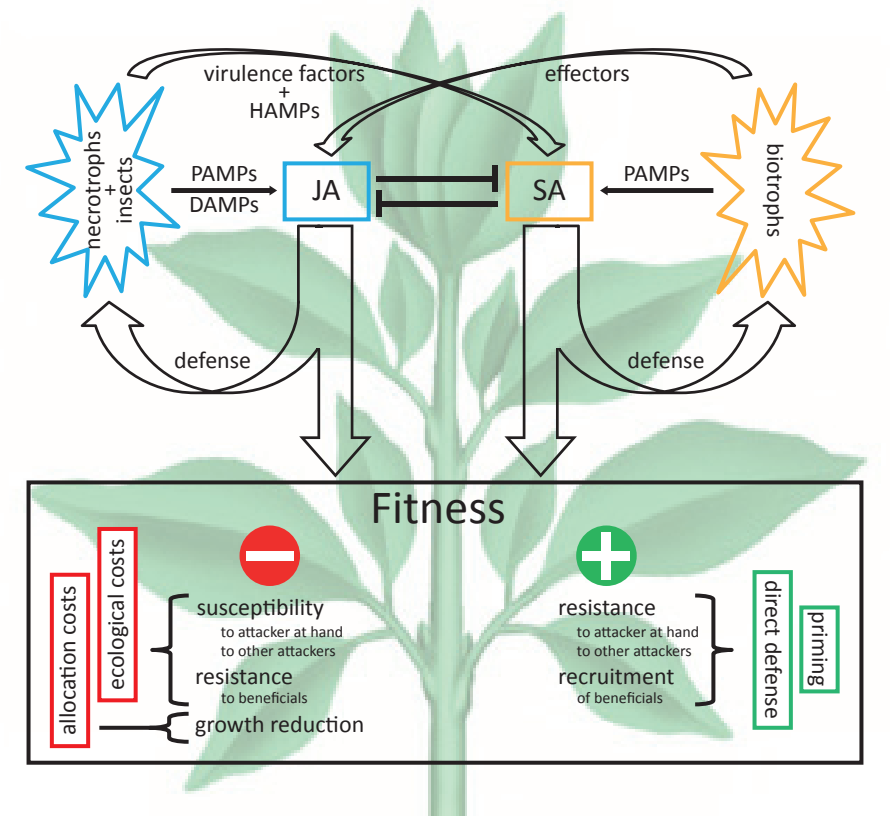


Figure 1: Schematic overview of hormone-regulated inducible defense responses and their effects on plant fitness. Upon attack by a necrotrophic pathogen or herbivorous insect, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are recognized, leading to activation of jasmonic acid (JA)-dependent defense responses. Upon attack by a (hemi-)biotrophic pathogen, its PAMPs are recognized and salicylic acid (SA)-dependent defense responses are activated. These SA- and JA-dependent defense signaling pathways antagonize each other. The induced defense mechanisms have positive effects on the plant's fitness by enhancing resistance through direct activation and priming of defense and through recruitment of beneficial microorganisms. Negative effects of induced plant defenses on plant fitness occur as well. Ecological costs are incurred via pathway crosstalk, through which an increase in resistance to one attacker leads to an increase in susceptibility to another attacker. In addition, pathway crosstalk can be hijacked by an attacker to antagonize effective defenses, resulting in increased susceptibility. Necrotrophic pathogens and herbivorous insects can produce virulence factors and herbivore associated molecular patterns (HAMPs), respectively that activate the SA pathway leading to suppression of effective JA-dependent defense responses. (Hemi-)biotrophic pathogens can produce effectors that activate JA or other hormone signaling pathways that act antagonistically on SA-dependent defenses. Furthermore, as ecological costs, beneficial microorganisms can be warded off by the plant's own defense mechanism. Allocation costs are incurred during activation of the plant's defense mechanism, because valuable resources are used for defense rather than for growth and reproduction. Allocation costs during direct activation of defenses are considerably larger than during priming of defenses.

The benefits of plant defenses are obvious; they help the plant to survive in the presence of harmful organisms (Figure 1). However, the inducible character of plant defenses leaves a time slot between attack and the expression of defenses in which the plant is vulnerable to the invading organism. Constitutive expression of defense traits does not have this drawback, making it probable that inducible defenses have other selective advantages over constitutive defenses (Heil & Baldwin, 2002). Fitness costs that are associated with defenses have been postulated to be a driving force behind the evolution of inducible defenses (Simms & Fritz, 1990). There are also costs associated with the genetic maintenance of inducibility, such as receptors and defense signal transduction routes, which all constitutively require energy and resources (Purrington, 2000; Cipollini *et al.*, 2003). These maintenance costs may be minimal, because many inducible pathway components have been co-opted from other processes, such as growth and development (Pieterse *et al.*, 2009).

The actual induced resistance status entails direct and indirect fitness costs. Direct resistance costs include allocation and autotoxicity costs (Heil & Baldwin, 2002; Strauss *et al.*, 2002). The latter is inflicted on plants by induced secondary chemicals that are toxic to the plant itself as well (Baldwin & Callahan, 1993; Heil & Baldwin, 2002; Strauss *et al.*, 2002). Allocation costs occur when valuable resources are allocated to resistance instead of to growth and reproduction (Herms & Mattson, 1992; Heil & Baldwin, 2002; Strauss *et al.*, 2002; Walters & Heil, 2007). Re-allocation of plant resources has also been postulated as a means by which the plant starves the pathogen in order to halt the infection (Canet *et al.*, 2010). Once induced, the enhanced resistance status needs to be maintained, but this is less costly (Van Hulten *et al.*, 2006).

Indirect resistance costs, also known as ecological costs (Heil & Baldwin, 2002; Strauss *et al.*, 2002), occur as a result of the changed physiology of the plant that in turn affects interactions with other biotic and abiotic environmental factors, such as beneficial or harmful organisms, competing plants and resource availability (Heil, 2002; Cipollini *et al.*, 2003; Kessler & Halitschke, 2007; Poelman *et al.*, 2008; Traw & Bergelson, 2010). In this introduction, an overview of current knowledge on costs and benefits associated with inducible defenses that are controlled by plant hormones is provided and it is discussed how this knowledge can be applied for improved crop protection.

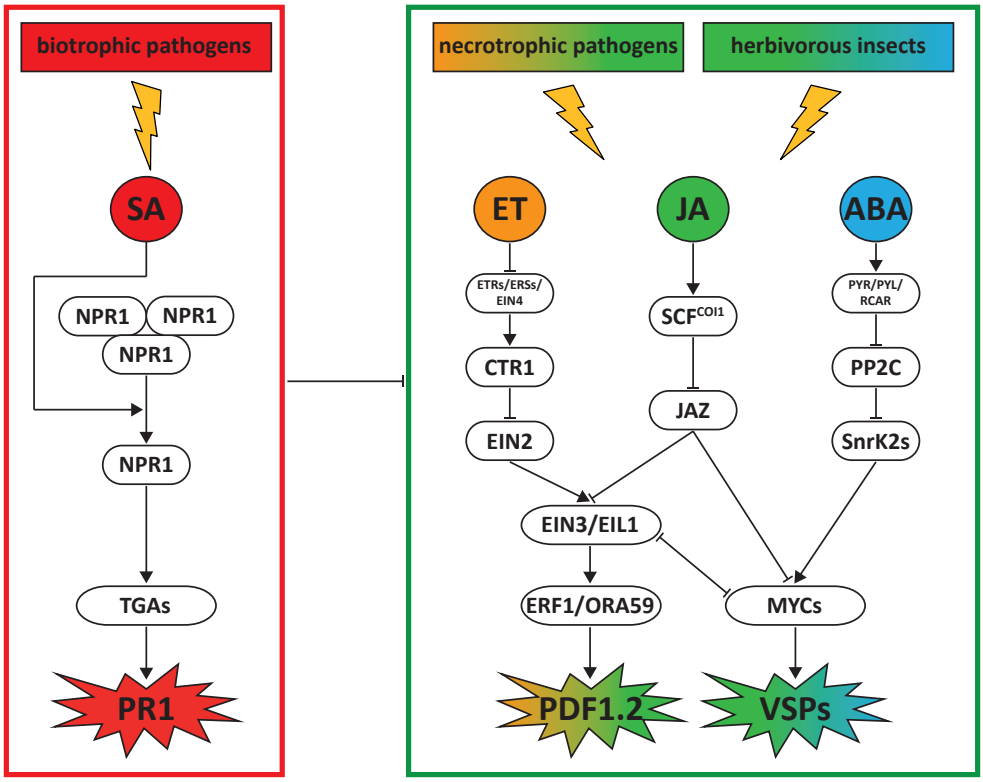


Figure 2: Networking by plant hormones in plant immunity.
Cross-communication between the different hormone signaling pathways (see text for details). Biotrophic pathogens induce the SA-dependent signaling pathway; necrotrophic pathogens induce JA- and ET-dependent signaling pathways; herbivorous insects induce JA- and ABA-dependent signaling pathways. The SA- and JA-dependent signaling pathways and the ET- and ABA-regulated branches of the JA pathway are mutually antagonistic. Arrows indicate an inducing effect. Blocked lines indicate a repressing effect.

SA-INDUCIBLE DEFENSES

SA is a phenolic compound that plays a key role in disease resistance signaling in plants (Mishina & Zeier, 2007; Vlot *et al.*, 2009). Besides its role in plant defense, SA also influences seed germination, vegetative growth, photosynthesis, respiration, thermogenesis, flower formation, seed production, senescence and responses to abiotic stress (reviewed in Rivas-San Vicente & Plasencia, 2011). Plants rapidly synthesize SA upon pathogen infection (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Vlot *et al.*, 2009). SA can be synthesized via two distinct enzymatic pathways that both require chorismate. The bulk of pathogen-induced SA is produced from isochorismate via ICS1, but chorismate can also be converted into SA via a series of enzymatic

reactions initially catalyzed by PAL (Wildermuth *et al.*, 2001; Vlot *et al.*, 2009). Defense signaling downstream of SA is largely regulated by the regulatory protein NPR1 (Dong, 2004). Activation of the SA signaling pathway leads to a change in the cellular redox state, which reduces NPR1 from its inactive oligomeric form to its active monomeric form. Monomeric NPR1 is then translocated to the nucleus where it interacts with TGA transcription factors (Mou *et al.*, 2003; Dong, 2004; Moore *et al.*, 2011), resulting in the activation of a large set of defense-related genes, amongst which are genes coding for PR proteins and WRKY transcription factors (Figure 2; Van Loon *et al.*, 2006a; Rushton *et al.*, 2010).

Benefits: SA triggers disease resistance

The first indication for a role of SA in disease resistance signaling came from White (1979), who showed that exogenous application of SA to tobacco plants enhanced resistance against tobacco mosaic virus (TMV). Nowadays, numerous examples exist that demonstrate the resistance-inducing capacity of SA in a wide variety of plants against (hemi-)biotrophic pathogens and some phloem-feeding insects (Klessig & Malamy, 1994; Walling, 2008; Vlot *et al.*, 2009). The significance of SA was further shown by the use of mutant or transgenic plants (mostly in *Arabidopsis thaliana* (Arabidopsis), tobacco and tomato) that are affected in the production or the perception of SA. For example, transgenic *NahG* plants, which are incapable of accumulating SA, and mutant *npr1* plants, which are impaired in SA signaling, are more susceptible to oomycete, fungal, bacterial and viral pathogens (reviewed in Glazebrook, 2005).

Hyaloperonospora arabidopsidis

An example of a biotrophic pathogen against which SA-inducible defenses are effective is the oomycete *Hyaloperonospora arabidopsidis* that causes downy mildew disease on Arabidopsis. Downy mildew diseases are responsible for significant yield losses in many crop species, such as lettuce, cucurbits and maize (Thines *et al.*, 2008; Lebeda & Cohen, 2011). *H. arabidopsidis* is an obligate biotroph and therefore dependent on living plant tissue to grow and reproduce (Coates & Beynon, 2010). Induction of SA biosynthesis and signaling is associated with and contributes to the hypersensitive response, which is characterized by the formation of necrotic lesions at the site of pathogen infection (Dangl *et al.*, 1996; Hammond-Kosack & Jones, 1996; Mittler & Lam, 1996). Since *H. arabidopsidis* is dependent on living plant tissue, timely SA production confines the pathogen to the site of infection. SA also facilitates the formation of reactive oxygen species (ROS), which play a role in the change in the cellular redox state during SA signaling (Caarls *et al.*, 2015). Furthermore, ROS can directly kill a pathogen or activate cell wall cross-linking and lignification to strengthen the cell wall, thereby contributing to resistance as well (Durner *et al.*, 1997; Dempsey *et al.*, 1999; Caarls *et al.*, 2015).

Trade-offs: allocation costs of SA-inducible defenses

Several studies investigated the costs of SA-inducible defenses. In general, exogenous application of SA or its chemical analogue benzothiadiazole (BTH) has been shown to reduce plant growth and seed production of different plant species (Heil *et al.*, 2000; Cipollini, 2002; Canet *et al.*, 2010). However, environmental conditions such as competition with neighboring plants and nutrient availability can influence these fitness effects and sometimes avert the growth costs associated with SA-inducible defenses (Heidel *et al.*, 2004; Dietrich *et al.*, 2005). Arabidopsis mutants constitutively expressing SA-inducible defenses, such as *cpr1, cpr5* and *cpr6*, were shown to be dwarfed and severely affected in seed production (Bowling *et al.*, 1994; Heil & Baldwin, 2002; Heidel *et al.*, 2004; Van Hulten *et al.*, 2006). Conversely, SA-deficient *NahG* and *sid2* (mutated in the SA biosynthesis gene *ICS1*) Arabidopsis plants had higher growth rates and seed production compared to wild-type plants under pathogen-free conditions (Cipollini, 2002; Abreu & Munné-Bosch, 2009), confirming the negative effects of SA on growth and reproduction. The decrease in growth that was observed after treatment with BTH was reduced in the SA signaling mutant *npr1*, implying a pivotal role of NPR1 in inhibiting plant growth when SA-dependent resistance mechanisms are activated (Van Hulten *et al.*, 2006; Canet *et al.*, 2010). However, after infection with the SA-inducing downy mildew pathogen *H. arabidopsidis*, *npr1* mutant plants displayed a lower fitness than wild-type plants (Heidel & Dong, 2006). This demonstrates that, although costly, SA-inducible defenses are beneficial when plants grow under pathogen pressure. The beneficial effect of SA-regulated defenses was particularly apparent under low nutrient conditions (Heidel & Dong, 2006), which supports the theory on allocation costs as driver of the evolution of inducible defenses. Mutants *cpr1* and *cpr5* that constitutively express SA-regulated defenses failed to show a fitness benefit under pathogen pressure, supporting the hypothesis that the inducible character of SA-dependent resistance prevents excessive fitness costs (Heidel & Dong, 2006; Van Hulten *et al.*, 2006).

Although negative effects of SA on fitness have mostly been ascribed to allocation costs (Heil *et al.*, 2000; Walters & Heil, 2007), toxic effects of SA may also contribute to reduced fitness (Bi *et al.*, 2010; Asaduzzaman & Asao, 2012). However, most studies focusing on autotoxicity costs of SA have not included plant genotypes that rule out effects of allocation costs, e.g. SA signaling mutants such as *npr1*, which makes claims on a role for SA in autotoxicity costs obscure. Moreover, most studies on allocation costs of SA signaling that have tested *npr1* made use of BTH as inducer of the SA pathway, which induces SA signaling and resistance without the toxic side effects of SA (Lawton *et al.*, 1996), thereby omitting autotoxicity effects of SA in their studies. One of the few studies that applied SA to *npr1* (Cipollini, 2002) found no decrease in seed set in comparison to non-treated *npr1* plants, whereas SA treatment did decrease seed set in wild-type plants, indicating that the costs incurred by SA are (mostly) allocation costs

and not autotoxicity costs. Besides direct allocation costs, SA-inducible defenses also inflict ecological costs. These ecological costs include crosstalk effects between the SA and JA signaling pathways, which are described in the section on crosstalk.

JA-INDUCIBLE DEFENSES

JA is a key regulator in the defense response against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Howe & Jander, 2008). Besides its essential role in regulating disease and pest resistance, JA has also been implicated in senescence, root growth, fruit ripening, tendril coiling, pollen development, tuberization and responsiveness to abiotic stress (Wasternack & Hause, 2013). JA is an oxylipin that accumulates rapidly in plants in response to infection with necrotrophic pathogens, wounding and herbivory (Creelman *et al.*, 1992; Penninckx *et al.*, 1996). The initial phase of JA formation takes place in the chloroplasts, where fatty acids of membrane lipids (e.g. linoleic acid) are metabolized by lipoxygenases to generate oxylipins including the JA precursor 12-oxo-phytodienoic acid (OPDA). Subsequently, OPDA is transported to the peroxisomes where it undergoes three steps of β-oxidation to generate JA (reviewed in Wasternack & Hause, 2013). JA can be conjugated to amino acids, such as L-isoleucine, resulting in JA-Ile, the most biologically active member of the JAs (Staswick & Tiryaki, 2004; Fonseca *et al.*, 2009). The F-box protein COI1 is a key regulator of the JA signaling pathway (Xie *et al.*, 1998), as it is part of the JA receptor complex (Yan *et al.*, 2009; Sheard *et al.*, 2010). Binding of JA to COI1 targets JAZ proteins for degradation via the 26S proteasome pathway (Chini *et al.*, 2007; Thines *et al.*, 2007). In the uninduced state, JAZ proteins repress JA-responsive gene expression by binding to transcriptional activators, such as MYC2, MYC3 and MYC4 (Fernández-Calvo *et al.*, 2011). Accumulation of JA triggers the degradation of JAZ proteins, resulting in derepression of JA-regulated genes (Figure 2). In Arabidopsis, there are two distinct branches of the JA signaling pathway that antagonize each other, the ERF- and the MYC-branch (hereafter referred to as such).

The ERF-branch

The ERF-branch of the JA response pathway is activated upon infection with necrotrophic pathogens and is regulated by the AP2/ERF-domain containing transcription factors ERF1 and ORA59 (Anderson *et al.*, 2004; Pré *et al.*, 2008). The ERF-branch also requires ET and results in the activation of the marker gene *PDF1.2* (Figure 2; Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003).

Ethylene

Besides the modulation of JA signaling, the gaseous hormone ET is foremost known as a regulator of developmental processes and responses to abiotic stresses, like germination, hypocotyl growth, apical hook formation, root growth, flowering, fruit ripening, leaf senescence, leaf abscission, root nodulation, programmed cell dead and freezing tolerance (Johnson & Ecker, 1998; Alonso & Stepanova, 2004). For the formation of ET, S-adenosyl-L-methione is converted by 1-aminocyclopropane-1-carboxylate synthase into 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is subsequently reduced to ethylene by ACC oxidase (Adams & Yang, 1979; Bleecker & Kende, 2000). In Arabidopsis, five ET receptors have been described: ETR1, ETR2, ERS1, ERS2 and EIN4 (Hua *et al.*, 1998). In the absence of ET, the receptors stimulate the negative regulator CTR1. Binding of ET to the receptors, inactivates the receptors and thereby CTR1 (Alonso & Stepanova, 2004; Stepanova & Alonso, 2009). Inactivation of CTR1 activates EIN2, which subsequently stabilizes the transcription factors EIN3 and EIL1. The stabilization of EIN3 and EIL1 results in the transcriptional activation of hundreds of genes (Figure 2; Alonso & Stepanova, 2004). ERF1 and ORA59 are direct targets of EIN3, and are also induced by JA (Lorenzo *et al.*, 2003; Pré *et al.*, 2008).

The MYC-branch

The MYC-branch of the JA response pathway is activated upon wounding and feeding by herbivorous insects and is regulated by the MYC transcription factors MYC2, MYC3 and MYC4 in synergistic action with ABA (Anderson *et al.*, 2004; Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011). Activation of the MYC-branch leads to induced expression of the marker genes *VSP1* and *VSP2* (Figure 2; Lorenzo *et al.*, 2004).

Abscisic acid

The hormone ABA is a metabolite belonging to the class of terpenoids (Nambara & Marion-Poll, 2005) and has been reported mostly to function in regulation of developmental processes, such as seed germination, senescence, dormancy and tolerance to abiotic stresses (Wasilewska *et al.*, 2008; Hauser *et al.*, 2011). In addition, ABA has been described to modulate plant defense responses (Asselbergh *et al.*, 2008; Feng *et al.*, 2012; Sánchez-Vallet *et al.*, 2012; Vos *et al.*, 2013b). Limited water supply is an important trigger for ABA biosynthesis (Raghavendra *et al.*, 2010). For the production of ABA, β-carotene needs to be converted to zeaxanthin, which is subsequently modified to neaxanthin and violacanthin. Nine-cis-epoxycarotenoid dioxygenase reduces these compounds to xanthoxin, which is converted by ABA2 to abscisic aldehyde. Abscisic aldehyde is then oxidized into ABA by AAO3 (reviewed in Nambara & Marion-Poll, 2005). RCAR, PYR and PYL proteins have been identified as ABA receptors (Ma *et al.*, 2009; Park *et al.*, 2009). Upon binding by ABA, RCAR/PYR/PYLs bind the PP2C negative

regulators of ABA signaling, such as ABI1 and ABI2 (Wasilewska *et al.*, 2008; Hubbard *et al.*, 2010). Binding of PP2Cs reduces PP2C-mediated suppression of SnRK2s, allowing their activation (Figure 2; Umezawa *et al.*, 2009), leading to phosphorylation of transcription factors, such as AREBs and ABFs (Hauser *et al.*, 2011). In addition, MYC transcription factors can act as positive ABA response regulators (Abe *et al.*, 2003; Raghavendra *et al.*, 2010; Vidhyasekaran, 2015).

Benefits: JA triggers disease resistance

Many JA-inducible defense responses and their effectiveness in plant resistance against diseases and pests were identified by exogenous application of JA and by the analysis of mutants with defects in JA signaling compounds such as COI1, MYCs and ERFs. This demonstrated that JA signaling is indispensable for resistance to a wide range of necrotrophic pathogens and herbivorous insects, whereby in general the ERF-branch is associated with resistance against necrotrophic pathogens (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003) and the MYC-branch with resistance against herbivorous insects (Lorenzo *et al.*, 2004; Howe & Jander, 2008; Kazan & Manners, 2012).

Botrytis cinerea

The fungal plant pathogen *Botrytis cinerea* is one of the most important plant pests. Since *B. cinerea* is able to attack over 200 plant species, it causes large yield losses and high costs of control (Windram *et al.*, 2012). *B. cinerea* is a necrotrophic pathogen and kills host cells by producing a variety of phytotoxins to obtain nutrients from the plant (Govrin & Levine, 2002; Glazebrook, 2005). Opposite to the production of ROS by the plant as a defense mechanism against *H. arabidopsidis*, *B. cinerea* might produce ROS as a virulence strategy (Govrin & Levine, 2002). Tolerance to ROS is therefore important for plant resistance against *B. cinerea* (Glazebrook, 2005). In fighting of infection with *B. cinerea*, the production of the inducible antimicrobial compound camalexin is also important (Ferrari *et al.*, 2003). Furthermore, ERF-branch-regulated defense responses play an important role in the defense against *B. cinerea*.

Pieris rapae

Infestation with the specialist chewing herbivore *Pieris rapae* (small cabbage white) can have detrimental effects on plant growth and fitness. Specialist insects feed on one or a few plant species from the same family, to which they have adapted (Howe & Jander, 2008). Plants can defend themselves against insect attack in several ways. Firstly, they can produce polyphenol oxidases, amino acid deaminases and proteinase inhibitors that lower the nutritive value of the consumed plant tissue (Kessler & Baldwin, 2002; Lawrence & Koundal, 2002; Chen *et al.*, 2005). Secondly, production of toxins and defense proteins either kill the insect or target physiological processes in the

insect. For example, glucosinolates accumulate in brassicaceous species after feeding by herbivorous insects. However, the specialist *P. rapae* contains a nitrile specifier protein in its guts, which enables it to breakdown glucosinolates to the less toxic nitriles (Wittstock *et al.*, 2004). Lastly, plants can produce volatile compounds or extrafloral nectar to attract predators of the insect (Dicke *et al.*, 1990; Kessler & Baldwin, 2002; Howe & Jander, 2008; Mithöfer & Boland, 2012; Heil, 2015). MYC-branch signaling plays an important role in the regulation of inducible defenses against *P. rapae*.

Trade-offs: allocation costs of JA-inducible defenses

Studies on the costs of JA-inducible defenses have mostly been executed with plants that were infested with insects. In contrast, cost studies on JA-inducible defenses that are associated with infection with necrotrophic pathogens are scarce. Infestation by insects and exogenous application of JA comes with costs, which is apparent from a decreased seed set and delayed flowering and fruit ripening (Agrawal *et al.*, 1999; Redman *et al.*, 2001; Van Dam & Baldwin, 2001). In addition, the Arabidopsis mutant *cev1* and the transgenic line overexpressing JA carboxyl methyltransferase (JMT), both constitutively expressing JA-dependent defenses, showed reduced growth phenotypes (Ellis & Turner, 2001; Cipollini, 2010). The effect on delayed flowering of JMT-overexpressing lines was especially apparent under low nutrient conditions (Cipollini, 2010). Furthermore, competition with neighboring plants increased JA-induced fitness costs in tobacco (Van Dam & Baldwin, 2001). The JA-associated trade-offs in reduced plant performance have therefore been mostly explained by allocation costs. However, JA is also known to directly regulate several plant developmental processes such as growth and seed production (Creelman & Mullet, 1997; Yang *et al.*, 2012), which complicates the assignment of the origin of the fitness decrease detected in plants expressing JA-dependent responses. Despite the fact that JA-induced responses are costly, they benefit plants when under attack, even in field situations (Baldwin, 1998). To our knowledge no studies on autotoxicity costs of JA have been described so far. Ecological costs of JA-inducible defenses include crosstalk effects between the ERF- and the MYC-branch of the JA signaling pathway, which are described in the section below on crosstalk.

HORMONAL CROSSTALK IN DEFENSE SIGNALING

Plant hormones are integral to plant immune responses and are differentially effective against different types of attackers. During plant-attacker interactions, multiple hormones are induced that together steer the immune response of the plant (De Vos *et al.*, 2005). The hormonal signal signature is vital for a successful immune response upon attack, as extensive cross-communication between defense signaling pathways

allows the plant to fine-tune the defense response to the attacker at hand (Reymond & Farmer, 1998). Hormonal crosstalk has often been interpreted as a cost-saving strategy and may have evolved as a means of the plant to reduce allocation costs by repression of unnecessary defenses that are ineffective against the attacker that is encountered (Pieterse & Dicke, 2007; Thaler *et al.*, 2012). However, proof for this hypothesis has not been demonstrated yet, as to the authors' knowledge there has been no study that measured the fitness levels of plants exhibiting hormonal crosstalk in comparison to that of crosstalk mutant plants. In this chapter, crosstalk between SA and JA signaling, and between the ERF- and the MYC-branch of the JA signaling pathway is covered, but other hormones have also been reported to modulate hormone-controlled immune signaling (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012).

Crosstalk between the SA and JA pathways

The first indication of crosstalk between SA and JA signaling came from Doherty *et al.* (1988), who showed that SA and its acetylated derivative aspirin are strong antagonists of the JA pathway. Many cases of crosstalk between the SA and JA pathways have been reported since (Bostock, 2005; Stout *et al.*, 2006; Pieterse *et al.*, 2012). Pharmacological experiments with Arabidopsis revealed that the JA-responsive genes *PDF1.2* and *VSP2* are highly sensitive to suppression by SA (Figure 2). The antagonistic effect of SA on JA signaling was observed in a large number of Arabidopsis accessions (Koornneef *et al.*, 2008) and was even reported to remain active in the next generation (Luna *et al.*, 2012), highlighting the potential significance of this phenomenon in the regulation of induced plant defenses in nature. Several important regulatory proteins of SA-JA pathway crosstalk have been identified, including NPR1, GRX480 and TGAs (Spoel *et al.*, 2003; Ndamukong *et al.*, 2007; Luna *et al.*, 2012; Pieterse *et al.*, 2012; Zander *et al.*, 2012; Caarls *et al.*, 2015). Timing and concentration of ET was shown to modulate the strength of SA-JA crosstalk and its NPR1-dependency (Leon-Reyes *et al.*, 2009). Recent data showed that in Arabidopsis SA does not affect JA biosynthesis but it affects JA signaling downstream of COI1, at the level of transcriptional regulation of JA-responsive genes (Leon-Reyes *et al.*, 2010b; Van der Does *et al.*, 2013). The ERF transcriptional activator ORA59, which regulates many JA-responsive genes, was identified as an important target for SA (Van der Does *et al.*, 2013; Zander *et al.*, 2014).

Many studies found an antagonistic effect of SA signaling on the JA pathway. However, in several cases JA signaling could suppress the SA pathway as well. A few molecular players have been reported to play a role in this JA-SA crosstalk, such as COI1 and MYC2 (Zheng *et al.*, 2012). Furthermore, neutral and synergistic interactions between SA and JA have also been reported (Schenk *et al.*, 2000; Van Wees *et al.*, 2000; Mur *et al.*, 2006). Timing, sequence of initiation and the relative concentration of each hormone are important for the outcome of the SA-JA crosstalk (Mur *et al.*, 2006;

Koornneef *et al.*, 2008; Leon-Reyes *et al.*, 2010a). Besides the aforementioned ET, other hormones can also modulate SA-JA crosstalk (Pieterse *et al.*, 2012). Under different plant-attacker conditions, simultaneous inductions of SA and JA signaling do not always lead to predicted outcomes, highlighting the complexity and context-dependency of the hormonal interactions.

Crosstalk between the ERF- and the MYC-branch of the JA pathway

As described above, defense responses to necrotrophic pathogens and herbivorous insects are regulated by distinct branches of the JA signaling pathway: the ERF- and the MYC-branch, respectively (Figure 2; Lorenzo & Solano, 2005; Kazan & Manners, 2008; Pieterse *et al.*, 2012). Transcriptional changes in response to these diverse types of attackers show limited overlap, suggesting that the context in which the induced JA signal is perceived is crucial in tuning the JA response (De Vos *et al.*, 2005). Recently, it has been shown that the MYC-branch transcription factors MYC2, MYC3 and MYC4 interact with the ERF-branch transcription factors EIN3 and EIL1 and repress each other's transcriptional activity (Figure 2; Song *et al.*, 2014). Activation of the ERF-branch resulted in reduced expression of the MYC-branch marker gene *VSP2*, whilst silencing of ORA59 or mutating the ET pathway caused enhanced levels of *VSP2* expression (Lorenzo *et al.*, 2004; Verhage *et al.*, 2011). Reciprocally, activation of the MYC-branch suppressed transcription of the ERF-branch marker gene *PDF1.2*, whereas mutation of MYC transcription factors genes or ABA signaling components led to enhanced expression of *PDF1.2* and the ERF transcription factor gene *ORA59* (Anderson *et al.*, 2004; Verhage *et al.*, 2011). Furthermore, a negative feedback of ABA on ET production has been found (LeNoble *et al.*, 2004). Taken together, these data clearly indicate a mutually antagonistic interaction between the different branches of the JA pathway (Figure 2).

ECOLOGICAL COSTS OF DEFENSE SIGNALING

Fitness costs associated with induced defense arise from allocation and ecological costs (Figure 1). Allocation costs are incurred when resources are allocated to resistance instead of to growth and reproduction (Heil & Baldwin, 2002) and have been described in the previous sections on SA- and JA-inducible defenses. Ecological costs arise when defense-induced plants have altered abilities to interact with their biotic and abiotic environment (adjusted from Heil, 2002). For example, induction of the JA defense pathway resulted in reduced numbers of visitations by beneficial pollinators (Strauss *et al.*, 2002), JA-induced extrafloral nectar production attracted flies that excluded beneficial ants (Heil *et al.,* 2004) and JA-regulated herbivore-induced plant volatiles (HIPVs) alter the interaction of a plant with herbivores, carnivores and competing

plants (Dicke & Van Loon, 2000). Also above-below-ground interactions with beneficial microbes can be affected by the activation of defense in foliar tissue. Exogenous application of SA to the soil inhibited the growth and formation of root nodules in the *Rhizobium*-legume symbiosis, whereas growth of *Rhizobium* cells itself was not affected by SA (Sato *et al.*, 2002; Mabood & Smith, 2007). Furthermore, De Román *et al.* (2011) found that foliar application of acibenzolar-S-methyl (ASM), a functional analogue of SA, to soybean led to a transient reduction in arbuscular mycorrhizal colonization of roots. Negative effects of foliar herbivory on colonization of the roots by mycorrhizal fungi have also been reported (Barber *et al.*, 2012). In contrast, recruitment of soilborne beneficial microbes upon stress induction in the leaves has also been shown (Figure 1). Beneficial *Bacillus subtilis* bacteria were recruited to the rhizosphere upon foliar infection of Arabidopsis with the bacterial pathogen *Pseudomonas syringae* (Rudrappa *et al.*, 2008). Moreover, foliar application of JA and wounding of the leaves of *Medicago truncatula* resulted in enhanced JA signaling and enhanced mycorrhization by *Rhizophagus irregularis* (formerly known as *Glomus intraradices*; Landgraf *et al.*, 2012). These results show the importance of testing effects of altered defenses in plants under realistic environmental conditions, because otherwise relevant ecological costs might be missed. The net effect of induced defense signaling on plant fitness strongly depends on the community context.

Ecological costs as a result of hormonal crosstalk are becoming increasingly recognized. Whereas hormonal crosstalk may be advantageous for the plant to keep allocation costs in check, evidence is accumulating that crosstalk at the level of gene expression is translated into crosstalk at the level of resistance. When plants encounter multiple attackers simultaneously or successively, the induction of a hormone signaling route might elevate the resistance to one attacker, but at the same time hormonal crosstalk can decrease the resistance to another attacker (Pieterse *et al.*, 2012). Furthermore, as a common virulence strategy, successful pathogens and insects can hijack crosstalk mechanisms by targeting plant hormone biosynthesis and perception to rewire immune signaling, rendering the plants more susceptible (Figure 1; Grant & Jones, 2009; Verhage *et al.*, 2010; Pieterse *et al.*, 2012). Ecological costs of crosstalk between different hormones are described in the next sections, focusing on crosstalk between SA and JA signaling, and between the ERF- and the MYC-branch of JA signaling.

Ecological costs of SA-JA crosstalk

Many examples of ecological costs of SA-JA crosstalk have been described. For instance, in Arabidopsis infection with the hemibiotrophic pathogen *P. syringae* leads to induction of the SA pathway, resulting in an effective resistance response against this pathogen. However, through SA-JA crosstalk mechanisms JA signaling is suppressed, which renders the infected leaves more susceptible to the necrotrophic

fungus *Alternaria brassicicola* (Spoel *et al.*, 2007). Similarly, induction of SA signaling in Arabidopsis by exogenous application of SA inhibited JA-induced resistance to the generalist herbivores *Spodoptera exigua* and *Trichoplusia ni* (Cipollini *et al.*, 2004; Cui *et al.*, 2005). In tobacco, *Manduca sexta* caterpillars consumed up to 2.5-times more leaf tissue from plants exhibiting increased SA signaling after inoculation with TMV than from mock-treated plants (Preston *et al.*, 1999). Furthermore, reduced SA signaling in Arabidopsis genotypes *NahG* and *npr1* was correlated with reduced feeding by *T. ni* in comparison to wild-type plants (Cui *et al.*, 2002). Crosstalk the other way around, namely of JA on SA, was also effective on the level of disease resistance, since the JA-insensitive mutant *coi1* showed enhanced expression of SA-dependent defenses and enhanced resistance to *P. syringae* (Kloek *et al.*, 2001).

Several pathogens have evolved ways to hijack host crosstalk mechanisms as a virulence strategy. One of the best studied examples is the production of coronatine by *P. syringae*. Coronatine is a pathogen-derived functional and structural mimic of JA-Ile that suppresses SA signaling, thereby promoting susceptibility to this pathogen (Kloek *et al.*, 2001; Brooks *et al.*, 2005; Cui *et al.*, 2005; Zheng *et al.*, 2012). Furthermore, the necrotrophic pathogen *B. cinerea* was shown to produce an exopolysaccharide that acts as an elicitor of the SA pathway and causes suppression of the JA pathway and consequently promotes pathogen growth (El-Oirdi *et al.*, 2011). Likewise, it has been suggested that phloem feeding insects can enhance SA levels and suppress JA-mediated defenses (Lazebnik *et al.*, 2014). For example, nymphs of the phloem-feeding silverleaf whitefly *Bemisia tabaci* activated SA-responsive gene expression in Arabidopsis, thereby suppressing the JA signaling pathway. This was shown to be associated with accelerated nymphal development, suggesting that the nymphs of *B. tabaci* can rewire the plant's immune signaling network to their own benefit (Zarate *et al.*, 2007). Additionally, eggs of *Pieris brassicae* butterflies have been reported to induce SA signaling upon oviposition, which suppresses JA signaling and provides an advantage for the freshly hatched caterpillars (Bruessow *et al.*, 2010).

Ecological costs of crosstalk between the ERF- and the MYC-branch

Indications of effects of crosstalk between the ERF- and the MYC-branch on the level of resistance to insects and necrotrophic pathogens have come mainly from studies with Arabidopsis mutants affected in one of the branches. In a two-choice setup, *P. rapae* caterpillars preferred to feed from Arabidopsis genotypes that highly expressed the ERF-branch of the JA pathway, such as *MYC2*-impaired *jin1* plants and ORA59 overexpressing plants, over wild-type plants that highly expressed the MYC-branch upon feeding by the caterpillars (Verhage *et al.*, 2011). Furthermore, the *jin1* mutant and the ABA biosynthesis mutant *aba2-1* were more resistant to the necrotrophic pathogens *B. cinerea*, *Plectosphaerella cucumerina* and *Fusarium oxysporum* due to a potentiated

expression of the ERF-branch in these mutants (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Nickstadt *et al.*, 2004; Adie *et al.*, 2007). A comparable mechanism may underlie findings from the early 1990s with black bean aphids *Aphis fabae* that displayed a higher growth rate and fecundity on bean leaves infected with the necrotrophic pathogen *Botrytis fabae*, compared to uninfected leaves (Zebitz & Kehlenbeck, 1991).

Indications of hijacking of hormonal crosstalk mechanisms by attackers have been found for ERF-MYC crosstalk as well. For example, application of the oral secretion of *P. rapae* caterpillars into wounded leaf tissue stimulated expression of the ERF-branch, suggesting that insecticidal compounds have the potential to manipulate the plant response towards the caterpillar-preferred ERF-branch (Verhage *et al.*, 2011). Together, these data support the existence of high ecological costs of crosstalk on the level of resistance to pathogens and pests.

PRIMING FOR ENHANCED DEFENSE

Besides the fact that inducible defenses involve fitness costs, the inducibility of defenses comes with an unsafe time slot between attack and the expression of defenses. This might have been a driving force for the development of a sophisticated, cost-effective way to activate inducible defense responses, namely by priming (Figure 1). Plants that are primed for enhanced defense do not express defenses in the absence of an attacker, but show a faster and stronger activation of cellular defense responses upon attack compared to non-primed control plants (Conrath *et al.*, 2002; Conrath *et al.*, 2006; Frost *et al.*, 2008). Prior activation of defenses is not a prerequisite for the primed state, which makes priming a cost-efficient form of induced immunity. Another benefit of priming is that it offers enhanced resistance against a broad spectrum of attackers. Multiple inducers of priming for defense have been identified, including beneficial microbes, pathogens and herbivorous insects, but also chemical elicitors and wounding (Conrath *et al.*, 2002; Conrath *et al.*, 2006). The diverse forms of priming are described in the sections below.

Various mechanisms underlying priming have been reported. Inactive cellular proteins that play a role in cellular signal amplification have been shown to accumulate in primed plants where they remain dormant until activation by stressors, resulting in an accelerated response. Examples of such dormant signal transducers implicated in priming are transcription factors and mitogen-activated protein kinases (Pozo *et al.*, 2008; Beckers *et al.*, 2009; Van der Ent *et al.*, 2009a). Chromatin modifications at the promoters of priming-associated genes have also been implicated in the regulation of the primed state (Jaskiewicz *et al.*, 2011; Luna *et al.*, 2012; Rasmann *et al.*, 2012). Priming has in several cases been demonstrated to be transferred to the plant's offspring, which in some cases was associated with epigenetic changes, allowing plants to retain

memory of a threatening situation into one or more successive plant generations (Luna *et al.*, 2012; Pieterse *et al.*, 2012; Rasmann *et al.*, 2012; Slaughter *et al.*, 2012).

SA-dependent systemic acquired resistance

Systemic acquired resistance (SAR) is a well-studied form of induced resistance in which priming is thought to play an important role (Conrath *et al.*, 2002; Durrant & Dong, 2004; Conrath *et al.*, 2006; Vlot *et al.*, 2009). SAR is activated locally and systemically upon infection with a (hemi-)biotrophic pathogen and enhances resistance of uninfected plant parts to subsequent infection with the same or a broad range of other pathogens. SAR was first described by Ross (1961), who demonstrated that uninfected leaves of TMV-infected tobacco plants became more resistant to subsequent infection with TMV. SAR is associated with endogenous accumulation of SA, both at the site of infection and in healthy systemic tissues. Mutant plants that are impaired in SA signaling, including the *npr1* mutant, are incapable of developing SAR, indicating that SAR requires SA signaling (Durrant & Dong, 2004). Recently, several long-distance signals involved in the communication between SAR-induced tissue and systemic SAR-expressing tissue have been identified (Vlot *et al.*, 2009; Dempsey & Klessig, 2012; Shah & Zeier, 2013). SAR is accompanied by priming of SA-dependent defenses, resulting in potentiated expression of SA-responsive genes, such as *PR1* (Mur *et al.*, 1996; Van Wees *et al.*, 1999). Additionally, SA-independent callose deposition is primed during SAR, resulting in accelerated strengthening of the cell wall at the site of pathogen penetration (Ton & Mauch-Mani, 2004). Exogenous application of low concentrations of SA or BTH does not directly activate defenses, but primes plants for enhanced expression of cellular defenses after pathogen attack (Conrath *et al.*, 2002; Conrath *et al.*, 2006). This indicates that SA-mediated priming is an intrinsic part of pathogen-activated SAR.

JA/ET-dependent induced systemic resistance

Induced systemic resistance (ISR) triggered by nonpathogenic microbes is another well-studied form of induced resistance in which priming plays an important role (Pieterse *et al.*, 2014). Plant roots contain a large number of rhizosphere-associated microbes, called the root microbiome, that aid in plant growth and reproduction (Berendsen *et al.*, 2012). Beneficial ISR-inducing microbes include soilborne plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF) as well as mycorrhizal fungi (Van Hulten *et al.*, 2010; Zamioudis & Pieterse, 2012). Like SAR, ISR offers a broad-spectrum resistance to foliar and root pathogens, but on top of that it is also effective against certain herbivores (Van Wees *et al.*, 2008; Pineda *et al.*, 2010). In contrast to SAR, rhizobacteria-mediated ISR was shown to be independent of SA and instead requires JA, ET and ABA signaling (Van Wees *et al.*, 2008; Van der Ent *et al.*, 2009a; Pieterse *et al.*, 2012). Most SA-signaling components in Arabidopsis proved to be dispensable for ISR, except the NPR1 protein

that acts downstream of JA and ET in ISR (Pieterse *et al.*, 1998; Van Wees *et al.*, 2000).

Large scale gene expression analysis revealed that induction of ISR can occur with only minor changes in gene expression in the leaves (Verhagen *et al.*, 2004; Van Wees *et al.*, 2008; Van der Ent *et al.*, 2009a). However, upon encounter with an invader, ISR-expressing plants show enhanced and accelerated expression of JA- and ET-regulated genes and accumulation of callose-rich papillae at the site of infection (Verhagen *et al.*, 2004; Pozo & Azcón-Aguilar, 2007; Pozo *et al.*, 2008). Concordantly, ISR is predominantly effective against necrotrophic pathogens and herbivorous insects, but also offers protection against biotrophs that are sensitive to cell wall defenses (Ton *et al.*, 2002; Van Oosten *et al.*, 2008). In roots, the induction of ISR results in altered expression of several genes, including the transcription factor *MYB72* (Verhagen *et al.*, 2004) that emerged as an important component of ISR, as *myb72* mutants were abolished in their ability to express ISR (Van der Ent *et al.*, 2008; Segarra *et al.*, 2009; Zamioudis *et al.*, 2014).

Herbivore-induced resistance

Priming has most often been studied in the context of plant-pathogen interactions, but plants can also be primed by signals associated with herbivore feeding. Tissue damage can lead to herbivore-induced resistance (HIR), which can be induced in neighboring plants via HIPVs or in systemic leaves of the same plant via internal signals or externally via HIPVs (Heil & Bueno, 2007; Frost *et al.*, 2008). Grafting experiments with tomato plants provided evidence that JA is the internal signal for systemic expression of herbivore-induced resistance (Sun *et al.*, 2011). Moreover, in Arabidopsis it was recently shown that electric signaling by membrane depolarization is important for wound-induced JA-responsive gene expression in systemic leaves (Mousavi *et al.*, 2013). HIPVs prime the plant for JA/ABA-inducible defense mechanisms, or act as signals to attract parasitic and predatory insects to combat attacking herbivores (Baldwin *et al.*, 2006; Ton *et al.*, 2007). In addition to the effect on insect performance, HIR can also prime the plant for enhanced resistance against microbial pathogens (De Vos *et al.*, 2006).

Crosstalk during priming

Despite the shared dependency on the NPR1 protein, distinct signaling cascades are important for SAR and ISR, requiring SA or JA and ET, respectively (Pieterse *et al.*, 1998; Van Wees *et al.*, 2000). Crosstalk between the SA and JA pathways could entail high ecological costs, as described in a previous section, but is this also true for SA- and JA-dependent priming? Simultaneous induction of SAR and ISR was shown to result in an additive effect on the level of resistance against *P. syringae* (Van Wees *et al.*, 2000). In plants mutated in either the SA or JA signaling pathway, this additive effect was not found. Furthermore, induction of ISR did not affect expression of SAR-induced

PR1. Thus, there is no evidence for SA-JA crosstalk during simultaneous activation of SAR and ISR. The Arabidopsis accession Bur-0 is constitutively primed for both *PR1* and *PDF1.2* expression upon exogenous application of SA and JA, respectively. Consequently, Bur-0 is more resistant to the hemibiotrophic pathogen *P. syringae* and also to the necrotrophic pathogen *P. cucumerina* (Ahmad *et al.*, 2011). Together, these results suggest that there is no SA-JA crosstalk during priming. However, when SAR was inherited in the next generation, the progeny showed a weaker induction of the JA-inducible gene *PDF1.2*, which was accompanied by increased susceptibility to the necrotrophic pathogen *B. cinerea*. This was associated with a chromatin modification at the *PDF1.2* promoter that is associated with transcriptional silencing (Luna *et al.*, 2012). Mycorrhizal fungi and PGPR have been reported to induce resistance to leaf chewing insects but also to increase susceptibility to phloem feeders (Koricheva *et al.*, 2009; Pineda *et al.*, 2010). These findings were recently expanded with studies on the effects of the ISR-inducing *Pseudomonas fluorescens* on the attraction of parasitoids by volatiles of aphid- or caterpillar-infested plants. Aphid-infested ISR-expressing plants attracted less parasitoids (Pineda *et al.*, 2013), whereas caterpillar-infested ISR-expressing plants attracted more parasitoids (Pangesti *et al.*, 2015). It is unknown whether there is a role for SA-JA crosstalk in these differential ecological consequences of ISR.

Benefits: limited allocation costs of priming

Priming of Arabidopsis with low concentrations of β-amino-butyric acid (BABA) was shown to have only marginal effects on plant growth and seed production in the absence of pathogens, suggesting that there are no or only limited allocation costs associated with priming (Van Hulten *et al.*, 2006). In the presence of pathogens, a clear fitness advantage was observed for primed plants over non-primed plants and plants expressing constitutive defenses. Walters *et al.* (2008) also found that priming in barley by saccharin did not incur fitness costs, both in greenhouse and field conditions. Furthermore, there are several studies that show that PGPR not only prime for defense but also increase plant growth and seed production, although these traits are not causally related (Raupach & Kloepper, 1998; Zehnder *et al.*, 2001; Zamioudis *et al.*, 2013). These results indicate that there are fitness benefits for plants that interact with PGPR whilst no allocation costs are associated with this. Ahmad *et al.* (2011) found that the Arabidopsis accession Bur-0 is constitutively primed for enhanced defense against pathogens and insects, without growth restraints. Together these results show that the benefits of priming outweigh the marginal costs of it in environments in which disease occurs. Therefore, priming for enhanced defense seems to be a very useful tool for application in crop protection.

INDUCIBLE DEFENSES AND TRADE-OFFS WITH CROP PROTECTION

In complex natural environments, plants encounter a multitude of pathogens and pests. In agriculture this leads to tremendous annual crop losses, representing a total value of over €450 billion worldwide. Allocation and ecological costs of (induced) plant defenses are a major problem for the implementation of induced resistance in agriculture (Walters & Heil, 2007). To successfully use inducible defenses in crop protection a functional understanding of the physiological and ecological consequences of the induced state is indispensable and demands more research (Bostock, 2005; Koornneef & Pieterse, 2008). Hormonal pathway crosstalk presents a challenge for translating fundamental knowledge into crop disease resistance traits. Plants often have to deal with simultaneous or subsequent attack by very different attackers. Genetic traits that are associated with contrasting resistance mechanisms to different attackers, for example SA signaling that causes elevated resistance to biotrophs, but reduced resistance to necrotrophs, can greatly impact plant fitness and thus crop yield. The extensive interactions between different hormone signaling routes that are activated upon encounter of a plant with an attacker, and the concentration-, space- and time-dependent context in which this occurs need to be dissected. However, to fully comprehend the plant's immune system, so that this knowledge can be applied to sustainable agriculture, plants need to be studied in agricultural and natural environments as well, because predictions on hormonal interactions and fitness effects during the encounter of plants with their biotic and abiotic environment do not always lead to the predicted outcomes (Clarke *et al.*, 2009; Ritsema *et al.*, 2010; Ballaré, 2011; Cerrudo *et al.*, 2012).

Breeders usually select for plant traits, such as yield and quality, while disease resistance is rarely in the top three of selected traits (Brown, 2002). Elevated resistance is usually correlated with detrimental effects on yield, but genetically and physiologically it is possible to heighten disease resistance while conserving plant fitness (Bechtold *et al.*, 2010). Research into the mechanisms of how plants successfully combine high disease resistance and high yield could open up new possibilities for the development of valuable crop species. Furthermore, priming for enhanced defense provides also an opportunity to protect plant species while minimizing the costs of resistance (Van Hulten *et al.*, 2006). Simultaneous activation of ISR and SAR provides an attractive tool for the improvement of crop species (Van Wees *et al.*, 2000). Overall, understanding of the functioning of the complex defense signaling network and the fitness costs involved is necessary for successful application of defense traits in crops. Therefore, molecular biologists and ecologists should join forces to place molecular mechanisms of inducible plant defenses in an ecological perspective.

OUTLINE OF THE THESIS

Inducible plant defenses are regulated by diverse plant hormones. SA is an important regulator of induced plant defenses against biotrophic pathogens. On the other hand, JA together with ET are important regulators of induced plant defense responses against necrotrophic pathogens, whereas JA together with ABA regulate induced plant defenses against herbivorous insects (Glazebrook, 2005; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). The hormonal signal signature is vital for a successful immune response upon attack, as extensive cross-communication between defense signaling pathways allows the plant to activate effective over ineffective defenses (Pieterse *et al.*, 2009). Ecological research revealed that inducible defenses entail fitness costs, since valuable resources are directed to defense instead of to growth and there are trade-offs with resistance to multiple attackers (Heil & Baldwin, 2002). The main goal of this research was to unravel whether hormonal crosstalk, by prioritizing effectual defenses, contributes to plant fitness under conditions of attack by multiple pathogens and herbivorous insects. Furthermore, the precise role of ABA and ET in modulation of JA signaling was investigated.

In Chapter 2, we investigated the role of ABA and ET in the activation of specific JA-dependent defenses during the Arabidopsis - *P. rapae* interaction by using Arabidopsis mutants. We show that ABA is required for both the activation of the MYC-branch and the suppression of the ERF-branch of the JA pathway in *P. rapae*-infested leaves. This antagonistic effect seems to be caused by MYC-mediated production of ABA. ABA can suppress the ERF-branch at the level of transcriptional activation at the GCC-box. Furthermore, ET has the capacity to suppress the *P. rapae*-induced MYC-branch, however, it is not produced during the *P. rapae*-Arabidopsis interaction, and thus does not play a significant role in the balance between the MYC- and the ERF-branch upon herbivory. Together, in this Chapter we provide evidence that during herbivory the JA pathway becomes dominated by the ABA co-regulated MYC-branch, thereby maximizing defenses against insects.

In Chapter 3, we investigated the differential activation of the JA response pathway in undamaged systemic leaves of *P. rapae*-infested plants. We show that feeding by *P. rapae* leads to priming of the MYC-branch of the JA pathway in systemic leaves, without fully activating costly JA-dependent defenses. Production of ABA upon secondary herbivore attack leads to elevated activation of defenses, which is associated with ABA-dependent enhanced resistance to *P. rapae* caterpillars. Together, we provide evidence that ABA is a crucial regulator of herbivore-induced resistance by activating primed JA-dependent defense responses upon secondary herbivore attack in Arabidopsis.

In Chapter 4, we tried to elucidate how Arabidopsis plants cope with multiple attackers and if pathway crosstalk contributes to enhance plant fitness under these

conditions. Induction of SA- or JA/ABA-dependent defense responses by the biotrophic pathogen *H. arabidopsidis* or the herbivorous insect *P. rapae*, respectively, was shown to reduce the level of JA/ET-dependent defense against subsequent infection with the necrotrophic pathogen *B. cinerea*, resulting in reduced resistance to *B. cinerea*. Despite this reduced resistance, there were no additional long-term negative effects on plant fitness. Furthermore, when plants were grown in dense competition stands to enlarge fitness effects of induced defenses, treatment with a combination of SA and JA did not cause additional negative effect on plant growth in comparison to the single hormone treatments. Taken together, this suggests that crosstalk might indeed be a cost-saving strategy.

In a field study, we observed that Arabidopsis plants that were infected with the biotrophic pathogen *H. arabidopsidis* displayed enhanced fitness, as evidenced by a 70% increase in seed production. In Chapter 5 we studied how environmental factors and disease pressure influence growth and seed production of Arabidopsis plants when they are infected with *H. arabidopsidis*. Under low nutrient availability and long-day conditions, Arabidopsis plants showed increased fitness after *H. arabidopsidis* infection, likely due to enhanced allocation of resources from the root to the shoot. The effects seemed most pronounced when disease pressure was low. Altogether, we provide evidence that interactions between plants and pathogens do not necessarily lead to negative fitness effects for the plants, which in this case might be associated with the biotrophic lifestyle of *H. arabidopsidis*.

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

ACKNOWLEDGEMENTS

The authors of this review are supported by VIDI grant no. 11281 of the Dutch Technology Foundation STW, which is part of the Netherlands Organization of Scientific Research, and ERC Advanced Investigator Grant no. 269072 of the European Research Council.

CHAPTER 2

Abscisic acid is essential for differential regulation of jasmonic acid-dependent defenses during Arabidopsis-insect interactions

Irene A Vos[1], Adriaan Verhage[1], Lewis G Watt[1], Ido Vlaardingerbroek[1], Robert C Schuurink[2], Corné MJ Pieterse[1], Saskia CM Van Wees[1]

[1] Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 80056, 3508 TB Utrecht, the Netherlands
[2] Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, P.O. Box 94215, 1090 GE Amsterdam, the Netherlands

ABSTRACT

In *Arabidopsis thaliana*, the jasmonic acid (JA) signaling pathway consists of two antagonistic branches that are regulated by MYC- and AP2/ERF-type transcription factors, respectively. Upon herbivory by *Pieris rapae*, the insect performance inhibiting MYC-branch is activated, while the insect preferred ERF-branch is concomitantly suppressed. Here, we investigated the modulating role of abscisic acid (ABA) and ethylene (ET) in this process. We show that herbivore-induced production of ABA is required for both the activation of the MYC-branch and the suppression of the ERF-branch, as exemplified by the expression patterns of the marker genes *VSP2* and *PDF1.2*, respectively. Exogenous application of ABA suppressed ectopic AP2/ERF-mediated *PDF1.2* expression in *35S::ORA59* plants. Moreover, the GCC-box promoter motif, which is required for JA/ET-induced activation of the ERF-branch genes *ORA59* and *PDF1.2*, was sufficient for ABA-mediated repression, suggesting that the antagonistic action of ABA on the ERF-branch is targeted at the GCC-box. Although application of gaseous ET counteracted *P. rapae*-induced activation of the MYC-branch and repression of the ERF-branch, induction of ET biosynthesis during infection with the necrotrophic pathogen *Botrytis cinerea* did not. In accordance, *P. rapae* caterpillars performed similarly on *B. cinerea*-infected plants and control plants. Together, these data indicate that ABA and ET levels regulate the balance between the MYC- and the ERF-branch of the JA response and that during herbivory the JA pathway becomes dominated by the ABA co-regulated MYC-branch, possibly to maximize defenses against the insect herbivore.

INTRODUCTION

In nature and under agricultural conditions, plants are a food source for over one million herbivorous insect species (Howe & Jander, 2008). The evolutionary arms race between plants and their herbivorous insect enemies has provided plants with a highly sophisticated defense system that can recognize and respond to insect movement, and insect feeding, which causes vibrations, wounding and contact with insect oral secretions. Conversely, insects can estimate the quality and suitability of the plant as a food source by contact chemoreceptors on the insect mouthparts, antennae and tarsi (Howe & Jander, 2008; Appel & Cocroft, 2014). Because plant defenses are costly, they are often only activated in case of insect or pathogen attack (Walters & Heil, 2007; Vos *et al.*, 2013a). The immune response activated by the plant upon attack is shaped by the induced production of diverse plant hormones. The quantity, composition and timing of the hormonal signal signature tailors the defense response specifically to the attacker at hand, thereby prioritizing effective over ineffective defenses, which can minimize fitness costs (De Vos *et al.*, 2005; Pieterse *et al.*, 2012; Vos *et al.*, 2013a).

Infection with necrotrophic pathogens or infestation with chewing herbivores triggers the production of the plant hormone jasmonic acid (JA; Creelman *et al.*, 1992; Penninckx *et al.*, 1996). Binding of JA to the F-box protein COI1, which is part of the JA receptor complex (Xie *et al.*, 1998; Yan *et al.*, 2009; Sheard *et al.*, 2010), targets JAZ proteins for degradation via the 26S proteasome pathway (Chini *et al.*, 2007; Thines *et al.*, 2007). In the uninduced state, JAZ proteins repress JA-responsive gene expression by binding to transcriptional activators, such as MYC2. Degradation of JAZ proteins results in derepression of JA-regulated genes.

Within the JA signaling pathway, two distinct, antagonistic branches of transcriptional regulation are recognized; the MYC-branch and the ERF-branch (hereafter referred to as such). Feeding by herbivorous insects activates the MYC-branch (Verhage *et al.*, 2011; Vos *et al.*, 2013b). This branch is controlled by the basic helix-loop-helix leucine zipper transcription factor MYC2, leading to transcription of hundreds of JA-responsive genes, including *VSP1* and *VSP2* that are robust marker genes of the MYC-branch (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004). Previously, it was shown that the transcription factors MYC3 and MYC4, which are phylogenetically closely related to MYC2, can also interact with JAZ proteins and act additively with MYC2 in the activation of JA-induced responses (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011). Furthermore, the triple mutant *myc2,3,4* was as susceptible as JA-nonresponsive *coi1-1* plants to the generalist herbivore *Spodoptera littoralis* (Fernández-Calvo *et al.*, 2011). The plant hormone abscisic acid (ABA) has been implicated in the modulation of the JA response (Anderson *et al.*, 2004; Bodenhausen & Reymond, 2007; Verhage *et al.*, 2011; Sánchez-Vallet *et al.*, 2012; Vos *et al.*, 2013b). In the ABA-deficient mutant

aba2-1, *VSP1* expression was reduced upon feeding by caterpillars of *Pieris rapae* (small cabbage white; Vos *et al.*, 2013b). Furthermore, ABA has been found to activate expression of primed JA-responsive genes in systemic tissue after feeding by *P. rapae* (Vos *et al.*, 2013b), indicating that ABA plays a co-regulating role in activation of the MYC-branch. The ERF-branch is activated upon infection with necrotrophic pathogens. The AP2/ERF-domain transcription factors ERF1 and ORA59 activate the transcription of a large set of ERF-branch genes, including *PDF1.2*, which is a robust marker gene of the ERF-branch. The expression of *ERF1*, *ORA59* and *PDF1.2* is impaired in both JA- and ethylene (ET)-unresponsive mutants, indicating that joint activation of JA and ET signaling is necessary for full expression of the ERF-branch (Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003; Pré *et al.*, 2008).

Recently, it was shown that the MYC-branch transcription factors MYC2, MYC3 and MYC4 interact with the ERF-branch transcription factors EIN3 and EIL1 and repress each other's transcriptional activity (Song *et al.*, 2014). Upon infestation with *P. rapae* caterpillars, the MYC-branch is activated, while the ERF-branch is concomitantly suppressed (Verhage *et al.*, 2011; Vos *et al.*, 2013b). In two-choice assays with *P. rapae* caterpillars, it was shown that the caterpillars prefer to feed from plants that express the ERF-branch over plants that express the MYC-branch or plants that show reduced expression of both branches (Verhage *et al.*, 2011). In *MYC2*-mutated *jin1* plants, *ORA59* and *PDF1.2* expression was highly upregulated after feeding by *P. rapae*, indicating that in wild-type plants, MYC2 represses *ORA59* and *PDF1.2* expression after feeding by *P. rapae* (Verhage *et al.*, 2011; Vos *et al.*, 2013b). Hence, the antagonism between the MYC- and the ERF-branch of the JA pathway is active during the Arabidopsis-*P. rapae* interaction, which makes it an excellent model to study the molecular basis of crosstalk between the MYC- and the ERF-branch of the JA pathway.

ABA-deficient mutants have been reported to be more susceptible to herbivory (Thaler & Bostock, 2004; Bodenhausen & Reymond, 2007; Dinh *et al.*, 2013) and more resistant to necrotrophic pathogens (Anderson *et al.*, 2004; Sánchez-Vallet *et al.*, 2012). Additionally, exogenously applied ABA had a positive effect on expression of the MYC-branch after feeding by *P. rapae* (Vos *et al.*, 2013b) and caused suppression of *PDF1.*2 induction after exogenous application of JA (Anderson *et al.*, 2004). ET insensitive mutants are in general more susceptible to necrotrophic pathogens and more resistant to herbivorous insects compared to wild-type plants (Van Loon *et al.*, 2006b). Hence, the interplay between the MYC- and the ERF-branch may allow the plant to activate a specific set of JA response genes that is required for an optimal defense against the attacker encountered (Pieterse *et al.*, 2012).

Although it is well established that ABA and ET play important roles in the regulation of JA-dependent defenses, the underlying mechanisms of how the MYC/ERF balance is regulated under plant-attacker conditions are not known. In this study,

we investigated the role of ABA and ET in the rewiring of the JA pathway during the Arabidopsis-*P. rapae* interaction. We provide evidence that ABA plays an essential modulating role in the activation of the MYC-branch and concomitant suppression of the ERF-branch during the Arabidopsis-*P. rapae* interaction.

RESULTS

ABA is required for activation of the MYC-branch and repression of the ERF-branch during *P. rapae* feeding

The JA-dependent transcriptional response of Arabidopsis to *P. rapae* feeding is predominantly regulated through activation of the MYC-branch of the JA pathway and concomitant suppression of the ERF-branch (Verhage *et al.*, 2011). Here, we investigated the role of ABA and ET in the differential expression of the MYC- and the ERF-branch upon feeding by *P. rapae*. Expression of the MYC-branch marker gene *VSP2* and the ERF-branch marker gene *PDF1.2* was monitored in wild-type Col-0, *MYC2*-impaired *jin1-7* (hereafter called *myc2*), *MYC2*, *MYC3*, *MYC4* triple mutant *myc2,3,4*, ABA biosynthesis mutant *aba2-1* and ET response mutant *ein2-1*. First-instar *P. rapae* caterpillars were allowed to feed for 24 h on the different Arabidopsis genotypes, after which they were removed. Expression levels of *VSP2* and *PDF1.2* in *ein2-1* plants resembled those in Col-0, showing strong *P. rapae*-induced transcription of *VSP2* at 24 h and 30 h, while *PDF1.2* levels were very low (Figure 1). *VSP2* transcript levels decreased to basal levels at 48 h, suggesting that stimulation of the MYC-branch was effective until at least 6 h after removal of the caterpillars. The *ein2-1* plants did differ from Col-0 at 24 h, showing an enhanced transcription level of *VSP2* (Figure 1). In *myc2* and *aba2-1* mutants, the transcriptional patterns of *VSP2* and *PDF1.2* were opposite to those observed in Col-0, with low *VSP2* expression and high *PDF1.2* expression up to 30 h. In *myc2,3,4* mutants, the expression of *VSP2* was almost zero. *PDF1.2* levels in *myc2,3,4* plants were similar to Col-0 up to 30 h, but increased significantly at 48 h (Figure 1). Together these results indicate that the MYC transcription factors function as a rewiring switch between the two branches of the JApathway, whereby *myc2,3,4* plants show a delay in expression of the ERF-branch. Furthermore, ABA is essential for activation of the MYC-branch and repression of the ERF-branch upon *P. rapae* feeding, while ET signaling is minimally involved in steering the MYC/ERF-balance during herbivory.

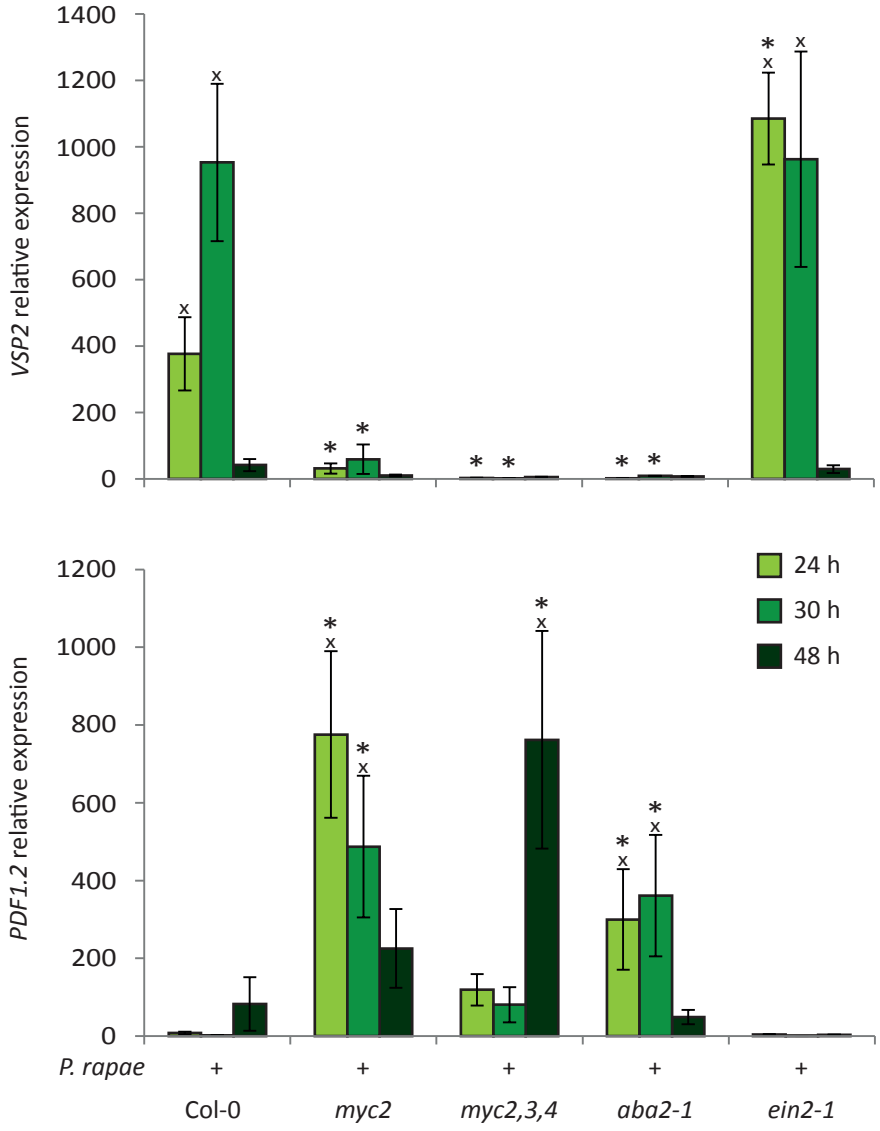


Figure 1: Differential JA-responsive gene expression in *P. rapae*-infested Arabidopsis plants.
RT-qPCR analysis of *VSP2* and *PDF1.2* gene expression in *P. rapae*-infested leaves of Col-0, *myc2*, *myc2,3,4*, *aba2-1* and *ein2-1* plants relative to non-infested Col-0 plants. First-instar *P. rapae* caterpillars were allowed to feed for 24 h after which they were removed. Infested leaves were harvested at the indicated time points. Crosses indicate a statistically significant difference with the non-infested control of the same line at the same time point (expression data of non-infested controls are not shown). Asterisks indicate a statistically significant difference with Col-0 at the same time point (two-way ANOVA; $P<0.05$). Error bars represent SE, $n=3$ plants

ABA antagonizes the ERF-branch by suppressing activation of the GCC-box

To further investigate the role of ABA in the regulation of the differential JA response upon feeding by *P. rapae*, we determined the effect of exogenously applied ABA on the *P. rapae*-induced expression levels of *VSP2* and *PDF1.2*. Application of 100 µM ABA alone did not induce or repress the expression of *VSP2* or *PDF1.2* in any of the tested lines or at any of the time points tested (Figure 2, Supplemental Figure 1 & data not shown). However, caterpillar-induced transcription levels of *VSP2* were significantly enhanced in Col-0, *aba2-1* and *ein2-1* plants when ABA was applied to the plants 24 h prior to *P. rapae* infestation (Figure 2). This ABA-mediated enhancement of *P. rapae*-induced *VSP2* expression was not observed in *myc2* and *myc2,3,4* plants. These results indicate that ABA acts positively on the *P. rapae*-induced MYC-branch, in a MYC-dependent manner. Conversely, ABA application diminished the high *P. rapae*-induced *PDF1.2* transcript levels in *myc2* and *aba2-1* plants. In *myc2,3,4* plants, *PDF1.2* levels were also significantly reduced by ABA at 48 h (Supplemental Figure 1), but not yet significantly at 30 h (Figure 2). This indicates that ABA antagonizes the activation of the ERF-branch in a MYC-independent manner.

Subsequently, we tested if ABA can suppress *PDF1.2* activation downstream of ORA59. To this end, we used a *35S::ORA59* overexpression line to drive constitutive expression of *PDF1.2*. The expression pattern for *VSP2* in the *35S::ORA59* line was similar to that in Col-0 after feeding by *P. rapae* and application of ABA (Figure 3A). *ORA59* levels were constitutively high in the *35S::ORA59* plants and were not influenced by *P. rapae* or ABA treatment. As expected, *PDF1.2* was constitutively expressed in untreated *35S::ORA59* plants and increased even further upon feeding by *P. rapae*, which likely can be ascribed to elevated JA levels in response to herbivory. Application of ABA significantly repressed *PDF1.2* levels in *35S::ORA59* in *P. rapae*-infested plants and, although not significant, also in non-infested plants (Figure 3A), suggesting that ABA antagonizes *PDF1.2* expression downstream of ORA59.

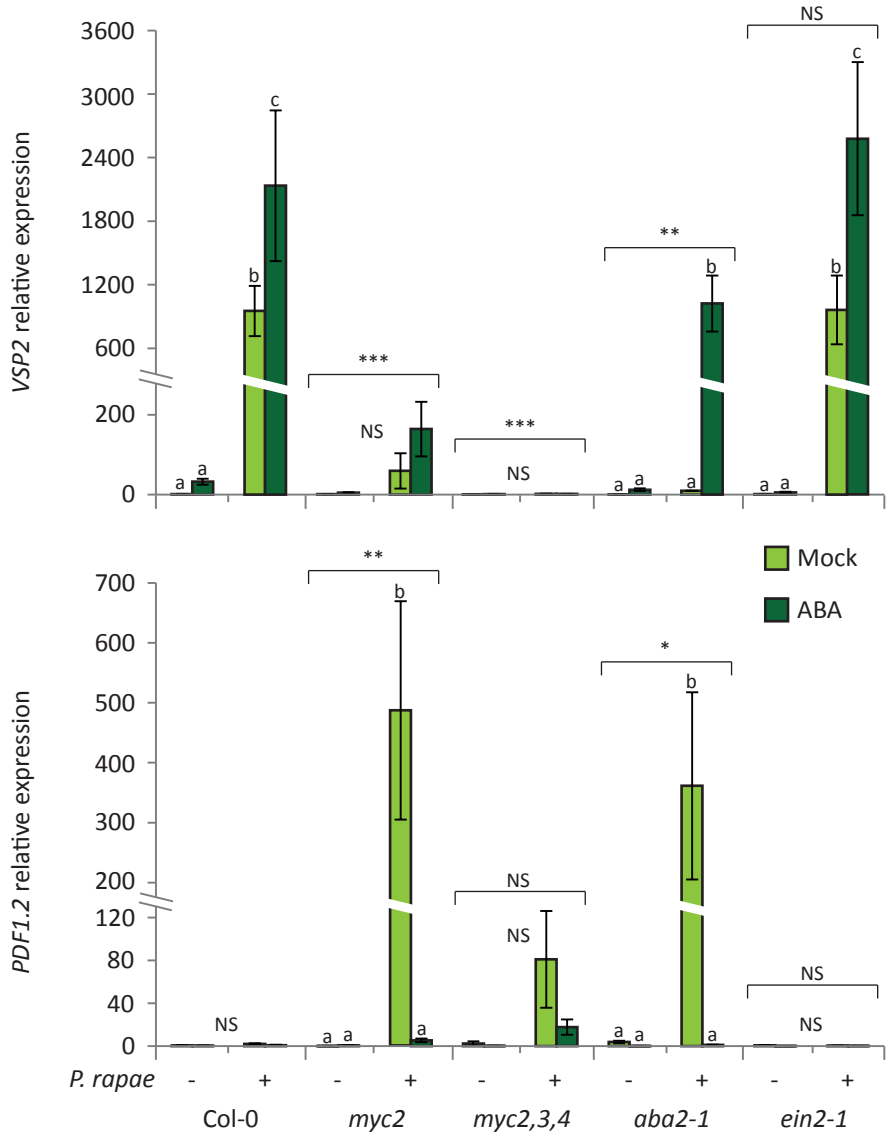
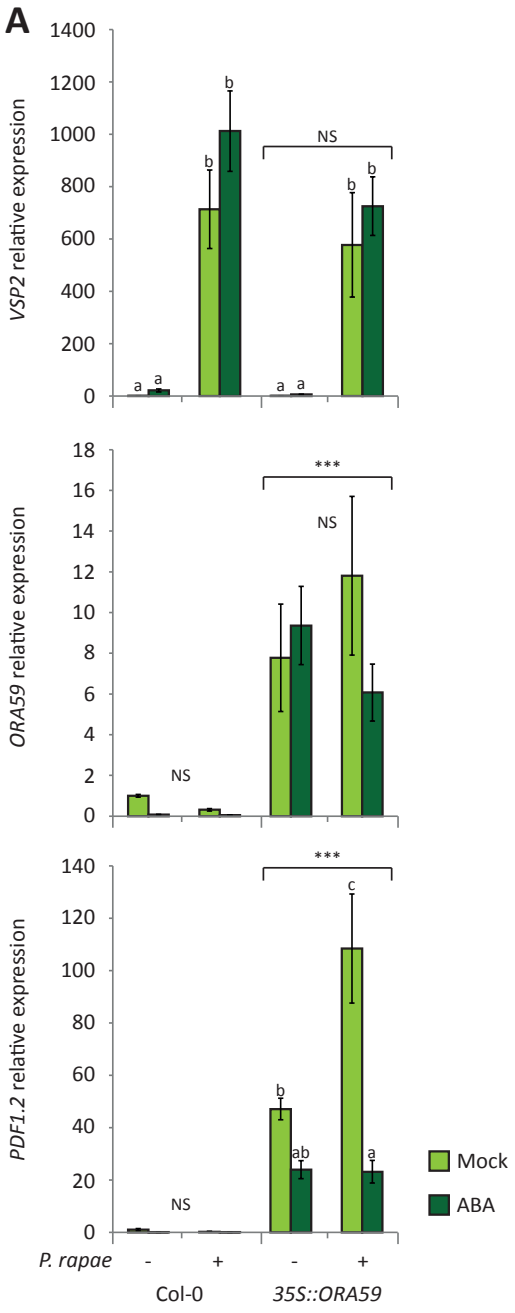
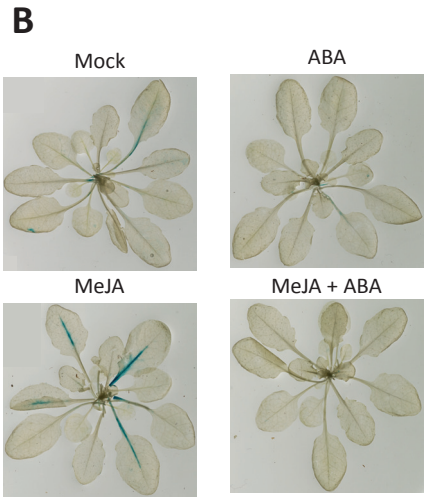
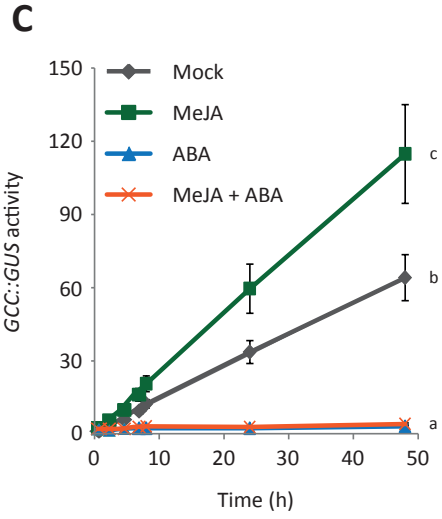


Figure 2: Effect of ABA treatment on *P. rapae*-induced *VSP2* and *PDF1.2* expression.
RT-qPCR analysis of *VSP2* and *PDF1.2* gene expression at 30 h in leaves of Col-0, *myc2*, *myc2,3,4*, *aba2-1* and *ein2-1* plants that were treated with a mock solution or with 100 µM ABA 24 h prior to infestation with *P. rapae.* Indicated are expression levels relative to non-infested Col-0 plants. Different letters indicate statistically significant differences between treatments of one line. Indications above the brackets specify whether there is an overall statistically significant difference between the mutant line and Col-0 (two-way ANOVA; *** = $P<0.001$; ** = $P<0.01$; * = $P<0.05$; NS = not significant). Error bars represent SE, $n=3$ plants.


A
VSP2 relative expression
ORA59 relative expression
PDF1.2 relative expression
NS
***
a
b
c
ab
Mock
ABA
P. rapae
Col-0
35S::ORA59


B
Mock
ABA
MeJA
MeJA + ABA


C
GCC::GUS activity
Time (h)
Mock
MeJA
ABA
MeJA + ABA

The GCC-box motif that is present in the promoter regions of *ORA59* and *PDF1.2* has previously been shown to be sufficient for transcriptional activation by JA and suppression by salicylic acid (SA; Van der Does *et al.*, 2013). Here, we tested if the GCC-box is also targeted for suppression by ABA. Therefore, we used a transgenic *GCC::GUS* line containing 4 copies of the GCC-box fused to a minimal *35S* promoter and the *GUS* reporter gene (Zarei *et al.*, 2011) and treated the plants with 100 µM MeJA, 100 µM ABA or a combination of MeJA and ABA. After 24 h, GUS activity was determined. Both the histochemical staining (Figure 3B) and the quantification of the GUS activity (Figure 3C) showed that ABA, like SA (Van der Does *et al.*, 2013), could suppress the MeJA-induced activation of the GCC-box. These results indicate that the GCC-box is sufficient for ABA-mediated suppression of JA-responsive gene expression.

ET antagonizes the MYC-branch

Although the impact of ET signaling on the expression of the MYC- and the ERF- branch upon *P. rapae* feeding is not merely as great as that of ABA, we did observe that in *ein2-1* plants *VSP2* transcription was significantly enhanced at 24 h compared to Col-0 (Figure 1). To further explore whether activation of ET signaling could influence the expression of the differential JA response during *P. rapae* feeding, we exogenously applied gaseous ET before and during infestation of Col-0 and *ein2-1* plants by the caterpillars. Gene expression was monitored using northern blots. The probe used for detection of *VSP* gene expression detected both *VSP1* and *VSP2* (designated as *VSP1/2)*. Treatment with 1 ppm of gaseous ET induced the expression of *PDF1.2* in Col-0 (Figure 4). In combination with *P. rapae* feeding, the ET-induced expression of *PDF1.2* was further enhanced, which is likely due to synergism between ET and *P. rapae*-induced JA signaling. Furthermore, ET treatment strongly reduced the level of *P. rapae*-induced expression of *VSP1/2*, indicating that induced ET signaling can antagonize the MYC-branch. Both the stimulating effect of ET on *PDF1.2* and the suppressive effect of ET on

Figure 3: Suppression of *P. rapae*-induced *PDF1.2* expression and MeJA-induced *GCC::GUS* activity by ABA.
A) RT-qPCR analysis of *VSP2*, *ORA59* and *PDF1.2* gene expression at 30 h in leaves of Col-0 and *35S::ORA59* plants that were treated with a mock solution or with 100 µM ABA 24 h prior to infestation with *P. rapae*. Indicated are expression levels relative to untreated Col-0 plants. Different letters indicate a statistically significant difference between treatments of one line. Indications above the brackets specify whether there is an overall statistically significant difference between *35S::ORA59* and Col-0 (two-way ANOVA; *** = P<0.001; NS = not significant). Error bars represent SE, n=3 plants.
B, C) GUS activity of the *GCC::GUS* line. Plants were dipped in a solution containing 100 µM MeJA, 100 µM ABA, a combination of both chemicals or a mock solution and harvested after 24 h. B) Rosettes were stained for GUS activity or C) GUS activity in the leaves was quantified for 48 h using a microplate reader. Different letters indicate statistically significant differences between treatments (regression analysis; P<0.05). Error bars represent SE, n=4 plants.

VSP1/2 were absent in *P. rapae*-infested *ein2-1* plants, indicating that both ET-mediated processes are dependent on EIN2 and thus regulated via the ET signaling pathway.

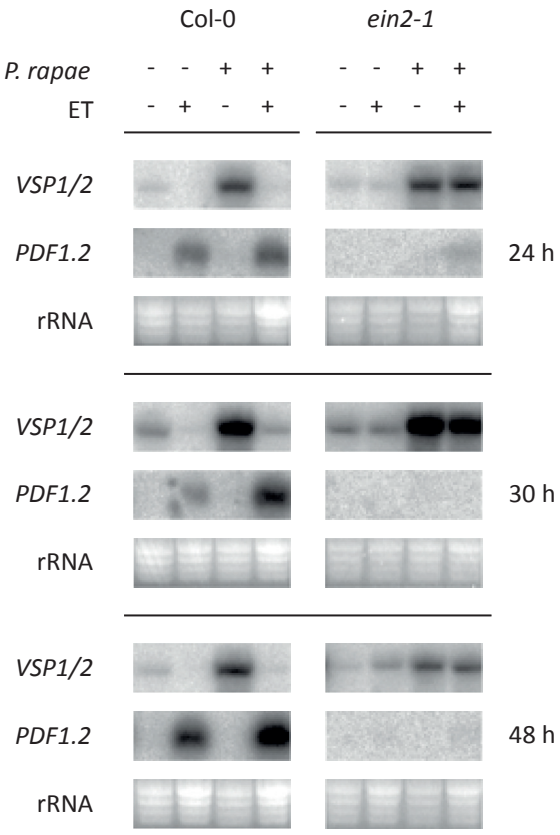


Figure 4: Effect of gaseous ET treatment on *P. rapae*-induced *VSP1/2* and *PDF1.2* expression
Northern blot analysis of *VSP1/2* and *PDF1.2* gene expression in leaves of Col-0 and *ein2-1* plants that were infested with *P. rapae* and treated with a continuous flow of gaseous ET (1 ppm) or ambient air (starting 24 h prior to infestation and continuing until tissue was harvested). First-instar caterpillars of *P. rapae* were allowed to feed for 24 h after which they were removed. Infested leaves were harvested at the indicated time points.

ABA and ET signaling differentially influence preference and performance of *P. rapae* caterpillars

Previously, Verhage *et al.* (2011) showed that *P. rapae* caterpillars prefer to feed from plants expressing the ERF-branch and are not deterred by plants expressing the MYC-branch. Here, we determined the effect of ABA and ET signaling on the preference of *P. rapae* by conducting two-choice assays. Two plants of each of the two genotypes tested were placed together in a two-choice arena. Leaves were inphysical contact with each other, which allowed the caterpillars to freely move from plant to plant. Two first-instar caterpillars were placed on each plant at the start of the assay (eight caterpillars per arena) and after 4 days the number of caterpillars per plant genotype was determined in 20-30 independent two-choice arenas. Significantly more *P. rapae* caterpillars were detected on *myc2* and *aba2-1* plants than on Col-0 wild-type (Figure 5A). This finding is in accordance with a preference of *P. rapae* caterpillars for plants that express the ERF-branch (Verhage *et al.*, 2011), like *myc2* and *aba2-1* do upon infestation by *P. rapae* (Figure 1). Mutant *ein2-1* plants that, like Col-0, expressed the MYC-branch and not the ERF-branch (Figure 1) accommodated a similar amount of caterpillars as Col-0 plants in a two-choice set-up. These results suggest that MYC2- and ABA-dependent suppression of the ERF-branch in wild-type Col-0 plants during feeding by *P. rapae* reduces the attraction to the caterpillars, whereas ET signaling is not influencing caterpillar preference.

To investigate whether the preference of *P. rapae* caterpillars for the ERF-branch-expressing *myc2* and *aba2-1* mutant plants coincides with increased performance of the caterpillars on these genotypes, we assessed their growth in no-choice assays with Col-0, *myc2*, *myc2,3,4*, *aba2-1*, *ein2-1*, and JA-nonresponsive *coi1-1* plants. One first-instar *P. rapae* caterpillar was placed on each plant and allowed to feed for 7 days, after which the caterpillar was weighed. Figure 5B shows that the caterpillars were significantly heavier when they fed from *myc2* than when they fed from Col-0 plants. On *aba2-1* mutants the caterpillars grew slightly better as well, but the difference with Col-0 was not significant. In contrast, on *ein2-1* mutants caterpillar growth was significantly inhibited. The growth of caterpillars on *myc2,3,4* and *coi1-1* mutants was highly increased, which corroborates that JA signaling is crucial for herbivore resistance. Next, we tested whether pretreatment of Col-0 plants with solutions of 100 µM MeJA, 100 µM ABA or 1µM of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) had an effect on caterpillar performance. MeJA or ABA pretreatment significantly reduced the weight of the caterpillars, whereas pretreatment with ACC did not have an effect (Figure 5C). These results indicate that, although caterpillars have a strong preference for the ERF-branch-expressing *myc2* and *aba2-1* plants, their performance is only slightly improved on these plants, which corresponds with the observation that the ERF-branch-activating ACC pretreatment had no effect on caterpillar performance. On the other hand, the MYC-branch is expressed in both Col-0 and *ein2-*

1 plants upon caterpillar feeding, but to a somewhat greater extent in *ein2-1* plants (Figure 1), which correlates with the reduced performance of the caterpillars on these plants. Furthermore, also the MYC-branch-activating MeJA and ABA pretreatments significantly reduced caterpillar performance (Figure 5C). In conclusion, enhancement of the ERF-branch results in caterpillar preference, whereas enhancement of the MYC-branch reduces caterpillar performance.

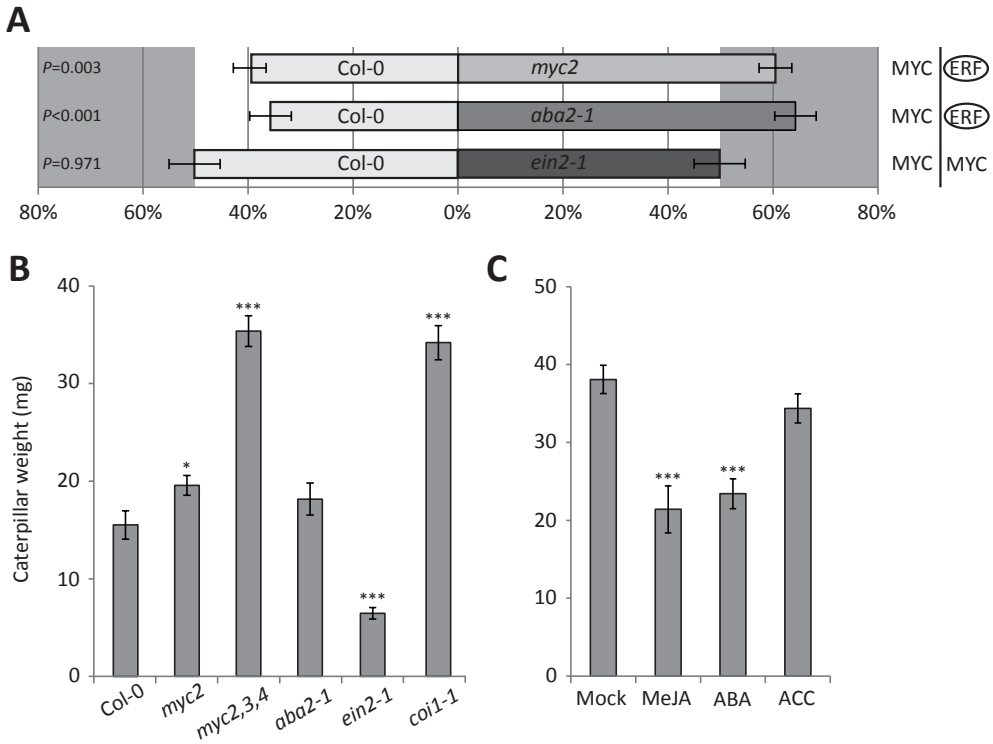


Figure 5: Effect of ABA and ET signaling on the preference and performance of *P. rapae.*

A) Caterpillar preference for Col-0 vs *myc2*, Col-0 vs *aba2-1* and Col-0 vs *ein2-1* plants. Two-choice arenas (*n*=20-30) consisted of two pots per genotype. In each two-choice arena, two first-instar *P. rapae* caterpillars were placed on the plants in each pot (total eight caterpillars per arena). After 4 days the number of caterpillars on each genotype was determined. The right panel displays which branch of the JA pathway is predominantly activated in the corresponding genotypes that are displayed in the left panel. Displayed are the average percentages (±SE) of the distribution of the *P. rapae* caterpillars over the two genotypes (x-axis). *P*-values indicate a statistically significant difference from the 50% percentile (Student's *t*-test). In cases of statistically significant differences (*P*<0.05), the preferred branch of the JA pathway is marked with a circle. Experiments were repeated with similar results.

B, C) Caterpillar performance on Col-0, *myc2*, *myc2,3,4*, *aba2-1*, *ein2-1* and *coi1-1* plants (B) and on Col-0 plants treated with a mock solution, 100 µM MeJA, 100 µM ABA or 1 µM ACC (C). The solutions were applied as root-drench at 5 and 2 days before caterpillar feeding. One first-instar caterpillar of *P. rapae* was placed on each plant and allowed to feed for 7 days after which the weight was determined. Asterisks indicate a statistically significant difference in comparison to Col-0 or mock-treated plants (ANOVA, Tukey post-hoc tests; *** = *P*<0.001; * = *P*<0.05). Error bars represent SE, *n*=8-17.

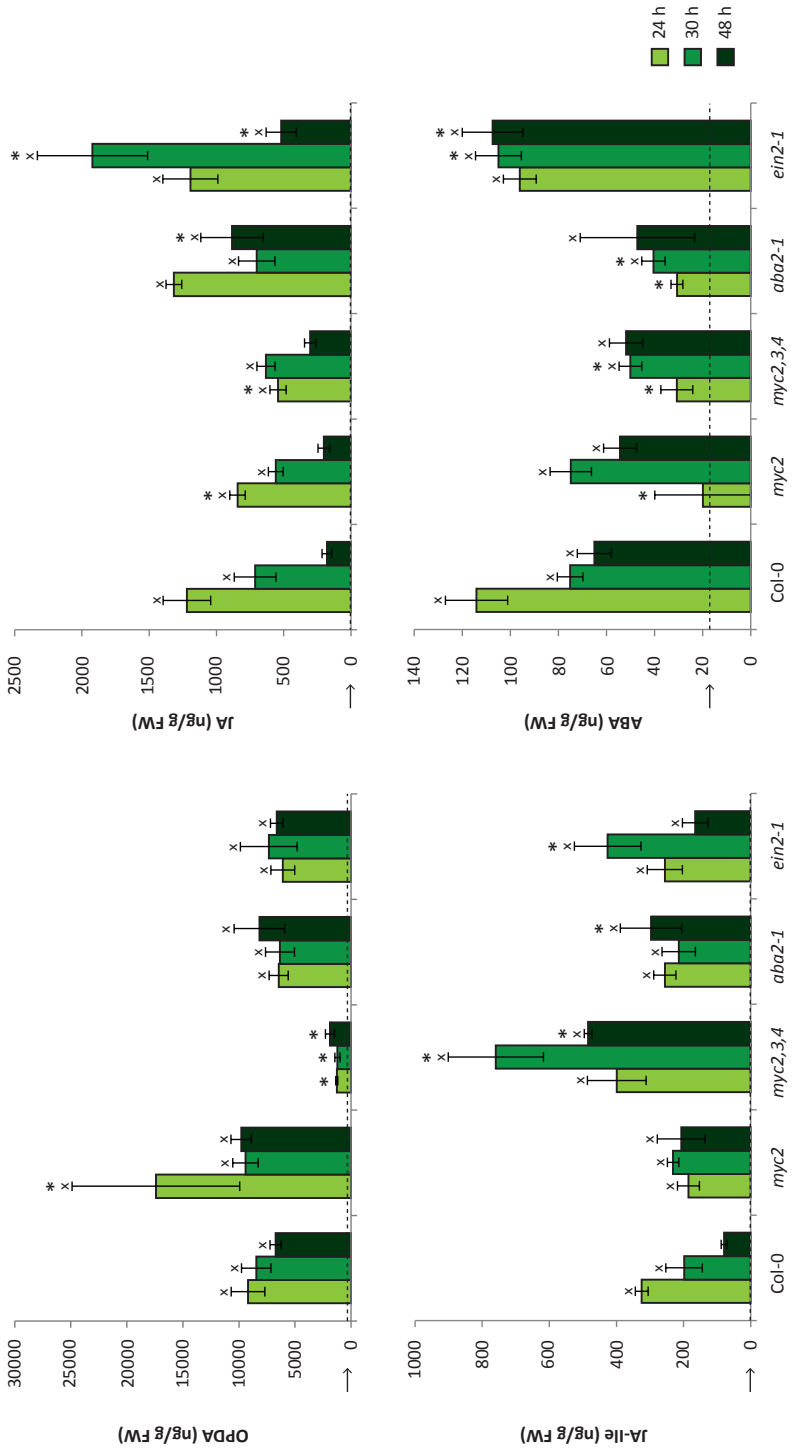
Signal signature of *P. rapae*-infested plant

To study whether the mutants used in this study are affected in herbivore-induced levels of JA, the biologically highly active conjugate JA-Ile, the JA-precursor OPDA or ABA, we monitored their accumulation in response to *P. rapae* feeding. Again, first-instar caterpillars were allowed to feed for 24 h after which they were removed from the leaves. Subsequently, hormone levels were measured in caterpillar-damaged leaves at different time points after caterpillar removal. Figure 6 shows that *P. rapae* feeding induced the accumulation of JA, JA- Ile, OPDA and ABA in Col-0 wild-type plants, confirming previous findings (Vos *et al.*, 2013b). In *ein2-1* plants OPDA and JA-Ile levels increased to a similar extent as in Col-0. However, *ein2-1* plants showed enhanced herbivore-induced levels of JA and ABA compared to Col-0, which correlates with the higher *VSP2* expression in this line upon *P. rapae* feeding (Figure 1). In *myc2* and *aba2-1* plants, the levels of JA, JA-Ile and OPDA raised to an almost similar extent as in Col-0 plants (Figure 6), indicating that the biosynthesis of JAs is only minimally affected by these mutations. In *myc2,3,4* plants, OPDA levels were significantly reduced at all time points after caterpillar feeding compared to Col-0. JA levels were also reduced in *myc2,3,4*, but only at 24 h. On the contrary, JA-Ile levels were significantly enhanced in *myc2,3,4* plants at 30 h and 48 h compared to Col-0. This suggests that the JA biosynthesis pathway is perturbed in the *myc2,3,4* plants, resulting in delayed biosynthesis of JAs. ABA levels were highly induced by *P. rapae* feeding in Col-0 at 24 h, but not in *myc2* and *myc2,3,4* plants. At later time points the ABA levels dropped in Col-0 and the differences between the *myc* mutants and Col-0 were no longer significant. These data suggest that herbivore-induced ABA biosynthesis is regulated via MYC transcription factors.

To monitor the emission of ET during *P. rapae* feeding, caterpillar-infested Col-0 plants were placed in 2-l air-tight cuvettes. The production of ET was 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 under climate chamber growth conditions. Figure7A shows that *P. rapae*-infested plants produced similar amounts of ET as non- infested plants, indicating that *P. rapae* feeding

Figure 6: Changes in the production of OPDA, JA, JA-Ile and ABA.

Absolute values (ng/ml/mg FW) of OPDA, JA, JA-Ile and ABA levels that were measured by Triple Quad LC/MS/MS in Col-0, *myc2*, *myc2,3,4*, *aba2-1* and *ein2-1* plants. First-instar *P. rapae* caterpillars were allowed to feed for 24 h after which hormone levels were determined in leaves of non-infested control plants and caterpillar-damaged leaves. Arrows and horizontal dashed lines indicate the average values of non-infested control plants. Crosses indicate a statistically significant difference with the non-infested control of the same line. Asterisks indicate a statistically significant difference with Col-0 at the same time point (two-way ANOVA; $P<0.05$). Error bars represent SE, $n=4$ plants.


24 h
30 h
48 h
ABA (ng/g FW)
JA (ng/g FW)
JA-Ile (ng/g FW)
OPDA (ng/g FW)
Col-0
myc2
myc2,3,4
aba2-1
ein2-1

had no effect on ET production. On the other hand, infection with the necrotrophic fungus *B. cinerea* strongly enhanced the production of ET (Figure 7B).

***B. cinerea* does not antagonize the MYC-branch**

Next, we investigated whether the antagonism between the MYC- and the ERF-branch is influenced when plants have encountered a previous stress prior to insect herbivory, a situation that can frequently occur in nature. To test this, six leaves per Col-0 plant were inoculated with *B. cinerea* and 24 h later first-instar caterpillars were placed on the leaves. Caterpillars were allowed to feed for 24 h, after which they were removed. *B. cinerea* infection strongly induced the expression of *PDF1.2* (Figure 8A), indicating that the ERF-branch was activated. *P. rapae* infestation activated the MYC-branch as evidenced by enhanced transcription of *VSP2* (Figure 8A). Surprisingly, infection with *B. cinerea* prior to *P. rapae* infestation did not antagonize the *P. rapae*-induced activation of *VSP2*. In contrast, *P. rapae* infestation subsequent to *B. cinerea* infection suppressed the *B. cinerea*-induced activation of *PDF1.2* (Figure 8A). Accordingly, *P. rapae* performance was not altered on *B. cinerea* infected plants, compared to control plants (Figure 8B). Together, these results suggest that in this set-up Arabidopsis plants prioritize their defense to *P. rapae* infestation, even when they were first conditioned by *B. cinerea* infection to express the ERF-branch of the JA pathway.

DISCUSSION

The complex plant immune regulatory network that is activated upon recognition of attackers is largely controlled by plant hormones (Pieterse *et al.*, 2012). JA has a decisive regulatory role in the defense responses against herbivorous insects and necrotrophic pathogens (Howe & Jander, 2008; Pieterse *et al.*, 2012). The MYC-branch of the JA signaling pathway is induced by feeding of herbivorous insects while necrotrophic pathogens activate the ERF-branch of the JA pathway. Several studies indicated that ABA co-regulates the JA-induced activation of the MYC-branch, while ET co-regulates activation of the ERF-branch (Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003; Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Pré *et al.*, 2008; Verhage *et al.*, 2011; Vos *et al.*, 2013b). ABA-deficient mutants are in general more susceptible to herbivory (Thaler & Bostock, 2004; Bodenhausen & Reymond, 2007; Dinh *et al.*, 2013) and more resistant to necrotrophic pathogens (Anderson *et al.*, 2004; Sánchez-Vallet *et al.*, 2012), while ET insensitive mutants are in general more resistant to herbivorous insects and more susceptible to necrotrophic pathogens (Van Loon *et al.*, 2006b). Previously, Verhage *et al.* (2011) showed that feeding of *P. rapae* caterpillars on Arabidopsis leads to activation of the MYC-branch while the herbivore-preferred ERF-branch is strongly suppressed.

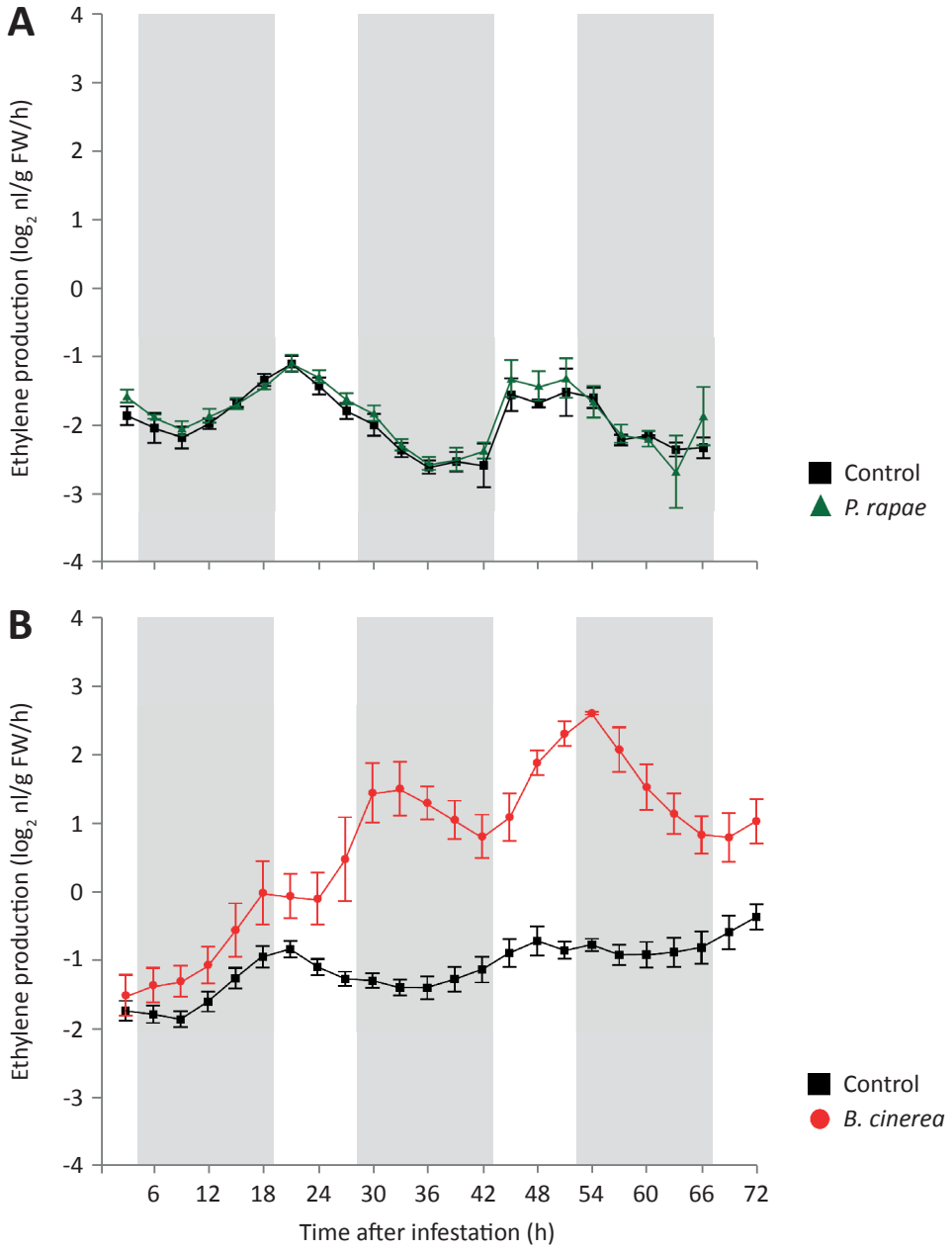


Figure 7: ET production of Col-0 plants during herbivory by *P. rapae* and infection with *B. cinerea*.
ET production was monitored in consecutive 3-h time intervals. Col-0 plants were infested with first-instar caterpillars of *P. rapae* (caterpillars fed on the leaves for the duration of the experiment) 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 represent SE, *n*=6. White areas indicate the light period, shaded areas indicate the dark period.

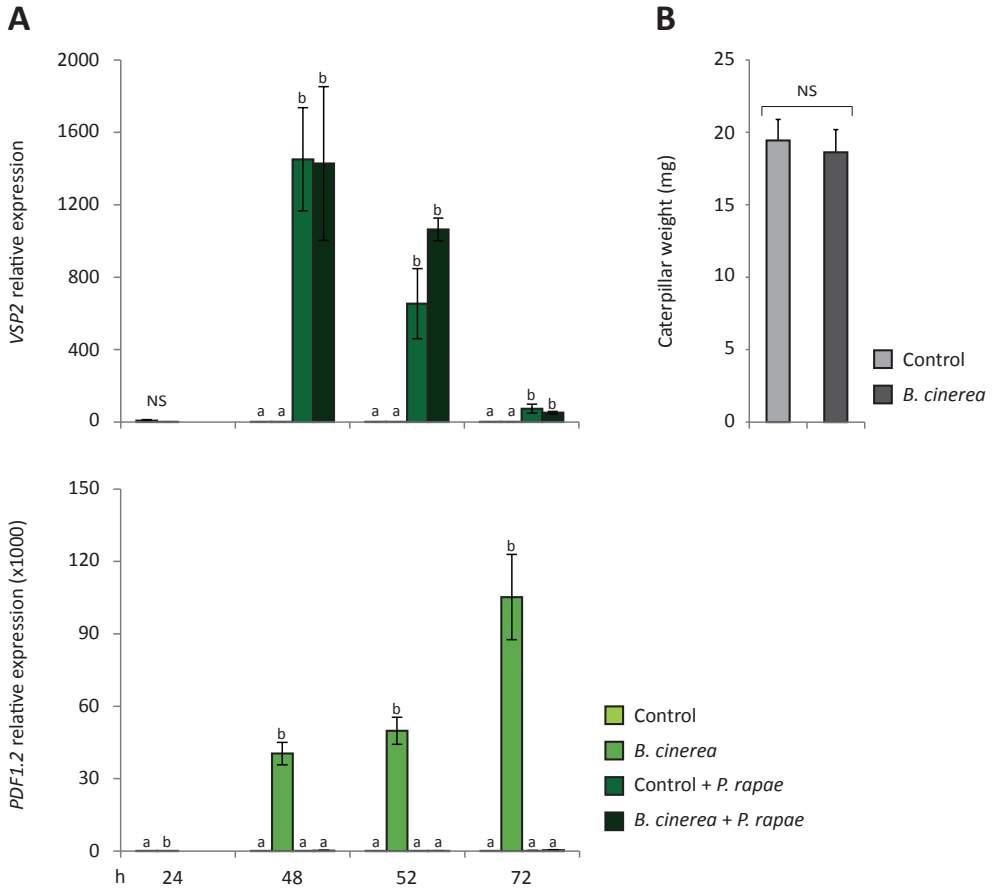


Figure 8: Effect of *B. cinerea* infection on *P. rapae*-induced gene expression.
A) RT-qPCR analysis of *VSP2* and *PDF1.2* gene expression in leaves of Col-0 control plants and leaves infected with *B. cinerea* 24 h prior to infestation with *P. rapae*. Indicated are expression levels relative to untreated Col-0 plants. Different letters indicate statistically significant differences between the treatments at the indicated time point (ANOVA, Tukey post-hoc tests; $P<0.05$; NS = not significant). Error bars represent SE, $n=3$ plants.
B) Caterpillar performance on Col-0 control plants or plants treated with *B. cinerea*. One first-instar caterpillar of *P. rapae* was placed on each plants and allowed to feed for 7 days after which the weight was determined (Student's *t*-test; NS = not significant). Error bars represent SE, $n=23$-28.

Here, we show that ABA and ET are important regulators of the antagonism between the MYC- and the ERF-branch during the Arabidopsis-*P. rapae* interaction.

The role of ABA in the balance between the MYC- and the ERF-branch during herbivory
In wild-type Col-0 plants, *P. rapae* feeding enhanced the production of JAs, as well as that of ABA (Figure 6 & Figure 9; Vos *et al.*, 2013b). Also in maize plants, increased

ABA biosynthesis has been demonstrated upon herbivory (Erb *et al.*, 2009). We show that *aba2-1* plants fail to activate the MYC-branch, measured as reduced activation of *VSP2* after *P. rapae* feeding (Figure 1). Importantly, *aba2-1* plants are also deficient in suppression of the ERF-branch, apparent from enhanced activation of *PDF1.2* after *P. rapae* feeding (Figure 1). Since *aba2-1* plants differ from Col-0 in the production of ABA but not of JAs (Figure 6), it seems plausible that in wild-type plants ABA is essential for shifting the MYC/ERF balance towards the MYC-branch upon herbivory. An important role for ABA in fine-tuning of the JA response during herbivory was confirmed by the observation that exogenous application of ABA to Col-0 plants stimulated the MYC-branch, while in *myc2* and *myc2,3,4* plants, that highly express the ERF-branch upon *P. rapae* feeding, ABA strongly inhibited the ERF-branch (Figure 2 & Supplemental Figure 1). In line with this, *B. cinerea* infection resulted in high expression of the ERF-branch, which was strongly suppressed by subsequent *P. rapae* feeding (Figure 8A), likely due to enhanced ABA levels upon *P. rapae* feeding. Exogenous ABA by itself did not alter the expression of the marker genes *VSP2* and *PDF1.2* at any of the time points investigated (Figure 2, Supplemental Figure 1 & data not shown), indicating that ABA alone is not sufficient for influencing the expression levels of these marker genes, but requires additional activation of the JA pathway.

Analysis of the *35S::ORA59* transgenic line showed that ABA is able to suppress *PDF1.2* even when ectopic *ORA59* expression levels are constitutively high (Figure 3A). Previously, Van der Does *et al.* (2013) investigated the suppressive effect of SA on JA-induced *PDF1.2* expression. They found that the GCC-box, which is present in the promoter of *PDF1.2* and *ORA59* and required for their JA-responsive expression, is essential and sufficient for transcriptional suppression by SA. SA was shown to suppress accumulation of the *ORA59* protein, which may contribute to SA/JA crosstalk via the GCC-box. Similarly, we show here that ABA completely inhibits the MeJA-induced activation of the GCC-box (Figure 3B & C). Furthermore, Zander *et al.* (2014) showed that *ORA59* transcript levels can be suppressed by SA. We found that, comparable to *PDF1.2* levels in *myc2* plants upon feeding by *P. rapae* and in Col-0 plants after *B. cinerea* infection (Figure 2 & Figure 8A), also *ORA59* transcript levels were high in these situations. These high *ORA59* transcript levels could also be suppressed by applying ABA to *myc2* plants (Supplemental Figure 2) or by *P. rapae* feeding on wild-type plants (Supplemental Figure 3). The latter is likely due to enhanced ABA levels that are induced upon *P. rapae* feeding. Together, these data point towards a similar mechanism for SA-dependent and ABA-dependent suppression of the ERF-branch at the level of transcriptional regulation at the GCC-box.

Interestingly, besides *myc2* plants also *myc2,3,4* plants showed suppression of the ERF-branch by exogenous application of ABA to *P. rapae*-infested plants (Figure 2 & Supplemental Figure 1). This suggests that ABA can down regulate ERF-dependent JA

responses independently of these three MYC transcription factors and independently of the previously reported MYC2/MYC3/MYC4-EIN3/EIL1 protein-protein interactions (Song *et al.*, 2014). The ABA biosynthesis that was induced upon *P. rapae* feeding was largely dependent on MYC transcription factors, as indicated by basal ABA levels in *P. rapae*-infested *myc2* and *myc2,3,4* plants compared to Col-0 at 24 h (Figure 6). Hence, the MYC-independent suppressive effect of ABA on the ERF-branch in *P. rapae*-damaged tissue might be partly regulated through MYC-mediated induction of ABA (Figure 9).

The role of ET in the balance between the MYC- and the ERF-branch during herbivory

Continuous monitoring of the production of ET in *P. rapae*-infested Arabidopsis plants revealed that *P. rapae* feeding did not induce changes in the emission of ET in this set-up (Figure 7 & Figure 9). At 24 h, *ein2-1* plants showed enhanced activation of the MYC-branch after *P. rapae* feeding, measured as increased activation of *VSP2* (Figure 1). The production of JA and especially ABA was enhanced in the *ein2-1* plants compared to Col-0 upon *P. rapae* feeding (Figure 6), suggesting that in wild-type plants basal ET signaling can suppress herbivory-induced production of JA and ABA, which tempers the activation of the MYC-branch.

Stronger evidence for a role for ET in rewiring of the MYC/ERF balance was provided by the experiment in which gaseous ET was applied to the plants. This ET treatment led to activation of the ERF-branch during *P. rapae* feeding, while the MYC-branch was suppressed (Figure 4). 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. Infection with the necrotrophic pathogen *B. cinerea* induced ET emission (Figure 7), but in contrast to exogenously applied gaseous ET, *B. cinerea* infection was not able to suppress the MYC-branch upon *P. rapae* feeding (Figure 8). A likely explanation for the discrepancy between the application of gaseous ET and infection with *B. cinerea* is that the activation of the ERF-branch by *B. cinerea* infection was not strong enough or too temporarily to completely suppress the *P. rapae*-induced activation of the MYC-branch.

ET has been found to play a role in many plant species in resistance to herbivores (Von Dahl & Baldwin, 2007). However, although ET signaling has the potential to modulate the balance between the MYC- and the ERF branch in Arabidopsis, ET levels do not change upon feeding by *P. rapae* and the ET inducing *B. cinerea* infection was not able to suppress the MYC-branch upon *P. rapae* feeding. Therefore, ET signaling is unlikely to play a major role in the defense response of Arabidopsis to *P. rapae* feeding (Figure 9).

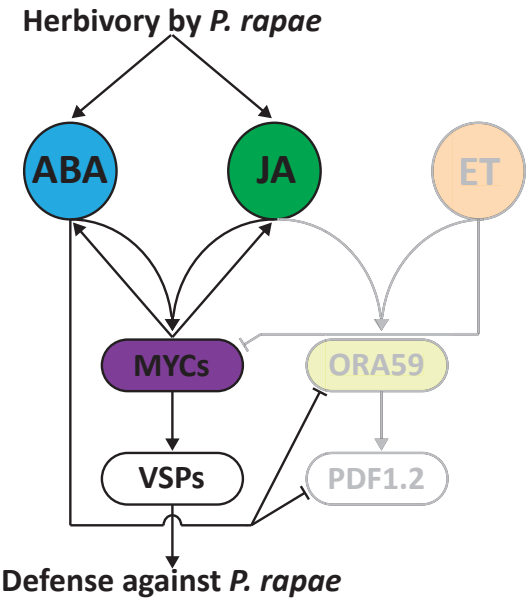


Figure 9: Model of differential regulation of JA responses during herbivory by *P. rapae*.
Feeding by *P. rapae* stimulates the production of JAs and ABA, resulting in activation of the MYC-branch and a concomitant defense response against *P. rapae*. Simultaneously, activation of the MYC-branch results in ABA-dependent suppression of the ERF-branch at the level of transcriptional activation at the GCC-box. This antagonistic effect seems to be caused by MYC-mediated production of ABA.
The ERF-branch components indicated in the model are shaded, because they are not activated during the Arabidopsis-*P. rapae* interaction. Although ET has the capacity to suppress the *P. rapae*-induced MYC-branch, it is not produced during the *P. rapae*-Arabidopsis interaction, and thus does not play a significant role in the MYC/ERF-branch interaction model during infestation. Arrows indicate a stimulating effect, whereas the blocked line indicates a suppressive effect.

The differential role of the MYC- and the ERF-branch on preference and performance of *P. rapae* caterpillars

The importance of ABA on herbivore resistance became further apparent from two-choice assays, in which *P. rapae* caterpillars were found to prefer to feed from the *aba2-1* and *myc2* plants over wild-type Col-0 plants (Figure 5A). These findings confirm previous results that *P. rapae* caterpillars prefer to feed from plants expressing the ERF-branch (Verhage *et al.*, 2011). Müller *et al.* (2010) showed that *P. rapae* caterpillars preferred to feed from plants containing glucosinolates. The *myc2* mutant was shown to have increased glucosinolate levels upon herbivory by *P. rapae* (Verhage *et al.*, 2011). On the contrary, the *myc2,3,4* mutant was recently found to contain <1% of wild-type levels of glucosinolates, which was associated with a preference for Col-0 over *myc2,3,4* by specialist *Pieris brassicae* caterpillars (Schweizer *et al.*, 2013). Therefore, beside activated ERF-signaling also glucosinolate accumulation might determine the feeding preference of specialist caterpillars.

The feeding preference of *P. rapae* caterpillars for *aba2-1* and *myc2* plants was not obviously correlated with enhanced performance on these mutants in no-choice assays (Figure 5B), which corresponds with the observation that the ERF-branch activating *B. cinerea* infection or ACC pretreatment had no effect on caterpillar performance (Figure 5C & Figure 8B). On the other hand, *P. rapae* caterpillars showed no preference for either *ein2-1* or Col-0 plants when given a choice between those two genotypes in a

two-choice assay (Figure 5A). However, performance of *P. rapae* caterpillars was highly reduced on *ein2-1* plants (Figure 5B), which corresponds with the observation that the MYC-branch activating MeJA or ABA treatment significantly reduced caterpillar performance (Figure 5C). Together, these results indicate that enhancement of the ERF-branch results in strong caterpillar preference, but caused only a minimal effect on caterpillar performance, whereas enhancement of the MYC-branch does not influence caterpillar preference, but has a strong negative effect on caterpillar performance.

All together, the results indicate that there is plasticity in the antagonistic MYC/ERF-branch interaction during JA-dependent defense signaling activated by attackers, which is regulated by ABA and ET levels and upon herbivory is dominated by the ABA co-regulated MYC-branch. This study highlights the interplay between JA on the one hand, and ABA and ET on the other hand, in shaping the outcome of the defense response that is triggered upon herbivory. By prioritizing the MYC-branch over the ERF-branch during insect herbivory, Arabidopsis is capable of focusing its JA-induced response to defenses that contribute to maximizing the chance of survival.

MATERIAL & METHODS

Plant material and cultivation

Seeds of *Arabidopsis thaliana* accession Col-0 and mutants *jin1-7* (*myc2*), *myc2,3,4*, *aba2-1*, *ein2-1* and *coi1-1* (Koornneef *et al.*, 1982; Feys *et al.*, 1994; Alonso *et al.*, 1999; Lorenzo *et al.*, 2004; Fernández-Calvo *et al.*, 2011) and the transgenic lines *35S::ORA59* and *GCC::GUS* (Pré *et al.*, 2008; Zarei *et al.*, 2011) were sown on river sand. Two weeks later, seedlings were transplanted into 60-ml 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 a 10-h day and 14-h night cycle at 70% relative humidity and 21°C. Plants were watered every other day and received 10 ml of half-strength Hoagland solution (Hoagland & Arnon, 1938) containing 10 µM sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Pieris rapae assays

Pieris rapae (small cabbage white) was reared on white cabbage plants (*Brassica oleracea*) as described (Van Wees *et al.*, 2013). First-instar caterpillars were used in all experiments. For gene expression analysis, two caterpillars were placed on fully expanded leaves of 5-week-old Arabidopsis plants using a fine paintbrush. Caterpillars were removed 24 h later and leaves were harvested at different time points after infestation. Throughout the ET production measurement, caterpillars remained on the leaves for the entire assay.

For the two-choice assays, two or three *aba2-1* and *ein2-1* mutant plants (instead

of one plant), were grown in one pot to compensate for their smaller size. Biomass and leaf area were measured from a representable subset of 6-week-old plants before the start of the assay to verify that the amount of leaf tissue was equal among the different genotypes tested. Two pots containing Col-0 wild-type plants and two pots with the mutant were placed together in an arena. Two first-instar caterpillars were released on the plants in each pot in the arena (n=20-30), so that there were eight caterpillars per arena. The plants in the arena were in physical contact with each other, allowing the caterpillars to freely move through the arena. After 4 days, the number of caterpillars present on each genotype was monitored and the frequency distribution of the caterpillars over the different genotypes was calculated.

To examine caterpillar performance, a single first-instar caterpillar was placed on a 5-week-old plant inside a plastic cup covered with an insect-proof mesh to contain the caterpillars. After 7 days of feeding, caterpillars were weighed to the nearest 0.1 mg on a microbalance.

***Botrytis cinerea* inoculation**

Botrytis cinerea inoculations were performed with strain B05.10 (Van Kan *et al.*, 1997) as described previously (Van Wees *et al.*, 2013). *B. cinerea* solution was made into a final density of $1 \cdot 10^5$ spores/ml and 5 µL droplets of the spores were applied to six leaves of 5-week-old plants. Plants were used immediately for measurement of ethylene production or were placed under a lid for 24 h to increase relative humidity and stimulate the infection, after which *P. rapae* caterpillars were placed on the plants.

Chemical treatments

Plants were treated with MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands) or ABA (Sigma, Steinheim, Germany) by dipping plants in a solution containing either 100 µM MeJA, 100 µM ABA or a combination of both chemicals and 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands) 24 h before caterpillar feeding. MeJA and ABA solutions were diluted from a 1000-fold concentrated stock in 96% ethanol. The mock solution contained 0.015% Silwet L77 and 0.1% ethanol.

For caterpillar performance, plants were treated with 100 µM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), 100 µM ABA (Sigma, Steinheim, Germany) or 1 µM ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma, Steinheim, Germany) by applying 20 ml of the solutions to the plants as a root drench, 5 and 2 days before caterpillar feeding. MeJA and ABA solutions were diluted from a 1000-fold concentrated stock in 96% ethanol. The mock solution contained 0.1% ethanol.

Application of gaseous ET to the plants was performed as described previously (Millenaar *et al.*, 2005; Leon-Reyes *et al.*, 2009). In short, gaseous ET (100 µl/l; 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 µl/l ethylene, which was flushed continuously through glass cuvettes (13.5 x 16.0 x 29.0 cm) at a flow rate of 75 l/h and then vented to the outside of the building. The concentration of ET in the airflow was verified using gas chromatography. Five-week-old plants were placed separately 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, RT-qPCR and northern blot analysis

Total RNA was isolated as described (Oñate-Sánchez & Vicente-Carbajosa, 2008). SuperScript™ III Reverse Transcriptase was used to convert DNA-free total RNA into cDNA. 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. 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 to 95°C with a ramp speed of 1.0°C/min. Transcript levels were calculated relative to the reference gene At1g13320 (Czechowski *et al.*, 2005) using the $2^{-\Delta\Delta CT}$ method described previously (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008).

For northern blot analysis, 15 µg of RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook *et al.*, 1989), electrophoretically separated on 1.5% agarose gel, and blotted onto Hybond-N^+ membranes (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. Equal loading was confirmed by staining rRNA bands with ethidium bromide. Northern blots were hybridized with gene-specific probes for *PDF1.2* and *VSP1/2* (Leon-Reyes *et al.*, 2010b). After hybridization with α-^{32}P-dCTP-labeled probes, blots were exposed for autoradiography.

The AGI numbers of the studied genes are At5g24780 (*VSP1*), At5g24770 (*VSP2*), At1g06160 (*ORA59*) and At5g44420 (*PDF1.2*).

Jasmonates and ABA analysis

For JA, JA-Ile, OPDA and ABA concentration analysis, 50-100 mg of *P. rapae*-infested damaged leaves as well as undamaged leaves from non-infested control plants were ground. The extraction and hormone analysis was performed as previously described (López-Ráez *et al.*, 2010). At the start of the extraction 1 ml of cold ethylacetate containing D_6-SA (25 ng/ml) and D_5-JA (25 ng/ml) was added to the samples as an internal

standard 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: JA 209/59 (fragmented under 12V collision energy), JA-Ile 322/130 (fragmented under 19V collision energy), OPDA 291/165 (fragmented under 18V collision energy) and ABA 263/153 (fragmented under 9V 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. Analytes were quantified using standard curves made for each individual compound.

Ethylene measurements

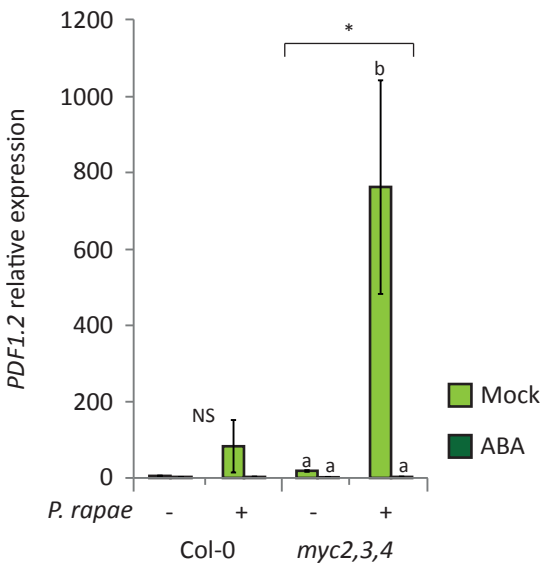
ET production was measured in a laser-driven photoacoustic detection system (ETD-300, Sensor Sense, Nijmegen, the Netherlands) connected to a 6-channel valve control box in line with a flow-through system (Voesenek *et al.*, 1990). Five-week-old plants were placed in 2-l air-tight cuvettes (four plants per cuvette), which were incubated under growth chamber conditions. After an acclimation time of 2 h, the cuvettes were continuously flushed with air (flow rate: 0.9 l/h), 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).

GUS assays

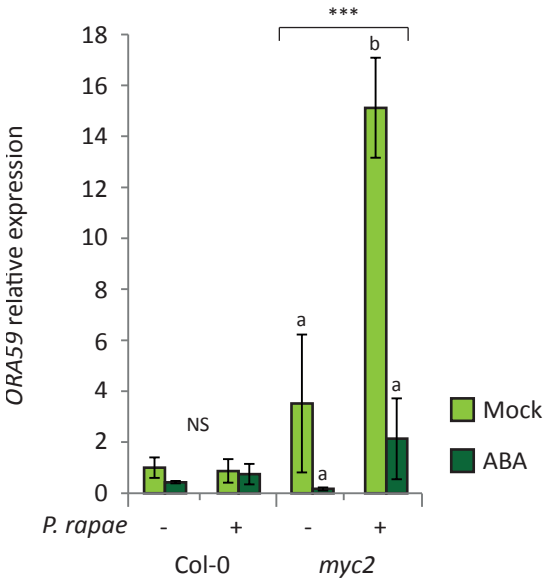
In the histochemical GUS assay, GUS activity was assessed by transferring plants to a GUS staining solution (1 mM X-Gluc, 100 mM NaPi buffer, pH 7.0, 10 mM EDTA and 0.1% [v/v] Triton X-100). After vacuum infiltration and overnight incubation at 37°C, the plants were destained by repeated washes in 96% ethanol (Spoel *et al.*, 2003). For the quantitative GUS assay, protein was isolated from frozen plant material and GUS activity was quantified using a microplate reader (BioTek Instruments, Inc., Winooski, United States of America) as described (Pré *et al.*, 2008).

ACKNOWLEDGEMENTS

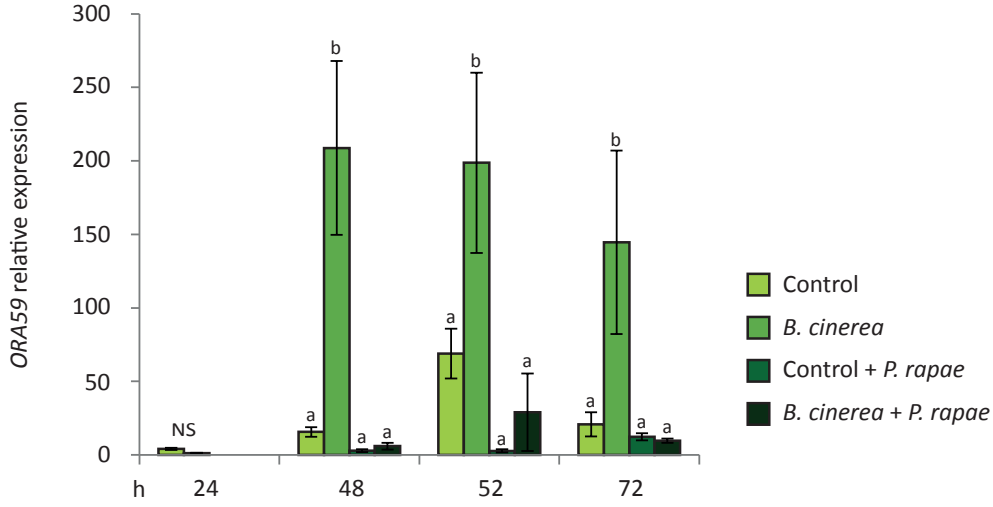
The authors thank Hans Van Pelt and Silvia Coolen for rearing of *P. rapae*, Rob Welschen and Ronald Pierik for their help with experiments on ET application and measurement and Michel de Vries for running the LC/MS/MS samples. This research was supported by VIDI grant no. 11281 of the Dutch Technology Foundation STW and VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which are part of the Netherlands Organization of Scientific Research (NWO), and ERC Advanced Investigator Grant no. 269072 of the European Research Council.



Supplemental Figure 1: Effect of ABA treatment on *P. rapae*-induced *PDF1.2* expression. RT-qPCR analysis of *PDF1.2* gene expression at 48 h in leaves of Col-0 and *myc2,3,4* plants that were treated with a mock solution or with 100 µM ABA 24 h prior to infestation with *P. rapae*. Indicated are expression levels relative to non-infested Col-0 plants. Different letters indicate statistically significant differences between treatments of one line. Indications above the brackets specify whether there is an overall statistically significant difference between *myc2,3,4* and Col-0 (two-way ANOVA; * = $P<0.05$; NS = not significant). Error bars represent SE, $n=3$ plants.



Supplemental Figure 2: Effect of ABA on *P. rapae*-induced *ORA59* expression. RT-qPCR analysis of *ORA59* gene expression at 24 h in leaves of Col-0 and *myc2* plants that were treated with a mock solution or with 100 µM ABA 24 h prior to infestation with *P. rapae*. Indicated are expression levels relative to mock-treated Col-0 plants. Different letters indicate statistically significant differences between treatments of one line. Indications above the brackets specify whether there is an overall statistically significant difference between *myc2* and Col-0 (two-way ANOVA; *** = $P<0.001$). Error bars represent SE, $n=3$ plants.



Supplemental Figure 3: Effect of *P. rapae* on *B. cinerea*-induced *ORA59* expression.
RT-qPCR analysis of *ORA59* gene expression in leaves of Col-0 control plants and leaves infected with *B. cinerea* 24 h prior to infestation with *P. rapae*. Indicated are expression levels relative to untreated Col-0 plants. Different letters indicate statistically significant differences between the treatments at the indicated time point (ANOVA, Tukey post-hoc tests; $P<0.05$; NS = not significant). Error bars represent SE, $n=3$ plants.

CHAPTER 3

Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid

Irene A Vos[1a], Adriaan Verhage[1a], Robert C Schuurink[2], Lewis G Watt[1], Corné MJ Pieterse[1], Saskia CM Van Wees[1]

[a] These authors contributed equally to this work

[1] Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 80056, 3508 TB Utrecht, the Netherlands

[2] Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, P.O. Box 94215, 1090 GE Amsterdam, the Netherlands

2013, *Frontiers in Plant Science* **4,** 539.

ABSTRACT

In *Arabidopsis thaliana*, the MYC2, MYC3 and MYC4 transcription factors on the one hand and the AP2/ERF transcription factors ORA59 and ERF1 on the other hand regulate distinct branches of the jasmonic acid (JA) signaling pathway in an antagonistic fashion, co-regulated by abscisic acid (ABA) and ethylene, respectively. Feeding by caterpillars of the specialist herbivore *Pieris rapae* results in activation of the MYC-branch and concomitant suppression of the ERF-branch in insect-damaged leaves. Here we investigated the differential JA signaling activation in undamaged systemic leaves of *P. rapae*-infested plants. We found that the *MYC2* transcription factor gene was induced both in the local insect-damaged leaves and the systemic undamaged leaves of *P. rapae*-infested Arabidopsis plants. However, in contrast to the insect-damaged leaves, the undamaged tissue did not show activation of the MYC-branch marker gene *VSP1*. Comparison of the hormone signal signature revealed that the levels of JA and (+)-7-*iso*-jasmonoyl-L-isoleucine raised to similar extents in locally damaged and systemically undamaged leaves, but the production of ABA and the JA precursor 12-oxo-phytodienoic acid was enhanced only in the local herbivore-damaged leaves, and not in distal undamaged leaves. Challenge of undamaged leaves of pre-infested plants with either *P. rapae* caterpillars or exogenously applied ABA led to potentiated expression levels of *MYC2* and *VSP1*, with the latter reaching extremely high expression levels. Moreover, *P. rapae*-induced resistance, as measured by reduction of caterpillar growth on pre-infested plants, was blocked in the ABA biosynthesis mutant *aba2-1*, that was also impaired in *P. rapae*-induced expression of *VSP1*. Together, these results suggest that ABA is a crucial regulator of herbivore-induced resistance by activating primed JA-regulated defense responses upon secondary herbivore attack in Arabidopsis.

INTRODUCTION

Plants possess sophisticated defense mechanisms to protect themselves against pathogens and herbivorous insects. These mechanisms include structural and chemical barriers that can be constitutively present in the plant or can be induced upon activation of the plant immune system. Recognition of the attacking organism induces local defense responses and the resistance induced is often extended to systemic tissue, thereby protecting undamaged distal plant parts against future attack. The plant hormones salicylic acid (SA) and jasmonic acid (JA) are major regulators of the induced defense signaling network controlling local as well as systemic resistance signaling events in roots and leaves (Pieterse *et al.*, 2012; Soler *et al.*, 2013). The SA-JA backbone of the immune signaling network can be modified by other hormones, such as ethylene (ET) and abscisic acid (ABA; Van Loon *et al.*, 2006b; Ton *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011). The hormone signal signature produced upon pathogen or insect attack depends on the stimuli perceived and determines the suite of attacker-specific defense responses that are activated in the plant (De Vos *et al.*, 2005; Pieterse *et al.*, 2009; Verhage *et al.*, 2010).

JA is an important hormone regulating the induction of defense responses to herbivorous insects and necrotrophic pathogens (Glazebrook, 2005; Howe & Jander, 2008). Infestation of *Arabidopsis thaliana* (Arabidopsis) with caterpillars of the specialist chewing herbivore *Pieris rapae* (small cabbage white) induces defense responses that inhibit *P. rapae* performance, resulting in reduced weight gain of the caterpillars on pre-infested plants (De Vos *et al.*, 2006). In many plant species it has been shown that this wound-induced resistance also extends systemically to undamaged plant parts (Howe & Jander, 2008). JA or one of its isoforms have been implicated as important signals in both root- and shoot-induced systemic defenses upon herbivory in various plant-herbivore interactions (Green & Ryan, 1972; Howe & Jander, 2008; Soler *et al.*, 2013). Depending on the hormonal context and the below- or above-ground origin of JA, different JA-dependent responses in systemic tissues are activated (Pieterse *et al.*, 2012; Tytgat *et al.*, 2013).

Disruption of plant tissue by herbivory triggers production of JA and its structurally related oxylipin derivatives (collectively called jasmonates (JAs); Mithöfer *et al.*, 2005). The F-box protein COI1 functions as a key regulator of JA signaling (Xie *et al.*, 1998). Mutant *coi1-1* plants are unresponsive to JAs and show alterations in the level of resistance to different herbivorous insects and necrotrophic pathogens (Van der Ent *et al.*, 2009b). (+)-7-*iso* jasmonoyl-L-isoleucine (JA-Ile) has been determined as the most biologically active form of JA (reviewed in Wasternack & Hause, 2013), however, the role of other oxylipin isoforms in activation of JA signaling has remained largely unknown. Work on OPR3-impaired *opr3* mutants revealed that the JA-precursor 12-oxo-phytodienoic acid

(OPDA) is a direct regulator of a distinct set of JA-responsive genes (Stintzi & Browse, 2000; Taki *et al.*, 2005; Böttcher & Pollmann, 2009), suggesting that additional oxylipins influence the final outcome of the JA response. Within the JA signaling pathway, two distinct, antagonistic branches of transcriptional regulation by JA are recognized; the MYC-branch and the ERF-branch (hereafter referred to as such). The MYC-branch, which is co-regulated by ABA, is activated upon feeding by herbivorous insects and is regulated by the basic helix-loop-helix leucine zipper transcription factors MYC2, MYC3 and MYC4, leading to the transcription of the *VSP1* and *VSP2* marker genes (Chapter 2; Anderson *et al.*, 2004; Thaler & Bostock, 2004; Lorenzo & Solano, 2005; Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011). The ERF-branch, which is co-regulated by ET, is activated upon infection with necrotrophic pathogens and is controlled by the AP2/ERF-domain transcription factors ERF1 and ORA59, leading to transcription of *PDF1.2*, a marker gene of the JA/ET-regulated ERF-branch (Penninckx *et al.*, 1998; Lorenzo *et al.*, 2004; Pré *et al.*, 2008).

ABA is known to have synergistic effects on the MYC-branch and antagonistic effects on the ERF-branch, as evidenced by effects of ABA on JA-induced transcriptional activation which was enhanced by ABA for *MYC2* and *VSP2* but suppressed by ABA for *PDF1.2* (Chapter 2; Anderson *et al.*, 2004). In line with these findings, ABA deficient mutants were reported to be more susceptible to herbivory (Thaler & Bostock, 2004; Bodenhausen & Reymond, 2007) and more resistant to necrotrophic pathogens (Anderson *et al.*, 2004; Sánchez-Vallet *et al.*, 2012). In *Nicotiana attenuata* plants, ABA has been shown to amplify JA-dependent defense responses as part of the signal transduction pathway that is elicited by oral secretions of *Manduca sexta* caterpillars (Dinh *et al.*, 2013). There have also been several reports on the role of ABA in systemic induced resistance triggered by diverse stimuli. It was hypothesized that ABA may function as a systemic signal in mediating above-ground resistance triggered by below-ground herbivory in maize (Erb *et al.*, 2009). However, it was proven in a subsequent study that the enhanced level of resistance was independent of ABA and instead was due to induced water stress in the plant upon root herbivory (Erb *et al.*, 2011). ABA was demonstrated to have a role in induced systemic resistance (ISR) that is elicited by below-ground beneficial rhizobacteria in Arabidopsis (Van der Ent *et al.*, 2009a). ABA signaling was involved in priming of above-ground defenses, as evidenced by enhanced callose deposition upon challenge of ISR-expressing tissue with the pathogen *Hyaloperonospora arabidopsidis*. A similar role for ABA was shown for induced resistance triggered by β-aminobutyric acid (BABA; Ton *et al.*, 2005).

In Arabidopsis, feeding by *P. rapae* caterpillars results in activation of the MYC-branch and concomitant suppression of the ERF-branch of the JA pathway in insect-damaged leaves (Chapter 2; Verhage *et al.*, 2011). In two-choice assays with *P. rapae* caterpillars and Arabidopsis plants it was shown that the caterpillars preferred plants

that express the ERF-branch of the JA pathway over plants that express the MYC-branch (Chapter 2; Verhage *et al.*, 2011). This suggests that suppression of the ERF-branch by activating the MYC-branch is part of the plant's defense strategy in this interaction. Here, we investigated the engagement of the MYC- and the ERF-branch in *P. rapae*-induced resistance in undamaged leaves. We provide evidence that undamaged leaves of herbivore-infested plants express elevated levels of *MYC2* mRNA, resulting in priming of the MYC-branch of the JA pathway. The enhancement in ABA levels, upon secondary herbivore attack, in the primed leaves mediates a potentiated expression of the MYC-branch resulting in enhanced expression levels of *VSP1*. This is associated with enhanced herbivore resistance in previously infested plants, which is shown to be ABA-dependent.

RESULTS

Effects of *P. rapae* feeding on differential JA-regulated responses in distal tissue

In Arabidopsis, *P. rapae* feeding locally activates the MYC-branch of the JA pathway while the ERF-branch is suppressed (Chapter 2; Verhage *et al.*, 2011). To investigate the expression of this differential JA response in undamaged (systemic) leaves of *P. rapae*-infested plants, we monitored the expression of the key transcription factor genes *MYC2* and *ORA59*, as well as their respective marker genes *VSP1/2*, and *PDF1.2*. The probe used for detection of *VSP* gene expression detected both *VSP1* and *VSP2* (designated *VSP1/2*). First-instar *P. rapae* caterpillars were allowed to feed for 24 h on Arabidopsis plants, after which the caterpillars were removed. Figure 1A shows that *MYC2* transcription was induced to high levels until 6 h after removal of the caterpillars (30 h), not only in locally damaged leaves that were eaten by the caterpillars, but also in systemic leaves that were not damaged. At later time points *MYC2* transcript levels decreased but remained elevated in comparison to non-infested control plants. The level of *P. rapae*-induced *MYC2* expression was strikingly similar in damaged and undamaged leaves. In locally damaged leaves of *P. rapae*-infested wild-type Col-0 plants, *MYC2* transcription coincided with activation of the MYC-branch marker genes *VSP1/2*, which peaked also at 6 h after removal of the caterpillars (Figure 1B). However, in systemic undamaged tissue *VSP1/2* transcription was remarkably lower. These results show that despite the fact that local and systemic tissues accumulated similar levels of *MYC2* transcripts, subsequent activation of the downstream target genes *VSP1/2* of the MYC-branch was severely reduced in systemic tissue.

In herbivore-damaged leaves, activation of the MYC-branch results in suppression of the ERF-branch (Chapter 2; Verhage *et al.*, 2011). *MYC2*-impaired *jin1-2* and *jin1-7* mutant plants thus displayed enhanced expression of the ERF-branch regulator *ORA59* and the ERF-branch marker gene *PDF1.2* in local insect-damaged leaves (Figure 1B &

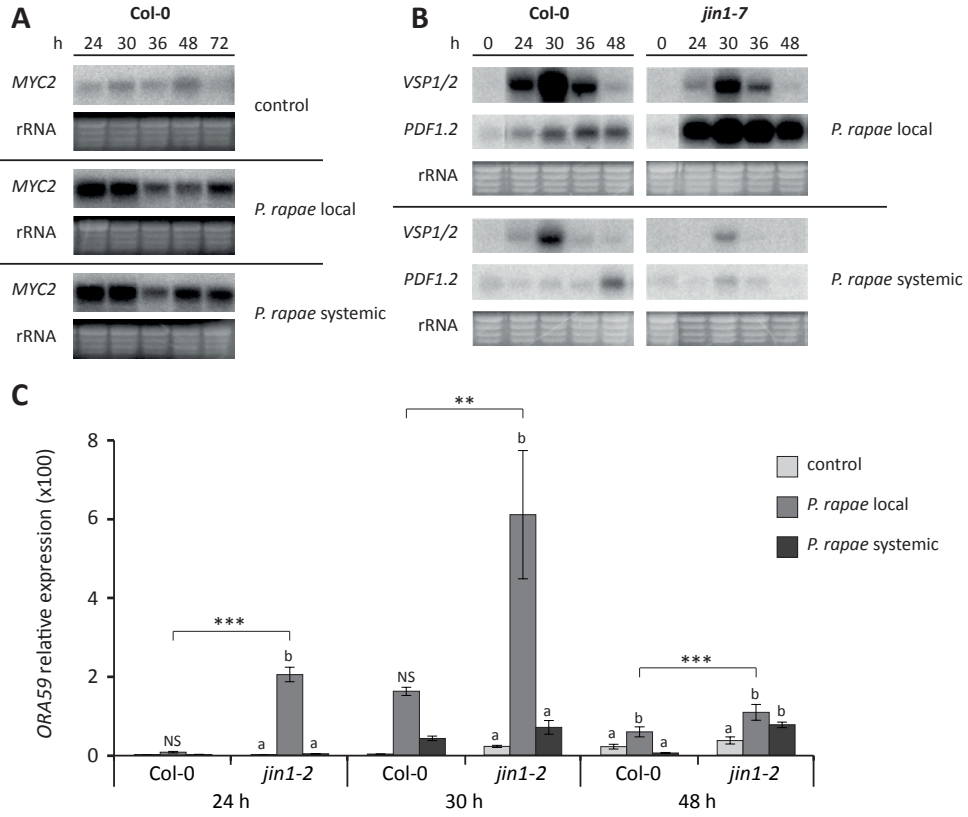


Figure 1: Differential JA responses in Col-0 and *MYC2*-impaired *jin1* plants in local damaged and systemic undamaged leaves upon herbivory by *P. rapae*.

(A) Northern blot analysis of *MYC2* transcript levels in non-infested (control) and *P. rapae*-infested Col-0 plants. First-instar caterpillars were allowed to feed for 24 h after which they were removed (24 h). Subsequently, damaged (*P. rapae* local) and undamaged (*P. rapae* systemic) leaf tissue was harvested for gene expression analysis.

(B) Northern blot analysis of JA-responsive *VSP1/2* and *PDF1.2* gene expression in damaged (*P. rapae* local) and undamaged (*P. rapae* systemic) tissue of *P. rapae*-infested Col-0 and MYC2-impaired *jin1-7* plants.

(C) RT-qPCR analysis of *ORA59* transcript levels (relative to non-infested Col-0 at 24 h) in non-infested (control) and *P. rapae*-damaged (*P. rapae* local) and –undamaged (*P. rapae* systemic) tissue of infested Col-0 and *jin1-2* plants. Asterisks indicate statistically significant differences between genotypes at specific time points (** $P<0.01$; *** $P<0.001$) and different letters indicate statistically significant differences between treatments within one genotype at specific time points ($P<0.05$; NS, not significant). Data were analyzed per time point using two-way ANOVA, error bars represent SE, $n=3$ plants.

C; Chapter 2; Verhage *et al.*, 2011). In systemic tissue of infested *jin1* mutant plants no elevation in ERF-branch activity was observed (Figure 1B & C). In fact, the levels of *PDF1.2* and *ORA59* expression were as low as in systemic tissues of Col-0 plants that were infested by *P. rapae*. Apparently, the ERF-branch of the JA pathway did not become activated in systemic leaves, neither in wild-type Col-0, nor in *jin1* mutant plants. Hence, the observed differences in JA-regulated gene expression in damaged versus undamaged leaves were confined to transcriptional activation of *MYC2*, without downstream consequences on *VSP1/2* induction and ERF-branch repression.

Different signal signatures in damaged versus undamaged leaves of *P. rapae*-infested plants

The arsenal of defense responses that is triggered by the JA pathway depends on the different isoforms of JA and on the hormonal context in which bioactive JAs are produced. 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, the biologically highly active amino acid conjugate JA-Ile, and ABA as it is a modulator of JA signaling and can mediate resistance to generalist herbivores. Again, first-instar caterpillars were allowed to feed for 24 h after which they were removed from the leaves. Hormone levels were measured 0, 6 and 24 h later (24, 30 and 48 h). JA, JA-Ile, OPDA and ABA levels increased significantly in locally *P. rapae*-damaged leaves (Figure 2). JA and JA-Ile levels also rose in systemic undamaged tissue of the same plants and reached similar levels as in herbivore-damaged leaves (Figure 2). In contrast, no rise in OPDA and ABA levels was detected in systemic undamaged tissue (Figure 2). These results demonstrate that the signature of JA, JA-Ile, OPDA and ABA as detected in damaged leaf tissue of herbivore-infested plants differs from that in distal undamaged tissue due to a lack in increase of ABA and OPDA.

P. rapae* feeding induces *MYC2* in undamaged leaves and primes for enhanced *P. rapae*- and ABA-induced *MYC2* and *VSP1

Previously, it has been demonstrated that systemic priming for enhanced JA-regulated defenses by ISR-inducing beneficial root-colonizing rhizobacteria is associated with enhanced expression of *MYC2* in above-ground plant parts, without a direct effect on the expression of downstream JA-responsive target genes (Pozo *et al.*, 2008; Van der Ent *et al.*, 2009a). Moreover, Abe *et al.* (2003) found that overexpression of *MYC2* primes the plants for enhanced sensitivity to ABA, resulting in enhanced expression of ABA-responsive genes upon exogenous ABA application. We therefore hypothesized that the observed systemic increase of *MYC2* transcripts in herbivore-damaged plants is part of a herbivore-induced priming response that may lead to an accelerated defense

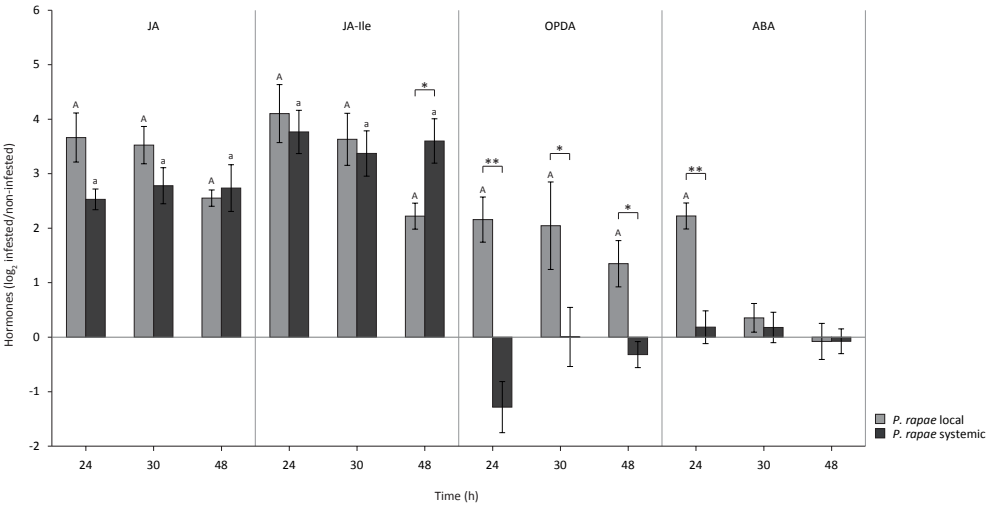


Figure 2: Production of JA, JA-Ile, OPDA and ABA in local damaged and systemic undamaged leaves of Col-0 plants upon herbivory by *P. rapae*.

Levels of JA, JA-Ile, OPDA and ABA in leaves of Col-0 plants on which *P. rapae* caterpillars had been allowed to feed for 24 h. Compound levels were measured by Triple Quad LC/MS/MS. Depicted in grey bars are log_2-transformed fold induction values of the compound levels (error bars represent SE, *n*=4 plants) in local damaged and systemic undamaged leaves of the same *P. rapae*-infested plants as compared to the levels in leaves of non-infested plants that were harvested at the same time points. Statistics were performed on log_2-transformed data. Letters indicate statistically significant differences in compound levels between leaves of non-infested control plants and damaged local leaves (capital letters) and undamaged systemic leaves (small letters) of *P. rapae*-infested plants. Data were analyzed per time point using Student's *t*-test ($P < 0.05$). Asterisks indicate statistically significant differences in compound levels between the damaged (local) and undamaged (systemic) leaves of *P. rapae*-infested plants. Data were analyzed per time point using Student's *t*-test (** $P < 0.01$; * $P < 0.05$).

response after secondary herbivore attack when ABA levels rise due to damage of the tissue. To investigate this, we monitored the expression of *MYC2* and *VSP1* at different time points in damaged and undamaged tissue of *P. rapae*-infested Col-0 leaves, before and after challenge with a secondary infestation by *P. rapae* or exogenous application of 10 µM ABA. Figure 3 shows that *MYC2* and *VSP1* genes were locally induced at 24 h after *P. rapae* caterpillars were placed on the leaves, after which transcription leveled off at 48 h. In systemic tissue, *MYC2* mRNA levels were increased 6-fold at 24 h and 20-fold at 48 h, whereas expression of the MYC2-regulated gene *VSP1* was not increased systemically. These findings are in line with the results shown in Figure 1. At 48 h, fresh *P. rapae* caterpillars were allowed to feed from the plants and this secondary infestation of previously undamaged leaves of infested plants resulted in enhanced expression of *MYC2* (2-fold) and especially *VSP1* (80-fold) compared to infestation of non- pre-infested plants. Also, when systemic leaves were challenge treated with exogenously

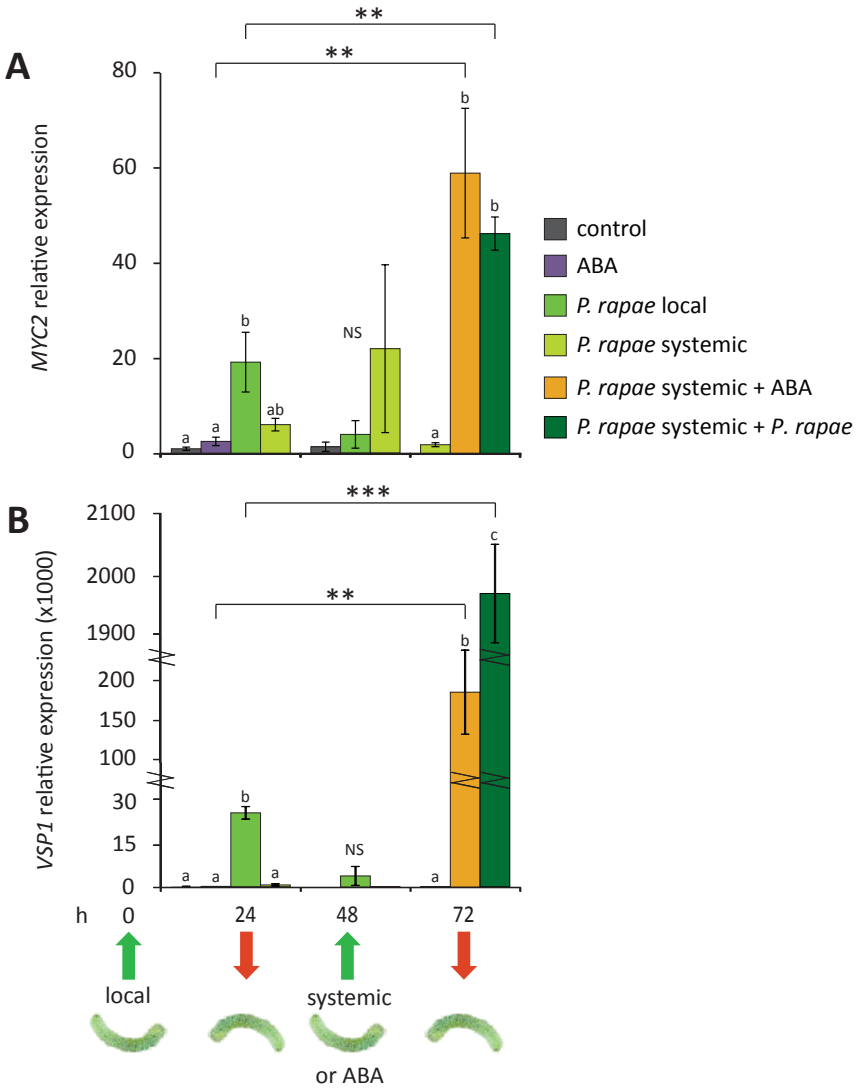


Figure 3: Effects of *P. rapae* feeding and ABA treatment on expression of the MYC-branch in undamaged leaves of pre-infested Col-0 plants.

RT-qPCR analysis of (A) *MYC2* and (B) *VSP1* transcript levels (relative to non-infested control at 24 h) in *P. rapae*-infested Col-0 plants. First-instar caterpillars were allowed to feed for 24 h after which they were removed (24 h). Damaged (*P. rapae* local) and undamaged (*P. rapae* systemic) leaves were harvested for analysis at different time points after removal of the *P. rapae* caterpillars. In addition, at 48 h, undamaged systemic leaves were challenged with fresh first-instar caterpillars or treated with 10 µM ABA. Treated systemic leaf tissue was harvested 24 h later (72 h) for gene expression analysis. For comparison, non-infested plants received ABA treatment as well. Different letters indicate statistically significant differences between treatments at specific time points (ANOVA, Tukey post-hoc test; $P<0.05$; NS, not significant). Asterisks indicate statistically significant differences between treatment of undamaged (*P. rapae* systemic) leaves of *P. rapae*-infested plants and leaves of non-pre-infested plants (Student's *t*-test; $^{**}P<0.01$; $^{***}P<0.001$). Error bars represent SE, $n=3$ plants.

applied ABA, the expression levels of *MYC2* and *VSP1* increased significantly (20-fold and 1600-fold respectively) compared to ABA treatment of control plants, that by itself already led to 2- and 100-fold induction levels, respectively, compared to uninduced control plants. These results indicate that undamaged tissue of *P. rapae*-infested plants is primed for enhanced expression of *MYC2* and *VSP1* and that ABA plays an important role in the onset of the potentiated expression pattern upon challenge.

***P. rapae*-induced resistance is blocked in *aba2-1* and *coi1-1* mutants**

To investigate the role of ABA in *P. rapae*-induced resistance in undamaged systemic tissue, we assessed the performance of *P. rapae* on uninduced and *P. rapae*-induced Col-0 plants, ABA biosynthesis mutant *aba2-1* and JA-nonresponsive mutant *coi1-1*. As an induction treatment, a first-instar caterpillar was placed on each plant and was allowed to feed for 24 h, which resulted in minor chewing damage on usually one leaf, after which the caterpillar was removed. Subsequently, a new first-instar caterpillar was placed on *P. rapae*-induced and on untreated control plants. After 7 days the weight of these caterpillars was determined. Figure 4A shows that the caterpillars weighed significantly less when fed on Col-0 plants that were pre-treated with *P. rapae* than on control Col-0 plants that were not pre-induced, confirming the findings of (De Vos *et al.*, 2006). The herbivore-induced reduction of *P. rapae* performance as observed in Col-0 plants was completely blocked in *aba2-1* and *coi1-1* mutant plants (Figure 4A). To investigate if the absence of this resistance effect in *aba2-1* and *coi1-1* plants coincides with reduced expression of the *VSP1* marker gene, we monitored *VSP1* transcript levels after infestation by *P. rapae*. Figure 4B shows that *VSP1* induction by *P. rapae* feeding is completely absent in both *aba2-1* and *coi1-1* mutant plants. Together, these results indicate that both ABA and JA play an important role in the expression of *P. rapae*-induced defenses.

DISCUSSION

Previously, we demonstrated that herbivory by *P. rapae* on Arabidopsis leads to activation of the MYC-branch of the JA pathway and concomitant suppression of the ERF-branch of the JA pathway in herbivore-damaged leaves (Chapter 2; Verhage *et al.*, 2011). Because *P. rapae* caterpillars have a preference to feed from leaf tissue that expresses the ERF-branch of the JA pathway (Chapter 2; Verhage *et al.*, 2011), 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 during herbivory is extended to systemic undamaged tissues. We demonstrated that *P. rapae* feeding induces similar levels of *MYC2* gene expression in damaged and undamaged leaves (Figure 1). However, in systemic undamaged leaves

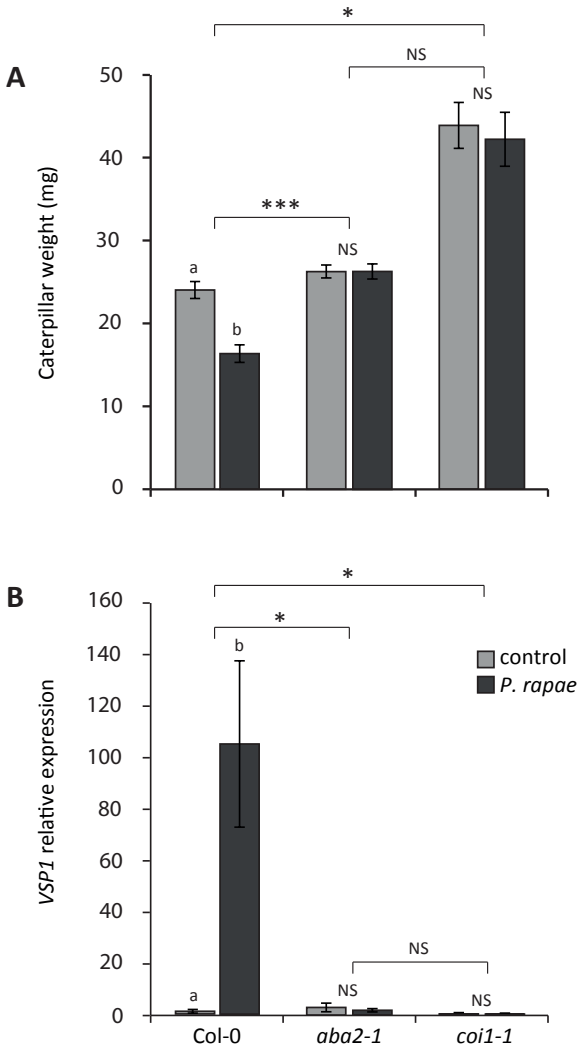


Figure 4: Effect of herbivory on *P. rapae* performance on Col-0, *aba2-1* and *coi1-1* plants.

(A) Growth of *P. rapae* caterpillars on herbivore-induced (*P. rapae*) and uninduced (control) Col-0, *aba2-1* and *coi1-1* plants. A first-instar caterpillar was allowed to feed for 24 h after which it was removed. Another 24 h later, a new first-instar caterpillar was placed onto uninduced plants and *P. rapae*-induced plants. Caterpillar fresh weight was measured after 7 days of feeding. Feeding of a single first-instar caterpillar for 24 h caused only minor chewing damage on usually one leaf, leaving ample tissue on all the plant genotypes for the subsequent caterpillar to feed for 7 days. The values presented are means (± SE) of 30-36 caterpillars that fed on similarly-treated plants. Different letters indicate statistically significant differences between performance on control (non-pre-infested) and herbivore-pretreated plants per genotype ($P<0.05$; NS, not significant); asterisks indicate statistically significant interaction between genotype x treatment (*$P<0.05$; ***$P<0.001$). Data were analyzed using two-way ANOVA.

(B) RT-qPCR analysis of *VSP1* transcript levels (relative to non-infested Col-0*)* in non-infested (control) and *P. rapae*-infested (*P. rapae*) Col-0, *aba2-1* and *coi1-1* plants. First-instar caterpillars were allowed to feed for 24 h after which they were removed and damaged leaves were harvested for analysis. Different letters indicate statistically significant differences between control and treatment of one genotype ($P<0.05$; NS, not significant); asterisks indicate statistically significant interaction between genotype x treatment (*$P<0.05$). Data were analyzed using two-way ANOVA, error bars represent SE, $n=3$ plants.

this did not lead to full induction of the MYC-branch of the JA-pathway, as evidenced by low *VSP1/2* induction levels and lack of MYC2-mediated suppression of the ERF-branch marker genes *ORA59* and *PDF1.2* (Figure 1B & C).

This observation resembles a phenomenon that is called priming for enhanced defense (Conrath *et al.*, 2006), which is also observed during rhizobacteria-mediated ISR. Upon root colonization by beneficial ISR-inducing rhizobacteria, aboveground plant tissue acquires an enhanced level of resistance that is effective against a broad spectrum of pathogens and herbivorous insects (Van Wees *et al.*, 2008; Pineda *et al.*, 2010). This type of systemic induced resistance is not associated with direct activation of defense-related genes, but a large set of predominantly JA-responsive genes becomes primed for accelerated expression after pathogen or insect attack (Van Wees *et al.*, 1999; Pozo *et al.*, 2008; Van Oosten *et al.*, 2008). Pozo *et al.* (2008) and Van der Ent *et al.* (2009a) demonstrated that induction of ISR elicited by *Pseudomonas fluorescens* WCS417r was associated with enhanced expression of *MYC2* in systemic leaf tissue, resembling our observation in undamaged leaves of *P. rapae*-infested plants. Moreover, MYC2-impaired *jin1* mutants were blocked in ISR and priming of JA-regulated defenses (Pozo *et al.*, 2008), highlighting the importance of MYC2 in this type of induced resistance and in priming for enhanced defense.

Analysis of the production of JA, JA-Ile, OPDA and ABA in local and systemic tissues revealed that the signature of these hormonal signals in undamaged leaves of *P. rapae*-infested plants is different from that observed in herbivore-damaged leaves (Figure 2). Levels of all four compounds rose significantly in herbivore-infested leaves, but in undamaged leaves only JA and JA-Ile levels were elevated, even to a similar extent as in damaged leaves. This correlates with the comparable levels of *MYC2* gene expression in local and systemic leaves. In contrast, neither ABA nor OPDA levels were upregulated in undamaged tissue. OPDA levels showed even a trend of decrease after 24 h of *P. rapae* feeding, which is in support of the findings of Koo *et al.* (2009), who showed a rapid depletion of OPDA levels in systemic tissue of Arabidopsis upon infliction of mechanical damage. Possibly, systemic OPDA is converted into JA and JA-Ile (Koo *et al.*, 2009).

The lack of an increase of ABA in undamaged tissue implies that ABA is not systemically translocated from insect-infested leaves. Erb *et al.* (2011) found that upon belowground herbivory in maize there was a local increase in JA, OPDA and ABA levels, comparable to our own findings on local herbivory. In systemic leaves they detected an increase in ABA levels, whereas JA and OPDA levels remained unaltered. However, this systemic increase in ABA levels is correlated to the general water stress that is inflicted by root herbivory, whereas in our setup increased levels of ABA seem strictly related to the relatively mild local wounding caused by insect feeding on generally only one leaf. Leaf wounding and herbivory are associated with leaf water loss (Aldea *et al.*, 2005; Consales *et al.*, 2012) and this abiotic stress may be the cause of the detected increase

in ABA. In combination with other wound-induced cues the increased ABA levels may activate effective anti-herbivore defenses. In systemic tissue of mechanically damaged soybean plants there is no water loss (Aldea *et al.*, 2005) and this may also be the case in undamaged tissue of *P. rapae*-infested Arabidopsis. The consequential lack of ABA increase may prevent the direct activation of costly defense responses, while instead the tissue becomes primed for the MYC-branch of JA signaling, which is a cost-efficient way of the plant to prepare itself for future attack (Van Hulten *et al.*, 2006; Vos *et al.*, 2013a). Upon subsequent damage of the primed systemic tissue, inflicted by secondary infestation, it is likely that local ABA levels rise due to water stress, which triggers full-blown anti-herbivore defenses (Figure 3). In this theory, water loss/ABA acts as a sensor for plants to trigger an appropriate local response or only prime systemic tissue for future induction. The difference in hormonal signal signature between local damaged and systemic undamaged tissue may be causally related to the strongly reduced activation of downstream MYC2-dependent target genes such as *VSP1/2* in the undamaged tissue.

As a regulator of the balance between the MYC- and the ERF-branch of the JA defense signaling pathway (Anderson *et al.*, 2004; Kazan & Manners, 2013), ABA impacts resistance against both pathogens and insects. Moreover, ABA plays a role in primed plant defenses against pathogens as triggered by resistance-inducing beneficial rhizobacteria and BABA (Ton *et al.*, 2005; Van der Ent *et al.*, 2009a). Arabidopsis lines that overexpress *MYC2* are hypersensitive to ABA (Abe *et al.*, 2003). Here, we demonstrate that the *aba2-1* mutant is blocked in *VSP1* induction upon *P. rapae* feeding, demonstrating that ABA signaling is required for activation of MYC-branch-regulated responses (Figure 4B). Since our plants show a systemic increase in *MYC2* expression, but no increase in *VSP1/2* expression, we tested if the enhanced *MYC2* transcript levels in the systemic leaf tissue were associated with enhanced sensitivity to ABA. Therefore, we applied 10 µM ABA to undamaged leaf tissue of *P. rapae*-induced plants. Indeed, the systemically primed leaves showed enhanced expression of the MYC-branch marker genes *MYC2* and *VSP1* in response to the ABA treatment (Figure 3), underlining the importance of ABA in the onset of the potentiated defense response in systemically primed leaves. Taken together, these findings indicate that ABA and JA are tightly interconnected and that regulation of ABA levels in response to herbivory can modulate JA-driven defense responses (Erb *et al.*, 2012). Besides ABA, additional signals could regulate systemic resistance induced by herbivores. Like ABA, OPDA was shown to increase only locally upon infestation and not systemically, rendering it a valid candidate for activating the primed MYC-branch in herbivory-induced systemic tissue.

Transcription factors can 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. To test if the undamaged tissue of *P. rapae*-infested plants is primed for future caterpillar attack, the effect of a succeeding infestation by *P. rapae* of these tissues was tested. *MYC2* gene expression was found to be induced at 2-fold higher levels after the second attack than after a first attack (Figure 3A). The expression levels of *VSP1* in *P. rapae*-challenged systemic leaves were a steep 80-fold higher compared to those observed in non-pre-infested *P. rapae*-damaged leaves (local). These results suggest that systemic undamaged tissues of *P. rapae*-infested plants are indeed primed for enhanced defense against future caterpillar attack.

Insect performance assays demonstrated that primary infestation by one *P. rapae* caterpillar for 24 h, which resulted in only minor chewing damage on usually one leaf, was sufficient to lead to reduced growth of a secondary infesting caterpillar on Col-0 plants, that was placed on the plant one day later and of whom the weight was determined 7 days later (Figure 4A). This result correlates with the *P. rapae*-induced priming of undamaged tissue, leading to enhanced activation of the MYC-branch upon challenge with *P. rapae*. This protective effect was completely blocked in the ABA biosynthesis mutant *aba2-1* and the JA response mutant *coi1-1*, that are both affected in MYC signaling (Figure 4B), indicating that functional JA and ABA pathways are both necessary for the onset of herbivore-induced resistance in undamaged systemic tissues. One could debate whether we can speak in this situation about induction of systemic resistance for two reasons. Firstly, during the pre-infestation the caterpillar might have crawled over more than one or two leaves and thereby caused invisible additional damage to the seemingly undamaged leaves. This cannot be excluded, but it is unlikely because *P. rapae* first-instar caterpillars usually stay on the leaf that they are placed on for the first day. Secondly, the challenge caterpillar that was allowed to feed for 7 days did not only feed from undamaged tissue but also from the pre-damaged tissue. The amount of pre-damaged tissue was, however, estimated to be less than 5% of the total amount of tissue, so this can unlikely explain the difference in caterpillars performance on pre-infested versus uninduced Col-0 plants. It is a fact that during the 7 days of the performance experiment there is a continuous induction of resistance and still, one could detect a difference in insect performance due to pre-infestation 8-9 days earlier, which indicates that the herbivore-induced effect on resistance that we detected is robust.

On the *coi1-1* mutant plants the caterpillars grew larger than on Col-0 and *aba2-1*, confirming previous findings that JA signaling is indispensable for insect resistance (Bodenhausen & Reymond, 2007; Fernández-Calvo *et al.*, 2011; Verhage *et al.*, 2011). The *aba2-1* mutant did not allow enhanced growth of the *P. rapae* caterpillars compared to Col-0, whereas caterpillars of the generalist herbivore *Spodoptera littoralis* grew significantly larger on *aba2-1* (Bodenhausen & Reymond, 2007). This suggests that ABA signaling is a critical component of the resistance response against generalists, but

we show that ABA also functions as an activator of primed defense responses against specialists (and possibly also generalists).

Priming has been demonstrated to entail limited fitness costs, especially in comparison to the higher costs associated with direct activation of defenses. Moreover, the fitness costs of priming were shown to be outweighed by the enhanced resistance benefits under pathogen pressure, which suggests that priming functions

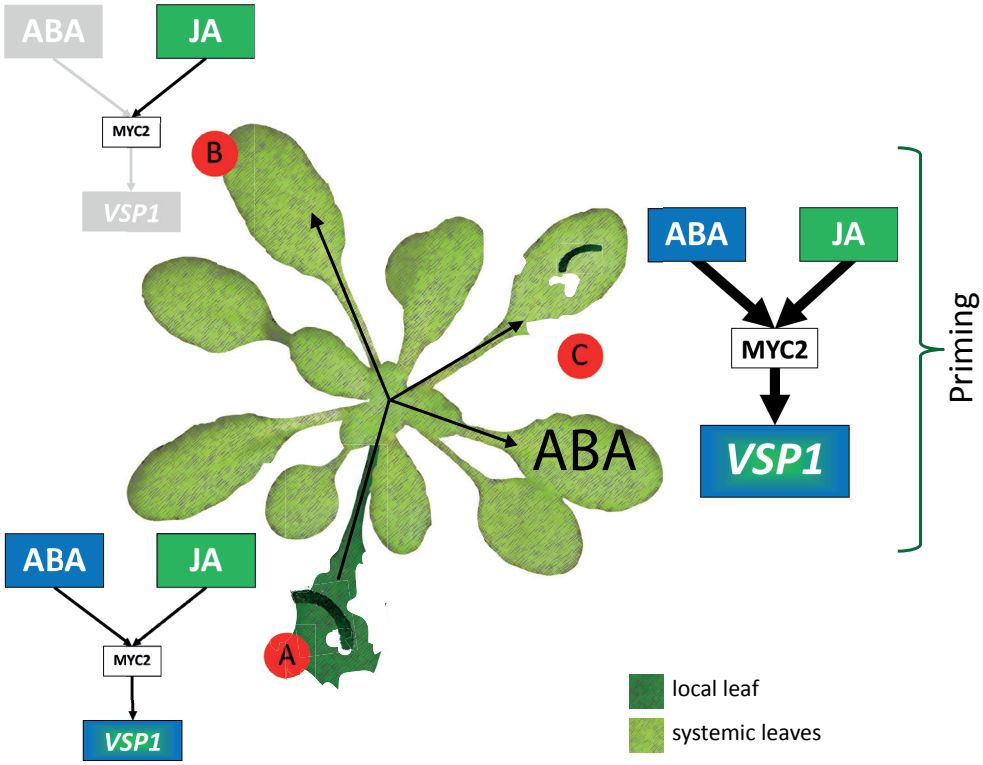


Figure 5: Model of differential JA responses in local damaged and systemic undamaged leaves upon *P. rapae* feeding.

(A) Upon *P. rapae* feeding, local damaged leaves show upregulation of JA/JA-Ile and ABA levels and of transcriptional activity of the MYC-branch regulator gene *MYC2* and its downstream response gene *VSP1*. (B) In systemic undamaged leaves of *P. rapae*-infested plants, the level of JA/JA-Ile and *MYC2* gene expression is upregulated. However, the level of ABA and MYC-regulated *VSP1* transcription is not increased. (C) Challenge of MYC-primed undamaged systemic leaves with either *P. rapae* caterpillars or exogenously applied ABA leads to potentiated transcript levels of *MYC2* and particularly *VSP1*, which are much higher than those observed in local infested leaves. Furthermore, *P. rapae* performance is reduced if plants were previously infested. This indicates that undamaged tissue of *P. rapae*-infested plants is primed for enhanced defense against subsequent *P. rapae* attack and that ABA production induced upon subsequent infestation is important for the onset of potentiated expression of the MYC-branch of JA signaling.

as an ecological adaptation of the plant to respond faster to a hostile environment (Van Hulten *et al.*, 2006; Walters *et al.*, 2008; Vos *et al.*, 2013a). The data presented here point to a model (Figure 5) in which herbivory leads to priming of the MYC-branch of the JA pathway in systemic undamaged leaves, without fully activating costly JA-dependent defenses, for which also a local increase of ABA is required, which is likely induced by local damage-induced leaf water stress. The primed state leads to elevated activation of MYC2-dependent defenses when undamaged systemic tissue is attacked by insect herbivores. ABA is identified as a regulator of herbivore-induced resistance by activating the potentiated expression of defense responses in previously undamaged tissue upon secondary herbivore attack.

MATERIAL & METHODS

Plant material and cultivation

Seeds of *Arabidopsis thaliana* wild-type Col-0 and mutants *jin1-2*, *jin1-7, aba2-1* and *coi1-1* (Koornneef *et al.*, 1982; Feys *et al.*, 1994; Lorenzo *et al.*, 2004) were sown on river sand and, after 2 weeks of growth, seedlings were transplanted into 60-ml 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 a 10-h day and 14-h night cycle at 70% relative humidity and 21°C. Plants were watered every other day and received half-strength Hoagland solution (Hoagland & Arnon, 1938) containing 10 µM sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Pieris rapae assays

Pieris rapae (small cabbage white) was reared on white cabbage plants (*Brassica oleracea*) as described (Van Wees *et al.*, 2013). In all experiments, first-instar caterpillars were used. For gene expression analysis, two caterpillars were placed separately on fully expanded leaves of 5-week-old Arabidopsis plants using a fine paintbrush. The caterpillars were removed 24 h later and leaves were harvested at different time points after introduction of the caterpillars. Leaves damaged by caterpillar feeding (local) were harvested separately from undamaged leaves (systemic) of infested and uninfested (control) plants. Undamaged systemic leaves that received a second treatment (*P. rapae* or ABA) were distinguished from locally damaged leaves by marking the local leaves before the second treatment. Even though *P. rapae* first-instar caterpillars commonly stayed on one leaf on which they were introduced, they incidentally crawled to other leaves. On leaves where they would not leave visible damage potential induction of addition unknown defenses cannot be excluded. Two fresh first-instar caterpillars were placed on fully expanded, undamaged leaves of *P. rapae*-infested plants at 48 h. At 72 h the caterpillars were removed and systemic damaged leaves were harvested for gene expression analysis.

For the *P. rapae* performance assay, 5-week-old plants were placed in Magenta GA-7 containers with a modified mesh lid. Plants were challenged with *P. rapae* by placing one fresh first-instar caterpillar on each plant, which was then allowed to feed for 7 days. The weight of each individual caterpillar was measured using a microbalance. To determine herbivore-ISR, each plant was exposed to herbivory by a single first-instar caterpillar for 24 h after which the caterpillar was removed. At 48 h, plants were challenged with one fresh first-instar caterpillar, which was allowed to feed on the plants for 7 days, as described above. In all experiments there was ample tissue for the caterpillars to feed from for 7 days.

ABA treatment

Undamaged control plants and *P. rapae*-infested plants were treated with ABA (Sigma, Steinheim, Germany) by dipping the plants in a solution containing 10 µM ABA and 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands). ABA was added to the solution from a 1000-fold concentrated stock in 96% ethanol.

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 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 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*, *VSP1/2* and *MYC2* as described (Leon-Reyes *et al.*, 2010b; Verhage *et al.*, 2011). After hybridization with α-[^{32}P]-dCTP-labeled probes, blots were exposed for autoradiography and signals were quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad, Veenendaal, The Netherlands). The AGI numbers for the genes studied are indicated in the primer table below. 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- or 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. 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 to 95°C with a ramp speed of 1.0°C/min. Transcript levels were calculated relative to the

reference gene At1g13320 (Czechowski *et al.*, 2005) using the $2^{-\Delta\Delta Ct}$ method described previously (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008).

The AGI numbers of the studied genes are At1g06160 (*ORA59*), At5g44420 (*PDF1.2*), At1g32640 (*MYC2*) and At5g24780 (*VSP1*).

Jasmonates and ABA analysis

For JA, JA-Ile, OPDA and ABA concentration analysis, 0.5 g of leaf tissue was ground in a mortar with liquid nitrogen. The samples of *P. rapae*-damaged leaves (local) and -undamaged leaves (systemic) originated from the same plants. The extraction and hormone analysis was performed as described (López-Ráez *et al.*, 2010); 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 microliters 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: JA 209/59 (fragmented under 12V collision energy), JA-Ile 322/130 (fragmented under 19V collision energy), OPDA 291/165 (fragmented under 18V collision energy), and ABA 263/153, ABA-D_6 269/159 (both isoforms of ABA fragmented under 9V 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 using standard curves made for each individual compound.

ACKNOWLEDGEMENTS

The authors thank Ido Vlaardingerbroek and Laurens Bakker for technical assistance, Piet van Egmond for running the LC/MS/MS samples and Hans van Pelt and Silvia Coolen for rearing of *P. rapae*. This research was supported by VIDI grant no. 11281 of the Dutch Technology Foundation STW and VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which are part of the Netherlands Organization of Scientific Research (NWO), and ERC Advanced Investigator Grant no. 269072 of the European Research Council.

Conflict of interest statement: The authors declare that the research was conducted in the absence of any commercial of financial relationships that could be construed as a potential conflict of interest.

CHAPTER 4

Impact of hormonal crosstalk on resistance and fitness of plants under multi-attacker conditions

Irene A Vos, Liselotte Moritz, Corné MJ Pieterse, Saskia CM Van Wees

Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 80056, 3508 TB Utrecht, the Netherlands

Submitted

ABSTRACT

The hormone salicylic acid (SA) induces plant defenses against biotrophic pathogens. Jasmonic acid and its oxylipin derivatives (JAs) together with ethylene (ET) are important hormonal regulators of induced plant defenses against necrotrophic pathogens, whereas JAs together with abscisic acid (ABA) regulate induced plant defenses against herbivorous insects. Hormonal crosstalk between the different plant defense pathways has often been hypothesized to be a cost-saving strategy that has evolved as a means of the plant to reduce allocation costs by repression of unnecessary defenses, thereby minimizing trade-offs between plant defense and growth. However, proof for this hypothesis has not been demonstrated yet. In this study the impact of hormonal crosstalk on disease resistance and fitness of *Arabidopsis thaliana* when under multi-species attack was investigated. Induction of SA- or JA/ABA-dependent defense responses by the biotrophic pathogen *Hyaloperonospora arabidopsidis* or the herbivorous insect *Pieris rapae*, respectively, was shown to reduce the level of induced JA/ET-dependent defense against subsequent infection with the necrotrophic pathogen *Botrytis cinerea*. However, despite the enhanced susceptibility to this second attacker, no additional long-term negative effects were observed on plant fitness when plants had been challenged by multiple attackers. Similarly, when plants were grown in dense competition stands to enlarge fitness effects of induced defenses, treatment with a combination of SA and MeJA did not cause additional negative effects on plant fitness in comparison to the single MeJA treatment. Together, these data support the notion that hormonal crosstalk in plants during multi-attacker interactions allows plants to prioritize their defenses, while limiting the fitness costs associated with induction of defenses.

INTRODUCTION

Plants can activate defense responses to protect themselves against a plethora of microbial pathogens and herbivorous insects. These defense responses are modulated by the induced production of a hormonal blend in the plant. The plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) are important regulators of induced defense mechanisms (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Vos *et al.*, 2013a). SA-dependent defenses are generally effective against biotrophic pathogens, while JA-dependent defenses are generally effective against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Howe & Jander, 2008). SA is rapidly synthesized upon infection with biotrophic pathogens (Malamy *et al.*, 1990; Métraux *et al.*, 1990). Defense signaling downstream of SA depends on the transcriptional regulator NPR1 (Dong, 2004), eventually resulting in the activation of a large set of defense-related genes, amongst which the robust marker gene of the SA signaling pathway, *PR1* (Van Loon *et al.*, 2006). In response to wounding, insect herbivory or infection with necrotrophic pathogens, JA and its oxylipin derivatives (collectively referred to as jasmonates (JAs)) rapidly accumulate in plants (Creelman *et al.*, 1992; Penninckx *et al.*, 1996). In *Arabidopsis thaliana* (Arabidopsis), there are two distinct branches of the JA response pathway that antagonize each other; the ERF-branch and the MYC-branch (hereafter referred to as such). The ERF-branch is activated upon infection with necrotrophic pathogens and is regulated by the AP2/ERF-domain transcription factors ERF1 and ORA59 (Anderson *et al.*, 2004; Pré *et al.*, 2008). The ERF-branch of the JA response is co-regulated by ET and results in activation of a large set of ERF-branch genes, including the marker gene *PDF1.2* (Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003). The MYC-branch is activated upon wounding or feeding by herbivorous insects and is regulated by the basic helix-loop-helix leucine zipper transcription factors MYC2, MYC3 and MYC4 in concerted action with ABA (Anderson *et al.*, 2004; Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011; Vos *et al.*, 2013b). Activation of the MYC-branch leads to transcription of a large set of JA-responsive genes, including *VSP1* and *VSP2* that are marker genes of the MYC-branch (Anderson *et al.*, 2004; Lorenzo *et al.*, 2004).

Activation of the different hormone-regulated defense responses is not without fitness costs. In several plant species it has been shown that exogenous application of SA or its chemical analogue benzothiadiazole (BTH) inhibited plant growth and seed production (Heil *et al.*, 2000; Cipollini, 2002; Canet *et al.*, 2010). Furthermore, under non-infected conditions, Arabidopsis mutants constitutively expressing SA-dependent defenses are dwarfed and severely affected in seed production (Bowling *et al.*, 1994; Heil & Baldwin, 2002; Heidel *et al.*, 2004; Van Hulten *et al.*, 2006). Conversely, SA-deficient Arabidopsis genotypes have higher growth rates and seed production

compared to wild-type plants (Cipollini, 2002; Abreu & Munné-Bosch, 2009). Activation of JA-dependent defense responses can also result in negative effects on plant fitness. Infestation with insects or exogenous application of JA decreased seed production and delayed flowering and fruit ripening (Agrawal *et al.*, 1999; Redman *et al.*, 2001; Van Dam & Baldwin, 2001). In addition, Arabidopsis plants constitutively expressing JA-dependent defenses, showed reduced growth phenotypes (Ellis & Turner, 2001; Cipollini, 2010). Together, this demonstrates the negative fitness effects of hormone-dependent defense activation.

Quantity, composition and timing of the hormonal blend and cross-communication between the hormone signaling pathways contributes to activation of effective over infective defenses (De Vos *et al.*, 2005; Pieterse *et al.*, 2012; Vos *et al.*, 2013a; Caarls *et al.,* 2015). Many cases of crosstalk between the SA and JA pathway have been reported (Bostock, 2005; Stout *et al.*, 2006; Pieterse *et al.*, 2012). Pharmacological experiments with Arabidopsis revealed that the JA-responsive genes *PDF1.2* and *VSP2* are highly sensitive to suppression by SA. The antagonistic effect of SA on JA signaling was observed in a large number of Arabidopsis accessions (Koornneef *et al.*, 2008) and was even reported to remain active in the next generation of plants (Luna *et al.*, 2012), highlighting the potential significance of this phenomenon in the regulation of induced plant defenses in nature. This antagonism between SA and JA signaling can affect plant resistance. For example, in Arabidopsis, induction of the SA pathway by exogenous application of SA or infection with the hemibiotrophic pathogen *Pseudomonas syringae* rendered the plants more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Spoel *et al.,* 2007; Leon-Reyes *et al.,* 2009). Furthermore, reduced SA signaling in Arabidopsis genotypes *NahG* and *npr1* was correlated with reduced feeding by the herbivorous insect *Trichoplusia ni* (Cui *et al.,* 2002).

Likewise, between the ERF- and the MYC-branch of the JA pathway a mutually antagonistic relationship exists (Lorenzo *et al.*, 2004; Verhage *et al.*, 2011; Vos *et al.*, 2013b). This antagonism between the ERF- and the MYC-branch can affect plant resistance against necrotrophs. For example, in *MYC2*-mutated *jin1* and ABA biosynthesis mutant *aba2-1* plants, the ERF-branch of the JA pathway is stimulated, resulting in enhanced resistance against necrotrophic pathogens, such as *Botrytis cinerea*, *Plectosphaerella cucumerina* and *Fusarium oxysporum* (Anderson *et al.,* 2004; Lorenzo *et al.,* 2004; Nickstadt *et al.,* 2004; Adie *et al.,* 2007; Sánchez-Vallet *et al.,* 2012). Furthermore, caterpillars of the insect herbivore *Pieris rapae* preferred to feed from *jin1* mutant plants and *ORA59*-overexpressing plants over wild-type plants (Verhage *et al.*, 2011), indicating that crosstalk between the ERF- and the MYC-branch also affects plant-insect interactions.

Extensive cross-communication between defense signaling pathways allows the plant to fine-tune the defense response to the attacker at hand (Reymond & Farmer,

1998). Since activation of inducible plant defenses is not without costs, there are trade-offs between plant defense and growth (Heil & Baldwin, 2002; Van Hulten *et al.*, 2006; Walters & Heil, 2007; Vos *et al.*, 2013a; Cipollini *et al.*, 2014). Hormonal crosstalk has often been interpreted as a cost-saving strategy and may have evolved as a means of the plant to reduce allocation costs by repression of unnecessary defenses that are ineffective against the attacker that is encountered (Pieterse & Dicke, 2007; Thaler *et al.*, 2012).

In this study the impact of hormonal crosstalk on disease resistance and fitness of Arabidopsis plants when under multi-species attack was investigated. Induction of SA- or JA/ABA-dependent signaling induced by a primary attacker was shown to negatively affect JA/ET-dependent defense responses activated by subsequent attack with a necrotrophic pathogen, resulting in reduced resistance to this attacker. However, although plants under multi-species attack became more susceptible to the second attacker, this did not lead to long-term negative fitness effect, providing preliminary support for the cost-saving character of hormonal crosstalk.

RESULTS

Multi-attacker conditions reduce resistance but not fitness of Arabidopsis plants

In this research, fitness costs associated with defense against multiple attackers were investigated. To this end, 5-week-old Arabidopsis plants were exposed to two attackers that induce antagonizing defense pathways. Firstly, the plants were either inoculated with the biotrophic pathogen *Hyaloperonospora arabidopsidis*, which induces the SA pathway, or infested with *P. rapae* caterpillars, which induce the MYC-branch of the JA pathway. Twenty four h later, the caterpillars were removed after which all plants were inoculated with the necrotrophic pathogen *B. cinerea*, which induces the ERF-branch of the JA pathway. Figure 1 shows the gene expression results from the defense inductions by the different combinations of attackers. When plants were infected with *H. arabidopsidis*, the SA pathway marker gene *PR1* was strongly induced (Figure 1A). In the combination treatment with *H. arabidopsidis* and *B. cinerea, PR1* expression was even higher at 28 h, probably because *B. cinerea* can induce the SA pathway as a virulence strategy (El-Oirdi *et al.*, 2011) and the tissue may be primed for SA responsiveness by the *H. arabidopsidis* infection. *PR1* expression leveled off again towards 72 h. Feeding by *P. rapae* induced the MYC-branch, as indicated by high *VSP2* expression (Figure 1B). *VSP2* expression returned to basal levels at 48 h and was not altered in the combination treatment with *B. cinerea* at any of the time points investigated. In all cases, the ERF-branch marker gene *PDF1.2* was activated in response to *B. cinerea* infection at 48 and 72 h, but was strongly repressed when plants were previously infected with *H. arabidopsidis* or infested with *P. rapae* (Figure 1A & B). Similar antagonistic effects on

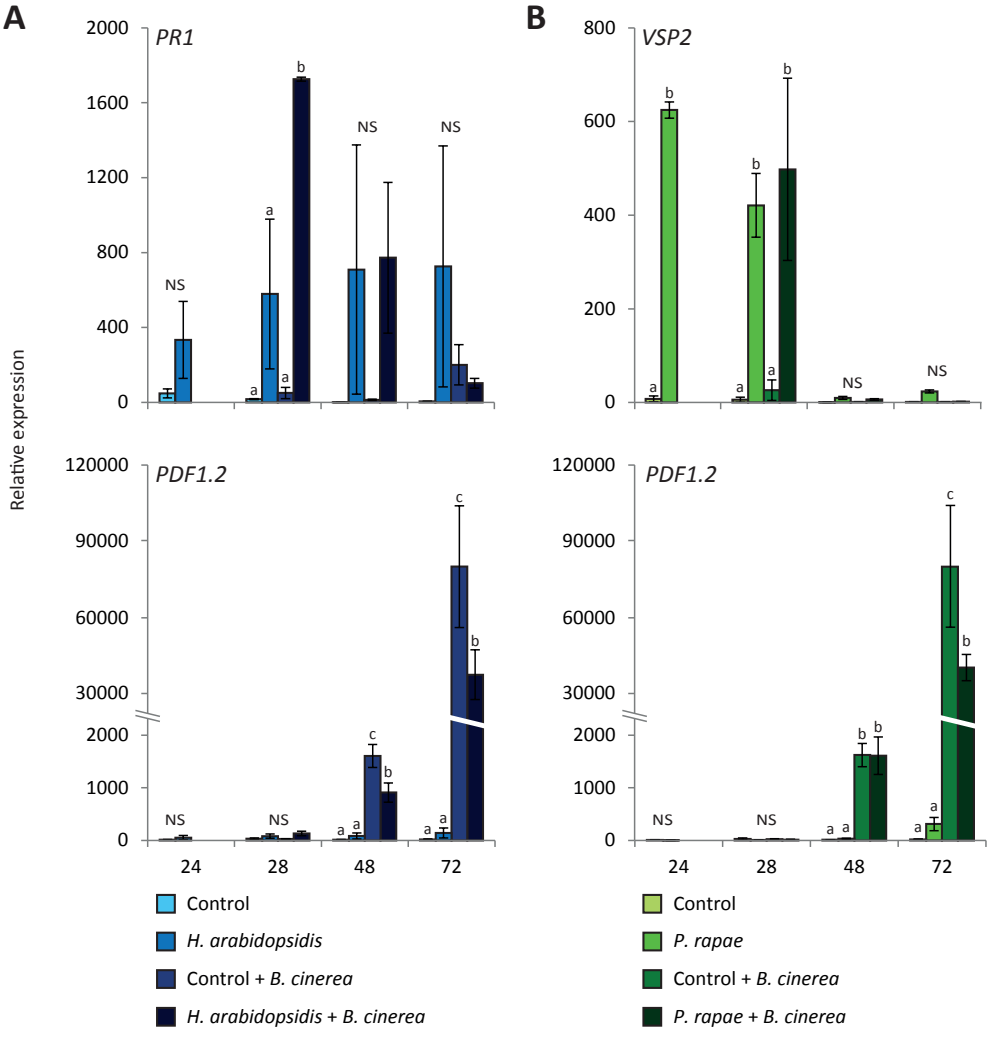


Figure 1: Differential expression of *PR1*, *VSP2* and *PDF1.2* in response to multiple attackers.
RT-qPCR analysis of *H. arabidopsidis*-responsive *PR1* expression (A), *P. rapae*-responsive *VSP2* expression (B) and *B. cinerea*-responsive *PDF1.2* expression (A & B). Plants were either inoculated with *H. arabidopsidis* or infested with *P. rapae* caterpillars. At 24 h the caterpillars were removed after which all plants were inoculated with *B. cinerea*. Samples were taken at the indicated time points after the first treatment. Different letters indicate a statistically significant difference between the different treatments within one time point (ANOVA, Tukey post-hoc test; $P<0.05$; NS = not significant). Error bars represent SE, $n=3$ plants.

PDF1.2 gene expression were found when, before *B. cinerea* infection, plants were induced by exogenous application of either 1 mM SA or a combination of 100 µM MeJA and 100 µM ABA (Supplemental Figure 1). This indicates that the activation of the SA pathway or the MYC-branch of the JA pathway prior to infection with *B. cinerea*

suppressed the *B. cinerea*-induced activation of the ERF-branch, providing evidence for hormonal crosstalk on the defense gene expression level induced by combinations of different attackers.

To investigate whether suppression of the ERF-branch by prior attack with either *H. arabidopsidis* or *P. rapae* is accompanied by a reduced level of resistance against *B. cinerea*, we performed disease resistance bioassays. Plants that were induced by *H. arabidopsidis* or *P. rapae* were significantly more susceptible to *B. cinerea* than control plants (Figure 2A & B). Accordingly, *B. cinerea Tubulin* transcript levels were significantly higher in induced plants than in control plants (Figure 2C). Plants that were treated with exogenous application of 1mM SA or a combination of 100 µM MeJA and 100 µM ABA were also more susceptible to subsequent *B. cinerea* infection (Supplemental Figure 2). Together, these results show that suppression of the ERF-branch of the JA pathway by either the SA inducer *H. arabidopsidis* or the MYC-branch inducer *P. rapae* coincides with a reduction in the level of resistance against *B. cinerea*.

To investigate whether these hormonal crosstalk-mediated effects on *PDF1.2* gene expression and resistance to *B. cinerea* impacted the fitness of the plants under multi-attacker conditions, the rosette size, flowering time and seed production were measured. Neither *H. arabidopsidis* infection nor *P. rapae* infestation affected any of these fitness parameters by themselves (Figure 3A & B), which could be explained by the non-optimal temperature for infection with *H. arabidopsidis* from 24 h onwards and the removal of the *P. rapae* caterpillars at 24 h. In contrast, *B. cinerea* infection had a strong negative effect on rosette size and seed production and prolonged the flowering time (Figure 3A & B). Prior attack with either *H. arabidopsidis* or *P. rapae* did not result in an additional effect on the fitness traits compared to *B. cinerea* infection alone. Similar results were found when plants were induced by exogenous application of 1 mM SA or a combination of 100 µM MeJA and 100 µM ABA (Supplemental Figure 3). Overall, it can be concluded that infection with *B. cinerea* led to reduced fitness. Nonetheless, although prior attack by *H. arabidopsidis* or *P. rapae* or induction by exogenously applied SA or a combination of MeJA and ABA resulted in enhanced susceptibility to *B. cinerea* infection, which was likely due to the suppression of the ERF-branch, this was not associated with additional fitness costs.

Fitness costs of SA and MeJA treatments in competition-grown plants

Since competition for light and nutrients can increase the probability of detecting fitness costs of activating different hormone signaling pathways (Dietrich *et al.*, 2005), Arabidopsis plants were grown in competition trays, consisting of separate small pots positioned very close together. This set-up led to competition of the above-ground plant parts, but not of the root-systems. Each tray consisted of 49 plants, of which 25 plants were supplied with a soil drench containing 500 µM SA, 50 µM MeJA, or a

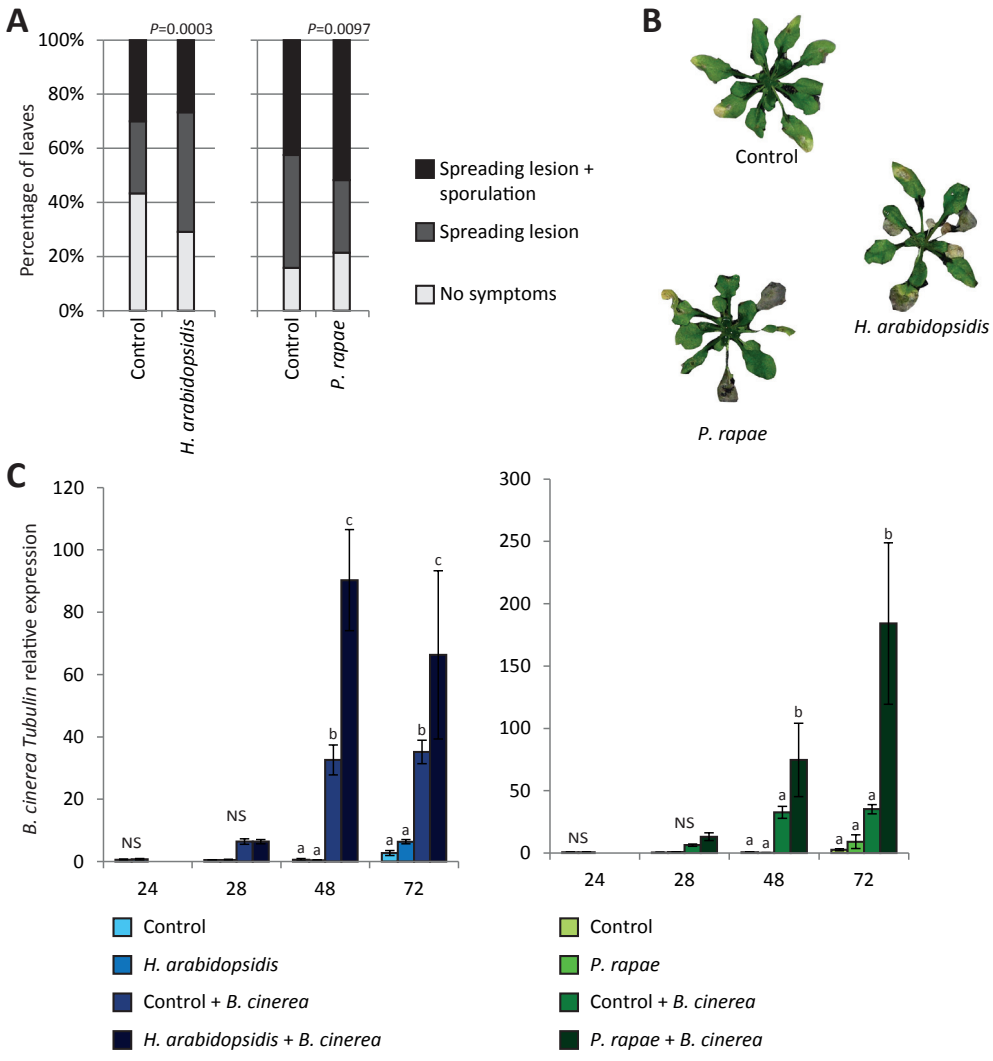


Figure 2: Effect of prior attack by *H. arabidopsidis* or *P. rapae* on disease resistance against *B. cinerea*.
(A) Quantification of disease symptoms of Arabidopsis Col-0 plants infected with *B. cinerea*. Twenty four h before inoculation with *B. cinerea*, plants were inoculated with *H. arabidopsidis* or infested with *P. rapae*. Disease severity of the inoculated leaves was scored in 3 classes. Percentage of leaves in each class was calculated per plant (X^2-test; n=20 plants).
B) Disease symptoms of *B. cinerea* infection in control plants, *H. arabidopsidis*-induced plants and *P. rapae*-induced plants.
C) RT-qPCR analysis of *B. cinerea Tubulin* levels relative to Arabidopsis reference gene mRNA levels after single and double treatments. Samples were taken at the indicated time points after the first treatment. Different letters indicate a statistically significant difference between the different treatments within one time point (ANOVA, Tukey post-hoc test; P<0.05; NS = not significant). Error bars represent SE, n=3 plants.

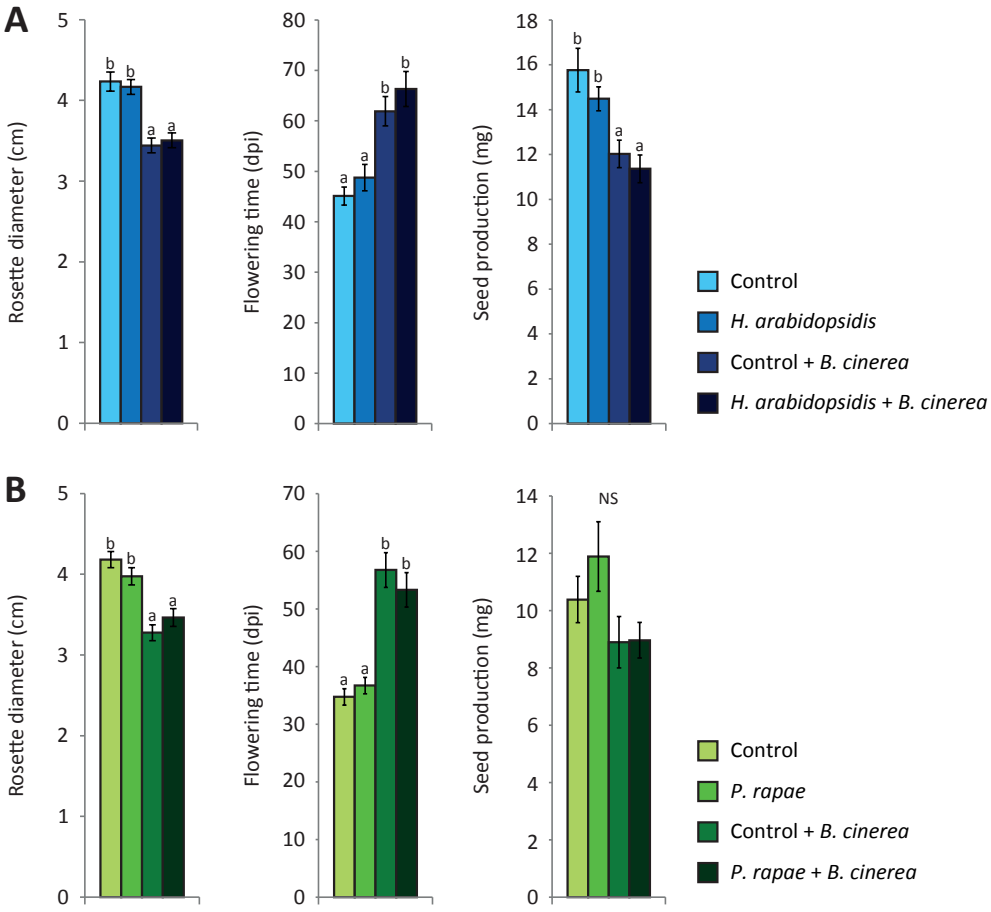


Figure 3: Growth and fitness parameters of single- and double-attacked plants.
Rosette diameter (cm), flowering time (days post inoculation) and total seed production (mg) of Arabidopsis plants. Plants were either inoculated with *H. arabidopsidis* or infested with *P. rapae* caterpillars. At 24 h the caterpillars were removed after which all plants were inoculated with *B. cinerea*. Different letters indicate a statistically significant difference between the different treatments (ANOVA, Tukey post-hoc test; $P<0.05$; NS = not significant). Error bars represent SE, $n=20$ plants.

combination of both hormones. The other 24 plants were treated with either a mock solution or a combination of both hormones (Figure 4). Only the inner nine plants were used for measurements, to circumvent any edge effect. In all trays, SA and SA/MeJA treatment induced *PR1* expression, whereas *VSP2* expression was only induced by the single MeJA treatment and not the SA/MeJA combination treatment (Figure 5), confirming that the hormone treatments induced the expected effects on SA- and JA-responsive gene expression.

When MeJA- or SA/MeJA-treated plants competed with mock-treated plants, leaf area and dry weight of the hormone-treated plants were reduced compared to the mock-treated plants (Figure 6). SA-treated plants did not show a significant reduction in leaf area or dry weight in competition with mock-treated plants, although a trend towards a reduction in leaf area and dry weight was detected. There was no significant difference in leaf area or dry weight when MeJA- and SA/MeJA-treated plants competed with each other, although a trend towards increased dry weight and leaf area was observed. Together, this indicates that there was no extra negative fitness effect of the double treatment compared to the MeJA treatment alone, but rather a trend towards a reduction of MeJA-induced fitness costs in the double treatment. On the other hand, when SA-treated plants competed with SA/MeJA-treated plants, SA/MeJA-treated plants had lower dry weight than SA-treated plants, but there was no significant difference in leaf area in this competition. Taken together, these data show that especially the activation of the JA pathway resulted in lower fitness and lower competitive ability. Activation of the SA pathway did not have major negative effects on fitness. Treatment with a combination of SA and MeJA reduced plant fitness, but did not result in an extra negative effect compared to the single MeJA treatment, indicating that also in dense competition stands, hormonal crosstalk might be a cost-saving strategy in induced plant immunity.

Figure 4: Schematic overview of the competition experiment set-up.
Arabidopsis plants were grown in competition trays, consisting of separate small pots positioned very close together. Each tray consisted of 49 plants, of which 25 plants were soil-drenched containing 500 µM SA, 50 µM MeJA, or a combination of both hormones. The other 24 plants were treated with either a mock solution or a combination of both hormones. Only the inner nine plants were used for measurements, to circumvent any edge effect.

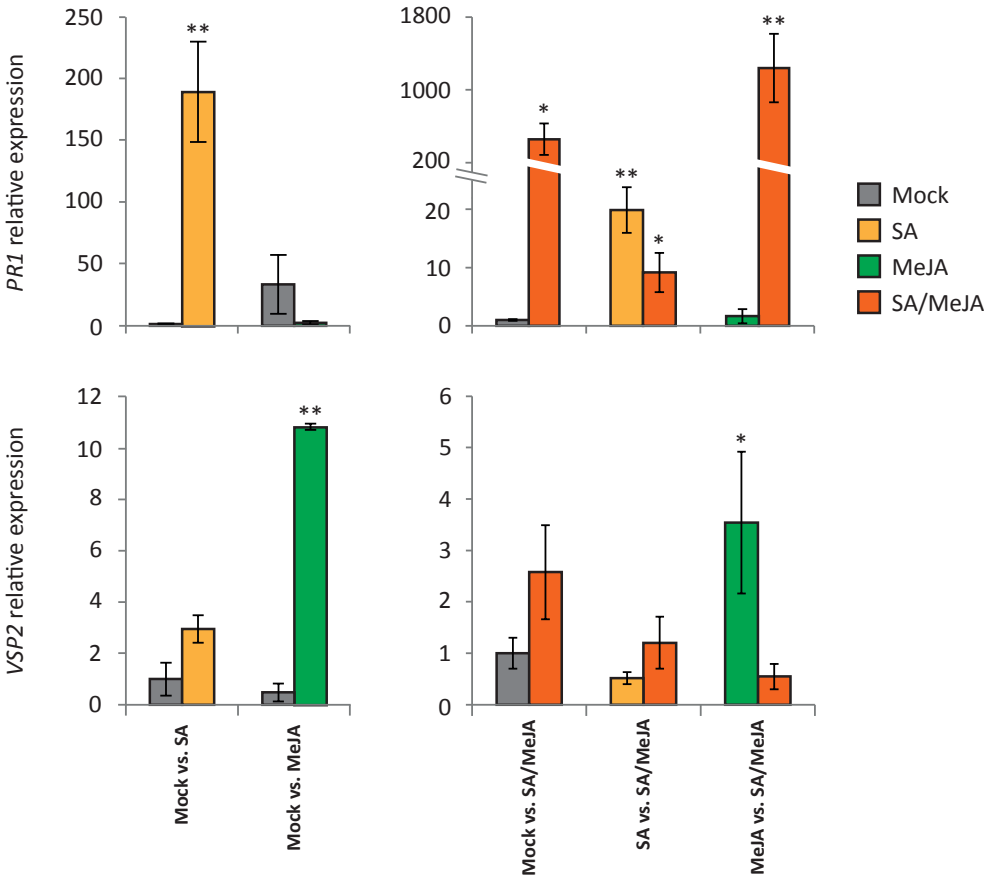


Figure 5: Differential gene expression in competition-grown plants.
RT-qPCR analysis of SA-responsive *PR1* expression and JA-responsive *VSP2* expression in competition-grown plants 24 h after treatment with a mock, SA, MeJA, or SA/MeJA solution. SA-, MeJA- and SA/MeJA-treated plants were grown in competition with mock-treated plants and SA- and MeJA -treated plants were grown in competition with SA/MeJA-treated plants. Indicated are expression levels relatively to those of mock-treated plants. Asterisks indicate a statistically significant difference between the indicated treatment and mock-treated plants (ANOVA, Dunnet post-hoc test; ** = $P<0.01$; * = $P<0.05$). Error bars represent SE, $n=3$ plants.

DISCUSSION

Crosstalk between hormone-regulated defense pathways is suggested to allow plants to fine-tune their defenses to optimize induced resistance to an attacker and reduce allocation costs (Heil & Baldwin, 2002; Pieterse & Dicke, 2007; Walters & Heil, 2007; Vos *et al.*, 2013a). Therefore, hormonal crosstalk has often been suggested to be a cost-saving strategy. Since individual plants are likely to be attacked by more than one

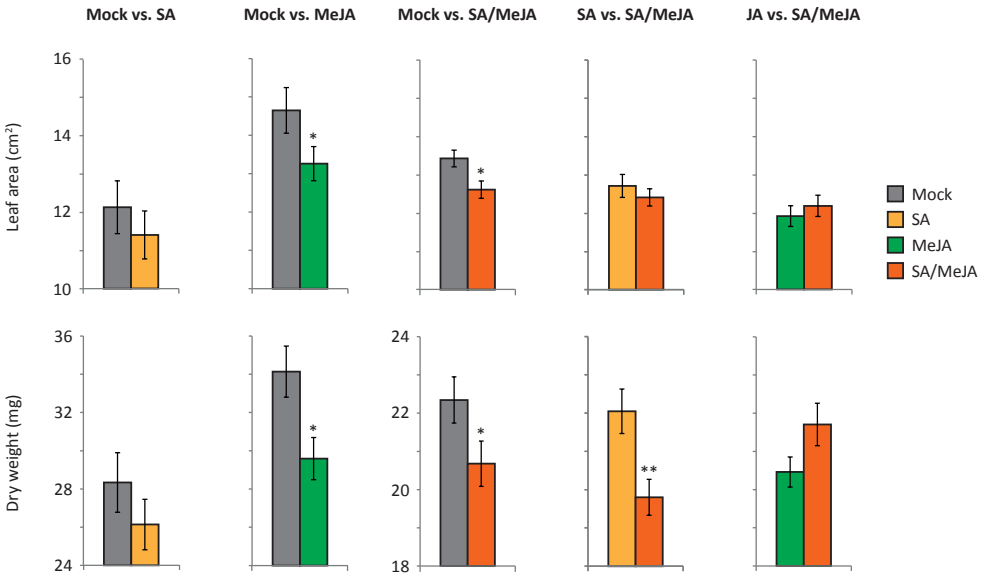


Figure 6: Growth parameters in competition-grown plants.
Leaf area (cm^2) and dry weight of the rosettes (mg) of competition-grown plants three weeks after treatment with a mock, SA, MeJA, or SA/MeJA solution. SA-, MeJA- and SA/MeJA-treated plants were grown in competition with mock-treated plants and SA- and MeJA-treated plants were grown in competition with SA/MeJA-treated plants. Asterisks indicate a statistically significant difference between the two treatments of the indicated competition tray (Students *t*-test; ** = $P<0.01$; * = $P<0.05$). Error bars represent SE, n=20-25 plants.

organism, hormonal crosstalk may coincide with ecological costs, leading to higher susceptibility to a subsequent attacker (Heil, 2002; Vos *et al.*, 2013a). Moreover, not much is known about the consequences of hormonal crosstalk on plant fitness under multi-species attack (Thaler *et al.*, 2012). In this research, costs of defense activation of Arabidopsis plants by multiple attackers or hormones were investigated.

Costs and benefits of Arabidopsis plants under multi-species attack

Several studies found a negative effect on resistance against a subsequent attacker when a plant was previously induced by another attacker (Vos *et al.*, 2013a). For example, in Arabidopsis, infection with the hemibiotrophic pathogen *P. syringae* resulted in higher susceptibility to a subsequent infection with the necrotrophic fungus *A. brassicicola* (Spoel *et al.*, 2007). Furthermore, feeding by the generalist herbivore *Spodoptera littoralis* led to increased growth of a virulent strain of *P. syringae* (Appel *et al.,* 2014). In tobacco, *Manduca sexta* caterpillars consumed up to 2.5-times more leaf tissue from plants previously infected with the SA-inducing tobacco mosaic virus than from mock-treated plants (Preston *et al.*, 1999) and black bean aphids had a higher growth

rate and fecundity on bean leaves infected with the necrotrophic pathogen *Botrytis fabae*, compared to uninfected leaves (Zebitz & Kehlenbeck, 1991). Comparably, in this study we found that when Arabidopsis plants were first induced by the SA pathway-inducing pathogen *H. arabidopsidis* or the MYC-branch-inducing caterpillar *P. rapae*, the ERF-branch of the JA pathway was suppressed and plants became more susceptible to a subsequent attack by the necrotrophic pathogen *B. cinerea* compared to non-induced plants (Figure 1 & Figure 2). Likewise, pretreatment with the hormones SA or a combination of MeJA and ABA, suppressed the induction of the ERF-branch and resulted in higher susceptibility to *B. cinerea* (Supplemental Figure 1 & Supplemental Figure 2).

Infection with *B. cinerea* or exogenous application of MeJA activated defense gene expression (Figure 1 & Figure 5). Furthermore, a negative effect on growth, flowering time and seed production was found after infection with *B. cinerea* (Figure 3) or MeJA treatment (Figure 6), suggesting that there were trade-offs between activation of defenses by these treatments and plant fitness (Heil & Baldwin, 2002; Van Hulten *et al.*, 2006; Walters & Heil, 2007; Vos *et al.*, 2013a; Cipollini *et al.*, 2014). However, there was no negative effect on growth, flowering time and seed production in response to *H. arabidopsidis* infection or infestation with *P. rapae*, while these attackers also induced defense gene expression (Figure 1 & Figure 3). This is probably caused by the fact that plants were only shortly exposed to these attackers, since 24 h after the first pathogen or insect treatment, plants were placed under conditions that inhibited further growth of the pathogen (*H. arabidopsidis*) or the inducer was removed (*P. rapae*), thereby reducing long-term effects of the primary induction treatments on plant fitness. Although these single inductions did not have a negative effect on the fitness parameters, still there were ecological costs of the double induction, as plants became more susceptible to *B. cinerea* infection (Figure 2). These ecological costs did not lead to additional negative effects on fitness compared to *B. cinerea*-infected plants that were not previously induced (Figure 3). Likewise, no additional fitness costs were incurred by the double treatment with SA or a combination of MeJA and ABA and *B. cinerea* (Supplemental Figure 3). This could be an indication that the hormonal crosstalk effect that we found at the level of gene expression (Figure 1 & Supplemental Figure 1) and disease resistance (Figure 2 & Supplemental Figure 2) is indeed a cost-saving strategy. Testing crosstalk mutants that are not affected in resistance to either of the attackers could give a definite answer (Thaler *et al.*, 2012; Vos *et al.*, 2013a). Alternatively, there might not be a linear relation between susceptibility and plant fitness, which could explain the lack of additional fitness costs in the double treated plants. However, Heidel *et al.* (2004) found that higher disease severity after *H. arabidopsidis* infection correlated with lower seed production. Furthermore, Cipollini (2002) found that seed production was significantly lower when plants were treated

with a high concentration of SA compared to a lower concentration. Together this suggests that there is a negative correlation between increased disease symptoms and lower fitness, but to our knowledge, this has never been shown for *B. cinerea* infection. However, from our own experience, we know that Arabidopsis can die from a *B. cinerea* infection, leading to a fitness level of 0.

Fitness effects of defense activation in plants grown in competition

Previously, it was shown in *Nicotiana attenuate* that allocation costs of induced defenses were only found when plants were grown with conspecific competitors (Van Dam & Baldwin, 2001). This can likely be explained by the fact that in dense competition stands, plants have to compete for light and nutrients in addition to the investment of resources in activation of induced defenses. Consequently, growing plants in a competition set-up can increase the probability of detecting fitness costs of activating the different defense signaling pathways (Dietrich *et al.*, 2005). Therefore, we tested the effect of hormone treatments on the fitness of plants grown in dense competition stands.

SA and/or MeJA solutions were supplied exogenously as a root drench. It has recently been shown that the effect of JA treatment on primary metabolism, development and defense specific traits depended on whether JA had been applied to the shoots or the roots of *Brassica oleracea* plants (Tytgat *et al.,* 2013). We observed that root drenching with SA and SA/MeJA resulted in activation of the SA marker gene *PR1* in the leaves, whereas *VSP2* expression was activated only by MeJA treatment and not by SA/MeJA treatment (Figure 5), indicating that application of the hormones as a root drench resulted in SA/JA crosstalk effects in the above-ground plant parts.

Treatment with MeJA led to a negative effect on leaf area and dry weight of the plants when competing with mock-treated plants (Figure 6), whereas when MeJA-treated plants competed against SA/MeJA-treated plants no significant differences in leaf area and dry weight were found, but a trend towards a reduction of the MeJA-induced fitness costs was visible. Activation of the SA pathway did not have major negative effects on fitness, although a trend towards reduced growth was detected. SA-treated plants showed higher dry weight in competition with SA/MeJA-treated plants, but no effect on leaf area was found. Taken together, these data show that activation of the JA pathway resulted in lower fitness and lower competitive ability, while the combination of MeJA with SA did not result in an extra negative effect, but rather a trend towards a positive effect was observed. Together, this indicates that also in dense competition stands, where costs of defense activation are likely to be higher (Van Dam & Baldwin, 2001; Dietrich *et al.,* 2005), hormonal crosstalk might be a cost-saving strategy in induced plant immunity.

Although growth rates and dry masses appear valuable phenotypic parameters to observe fitness effects, in some cases it failed to be valid predictors of effects on

life time seed production (Dietrich *et al.*, 2005). Therefore, it would be worthwhile to measure seed production in the competition set-up as well. Additionally, it could be interesting to use pathogens instead of hormones, to make the effect of induction last longer and to add a disease resistance effect, which could lead to ecological costs. Furthermore, environmental conditions such as nutrient availability and presence of competitors have been shown to impact the amount of fitness costs of induced defenses (Cipollini *et al.*, 2003; Dietrich *et al.*, 2005), making it likely that environmental conditions also impact the magnitude of fitness benefits of hormonal crosstalk.

CONCLUSION

In conclusion, our results show that hormonal crosstalk during multi-attacker interactions can shift the balance between SA- or JA/ABA-dependent defenses on the one hand and JA/ET-dependent defenses on the other hand. Despite the reduced JA/ET-dependent necrotroph resistance observed during the double attacker interactions, there were no additional long-term negative fitness effects of plants that were sequentially attacked by the different attackers. Furthermore, in most cases plants grown in competition stands did not show a negative effect on plant growth in response to treatment with both SA and MeJA in comparison to the single hormone treatments. Taken together, these results suggest that hormonal crosstalk might indeed be a cost-saving strategy that allows plants to prioritize their defenses and reduce fitness costs of defense activation.

MATERIAL & METHODS

Plant material and cultivation

Seeds of *Arabidopsis thaliana* accession Col-0 were sown on river sand. Two weeks later, seedlings were transplanted into 60-ml 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 a 10-h day and 14-h night cycle at 70% relative humidity and 21°C. Plants were watered every other day and received half-strength Hoagland solution (Hoagland & Arnon, 1938) containing 10 µM sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

Hyaloperonospora arabidopsidis inoculation

Hyaloperonospora arabidopsidis WACO9 was maintained on susceptible Arabidopsis *eds1* plants by weekly transfer to healthy 14-day-old seedlings as described (Koch & Slusarenko, 1990). Sporangia were obtained by washing diseased leaves in demineralized water. Debris was filtered out using Miracloth (Merck) and spores were resuspended

in demineralized water to a final density of 50 spores/µl. Five-week-old plants were inoculated by spraying the *H. arabidopsidis* spore suspension using a fine paint brush, after which the plants were kept at 100% RH at 17°C for 24 h to facilitate infection (Van Damme *et al.*, 2005).

***Pieris rapae* infestation**

Pieris rapae (small cabbage white) was reared on white cabbage plants (*Brassica oleracea*) as described (Van Wees *et al.*, 2013). First-instar caterpillars were used in all experiments. Two caterpillars were placed on fully expanded leaves of 5-week-old plants using a fine paintbrush. Caterpillars were removed 24 h later.

***Botrytis cinerea* inoculation**

Botrytis cinerea inoculations were performed with strain B05.10 (Van Kan *et al.*, 1997) as described previously (Van Wees *et al.*, 2013). *B. cinerea* suspension with a final density of $1 \cdot 10^5$ spores/ml was prepared and 5 µL droplets of the spores were applied to six leaves per plant per treatment. Plants were placed under a lid to increase relative humidity to 100% to stimulate the infection. Samples for gene expression analysis were harvested at the indicated time points. Four days after *B. cinerea* treatment, lids were removed.

Rosette diameter, flowering time and seed production

Rosette diameters were measured from pictures that had been taken at the indicated time points. Two opposing longitudinal measurements were taken of each rosette using ImageJ. On-picture rulers were used to convert measured pixels to realistic centimeters. Flowering time was noted in days after treatment when the first flower appeared. To determine seed production, plants were watered every other day until they stopped producing new flowers. Inflorescences were harvested when all plants had finished flowering and the seeds were weighed on a microbalance with a 0.0001 g resolution.

Competition experiment

For the competition experiment, seedlings were transplanted to trays consisting of 18-ml pots organized in a 7 x 7 format, so that plants experienced competition of the aboveground plant parts, but not of the roots. Hormone treatment was applied in a chess pattern to 4-week-old plants (Figure 4). Only the inner 9 plants were used for determining gene expression, leaf area and dry weight to circumvent an edge effect. Samples for gene expression were harvested 24 h after hormone application. Three weeks after treatment, plants were harvested and leaf area was measured using a LI-3100C Area Meter (LI-COR Environmental). Rosette dry weight was determined on a microbalance with a 0.001 g resolution when the leaves had fully dried in a 60°C stove.

Chemical treatments

Five-week-old plants were treated with SA (Malinkrodt Baker, Deventer, the Netherlands) or a combination of MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands) and ABA (Sigma, Steinheim, Germany) by dipping plants in a solution containing either 1 mM SA or a combination of 100 µM MeJA and 100 µM ABA and 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands). MeJA and ABA solutions were diluted from a 1000-fold concentrated stock in 96% ethanol. The mock solution contained 0.015% Silwet L77 and 0.1% ethanol.

For the competition experiment, 4-week-old plants were treated with 500 µM SA (Mallinckrodt Baker, Deventer, The Netherlands), 50 µM MeJA (Duchefa Biochemie BV, Haarlem, The Netherlands) or a combination of both by applying 3 ml of the solutions to the plants as a root drench. MeJA solution was diluted from a 1000-fold concentrated stock in 96% ethanol. The mock solution contained 0.1% ethanol.

RNA extraction and RT-qPCR

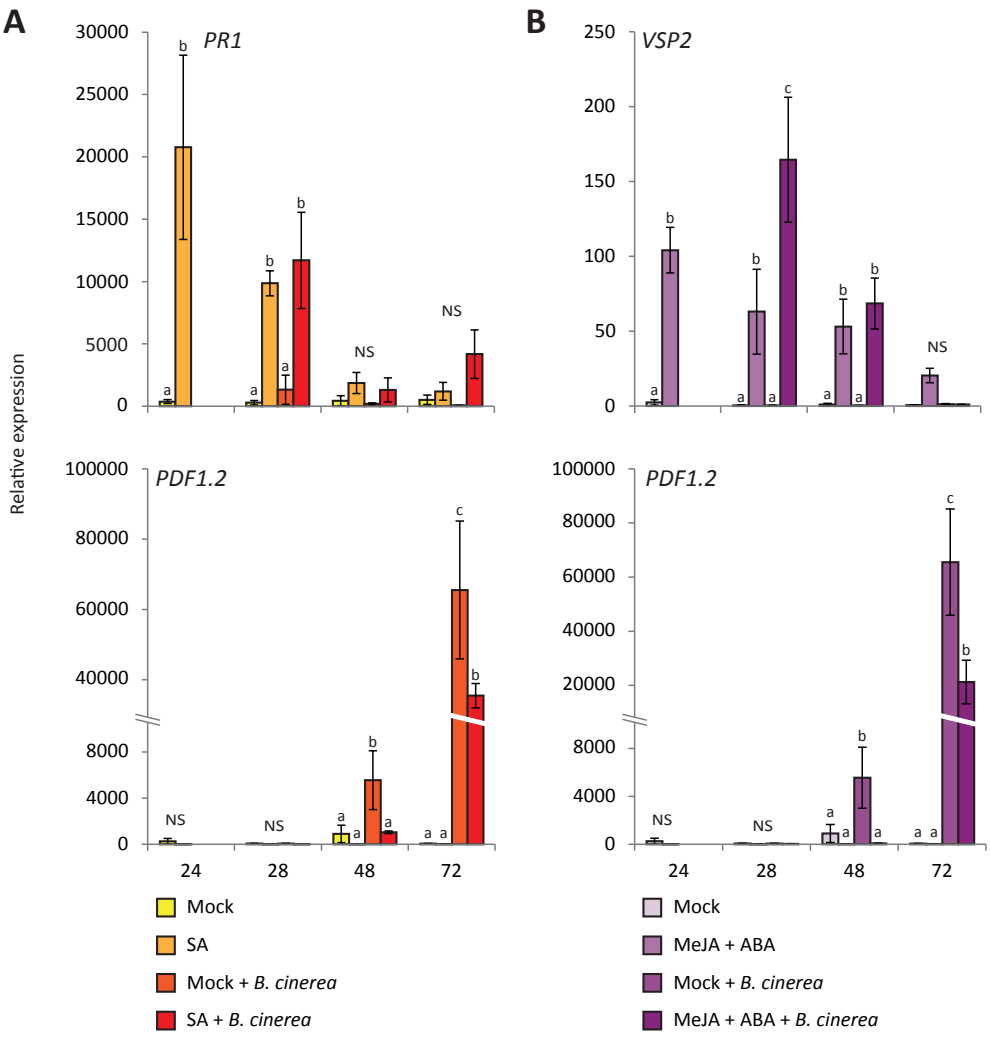
Total RNA was isolated as described (Oñate-Sánchez &Vicente-Carbajosa, 2008). SuperScript™ III Reverse Transcriptase was used to convert DNA-free total RNA into cDNA. PCR reactions were performed in optical 384-well plates (Applied Biosystems) with an ABI PRISM® 7900 HT sequence detection system using SYBR® Green to monitor the synthesis of double-stranded DNA. 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 to 95°C with a ramp speed of 1.0°C/min. Transcript levels were calculated relative to the reference gene At1g13320 (Czechowski *et al.*, 2005) using the $2^{-\Delta\Delta CT}$ method described previously (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Primer sequences were as described (Vos *et al.,* 2013b).

The AGI numbers of the studied genes are At2g14610 (*PR1*), At5g24770 (*VSP2*) and At5g44420 (*PDF1.2*).

ACKNOWLEDGEMENTS

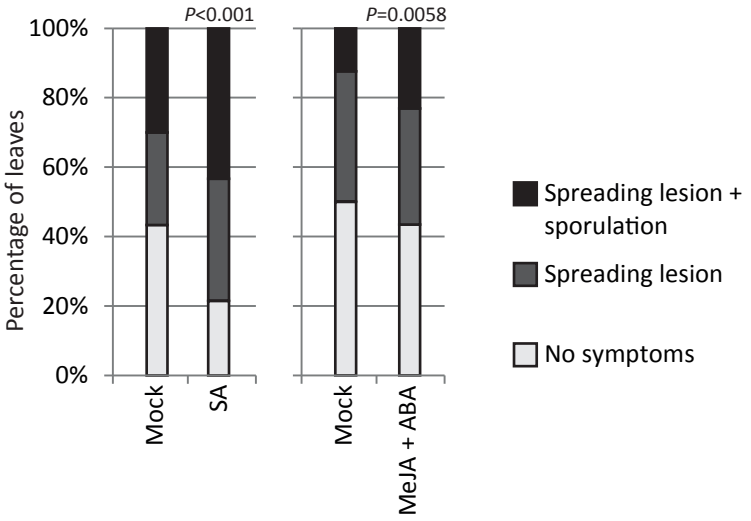
The authors thank Arjen Biere for critical reading of the manuscript and Hans Van Pelt and Silvia Coolen for rearing of *P. rapae*. This research was supported by VIDI grant no. 11281 of the Dutch Technology Foundation STW, which is part of the Netherlands Organization of Scientific Research (NWO), and ERC Advanced Investigator Grant no. 269072 of the European Research Council.

Conflict of interest statement: The authors declare that the research was conducted in the absence of any commercial of financial relationships that could be construed as a potential conflict of interest.

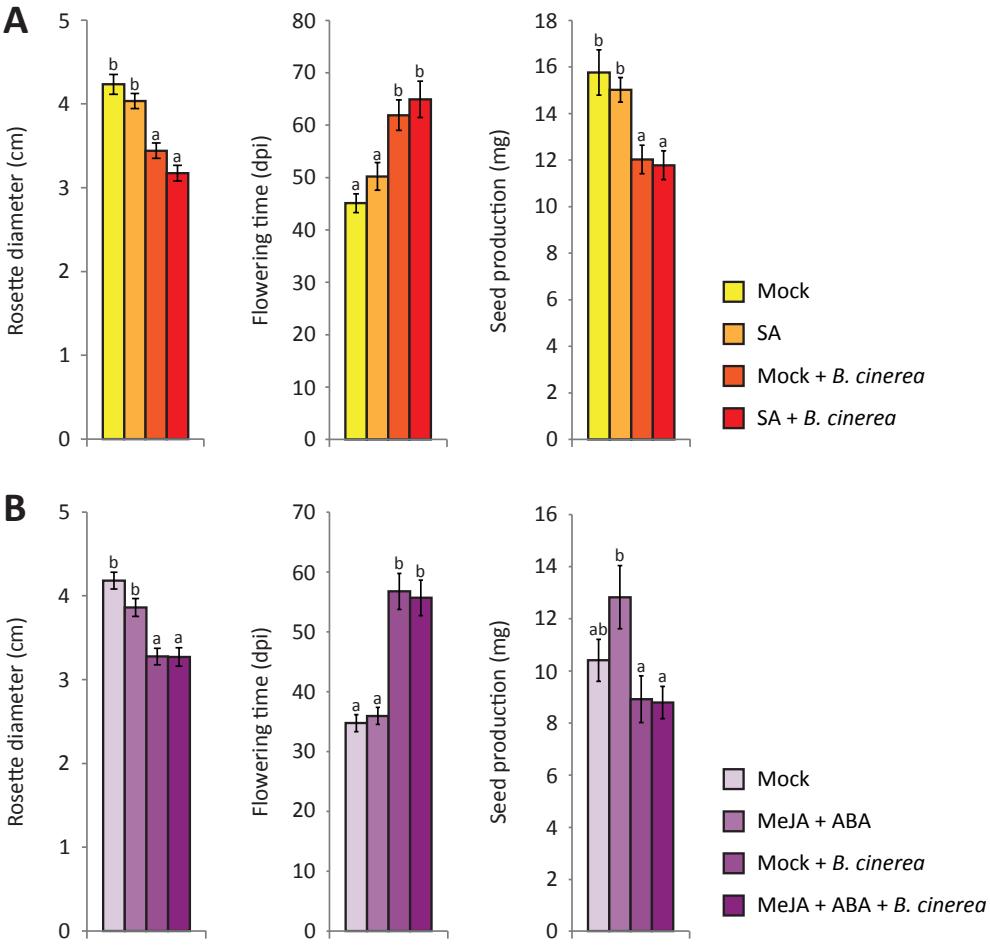


Supplemental Figure 1: Differential expression of *PR1*, *VSP2* and *PDF1.2* in response to hormone treatment and *B. cinerea* infection.

RT-qPCR analysis of SA-responsive *PR1* expression (A), MeJA/ABA-responsive *VSP2* expression (B) and *B. cinerea*-responsive *PDF1.2* expression (A & B). Plants were either treated with 1 mM SA or a combination of 100 µM MeJA and 100 µM ABA. At 24 h, all plants were inoculated with *B. cinerea*. Samples were taken at the indicated time points after the first treatment. Different letters indicate a statistically significant difference between the different treatments within one time point (ANOVA, Tukey post-hoc test; $P<0.05$; NS = not significant). Error bars represent SE, $n=3$ plants.



Supplemental Figure 2: Effect of hormone application on disease resistance against *B. cinerea*.
Quantification of disease symptoms of Arabidopsis Col-0 plants infected with *B. cinerea*. Twenty four h before inoculation with *B. cinerea*, plants were treated with 1 mM SA or a combination of 100 µM MeJA and 100 µM ABA. Disease severity of the inoculated leaves was scored in 3 classes. Percentage of leaves in each class was calculated per plant (X^2-test; *n*=20 plants).



Supplemental Figure 3: Growth and fitness parameters of single- and double-treated plants.
Rosette diameter (cm), flowering time (days post inoculation) and total seed production (mg) of Arabidopsis plants. Plants were either treated with 1 mM SA or a combination of 100 µM MeJA and 100 µM ABA. At 24 h the caterpillars were removed after which all plants were inoculated with *B. cinerea*. Different letters indicate a statistically significant difference between the different treatments (ANOVA, Tukey post-hoc test; $P<0.05$). Error bars represent SE, $n=20$ plants.

CHAPTER 5

Increased plant fitness upon infection with the biotrophic pathogen *Hyaloperonospora arabidopsidis*

Irene A Vos, Merel Steenbergen, Adriaan Verhage, Marieke Van Hulten, Corné MJ Pieterse, Saskia CM Van Wees

Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 80056, 3508 TB Utrecht, the Netherlands

ABSTRACT

Plants can protect themselves against attack by pathogens and insects by activating their inducible immune system. Activation of these inducible defenses entails fitness costs, since resources are allocated to resistance instead of to growth and reproduction. Environmental factors such as day length, competition with other plants and nutrient availability can influence the magnitude of these fitness costs or sometimes even avert them. In a field study, we observed that *Arabidopsis thaliana* plants that were infected with the biotrophic pathogen *Hyaloperonospora arabidopsidis* displayed enhanced fitness, as evidenced by a 70% increase in seed production. In this study, we investigated how environmental conditions influence the fitness of Arabidopsis after infection with this downy mildew pathogen. Generally, rosette growth was reduced in the first week after infection. However, under low nutrient availability, long-day conditions and low disease pressure, infected plants could compensate for this growth reduction in the second week by increasing their shoot growth at the expense of root growth. At the end of the vegetative growth phase, infected plants had grown as much or even more than control plants, indicating that they had compensated for the initial growth reduction. Moreover, *H. arabidopsidis*-infected plants could produce significantly more seeds than control plants. These results show that under certain environmental conditions, infection with *H. arabidopsidis* can positively influence plant fitness. As a biotroph, *H. arabidopsidis* benefits from keeping its host alive. Hence, it is tempting to speculate that the observed conditional plant fitness-promoting effect of *H. arabidopsidis* infection is a reflection of the plant-beneficial characteristics associated with the biotrophic lifestyle of this pathogen.

INTRODUCTION

To ward off the large diversity of attackers that plants encounter during their life time, they possess constitutive defenses, like rigid cell walls, thick cuticles, trichomes, thorns, and toxic or repellent compounds (Osbourn, 1996). In addition, recognition of an attacking pathogen or insect results in the activation of specific plant defense signaling pathways and the expression of appropriate inducible defense responses. These induced defenses are often controlled by a network of interacting plant hormones. The quantity, composition and timing of the hormonal signal signature that is produced upon pathogen or insect attack tailors the defense response specifically to the attacker at hand, which may help to prioritize effective over ineffective defenses (De Vos *et al.*, 2005; Pieterse *et al.*, 2012; Vos *et al.*, 2013a). Infection with a biotrophic pathogen, such as the oomycete *Hyaloperonospora arabidopsidis*, triggers the rapid synthesis of the plant hormone salicylic acid (SA; Malamy *et al.*, 1990; Métraux *et al.*, 1990; Zeilmaker *et al.*, 2015), leading to the activation of a large set of defense-related genes, amongst which the robust marker gene of the SA signaling pathway, *PR1* (Van Loon *et al.*, 2006a). The downy mildew pathogen *H. arabidopsidis* is an obligate biotroph and requires its host plant to remain alive in order to complete its life cycle and eventually reproduce (Coates & Beynon, 2010).

The activation of inducible plant defenses entails significant fitness costs, primarily because valuable resources are allocated to resistance instead of to growth and reproduction (Herms & Mattson, 1992; Heil & Baldwin, 2002; Walters & Heil, 2007; Vos *et al.*, 2013a). Exogenous application of SA or its chemical analogue benzothiadiazole (BTH) has been shown to reduce plant growth and seed production in different plant species (Heil *et al.*, 2000; Cipollini, 2002; Canet *et al.*, 2010). Furthermore, under non-infected conditions, *Arabidopsis thaliana* (Arabidopsis) mutants constitutively expressing SA-inducible defenses, such as *cpr1*, *cpr5* and *cpr6* are dwarfed and severely affected in seed production (Bowling *et al.*, 1994; Heil & Baldwin, 2002; Heidel *et al.*, 2004; Van Hulten *et al.*, 2006). Conversely, the SA-deficient *NahG* and *sid2* Arabidopsis genotypes have higher growth rates and seed production compared to wild-type plants (Cipollini, 2002; Abreu & Munné-Bosch, 2009), confirming the negative effects of SA on growth and reproduction. In SA-nonresponsive *npr1* plants, negative fitness effects of BTH treatment were minimized, indicating that NPR1-mediated signaling plays an important role in the shift from plant growth to plant defense when SA-dependent defenses are induced (Van Hulten *et al.*, 2006; Canet *et al.*, 2010). However, when *npr1* plants were infected with the biotrophic pathogen *H. arabidopsidis*, their fitness level was lower than that of wild-type plants (Heidel & Dong, 2006), demonstrating that, although costly, the induction of SA-dependent defenses is beneficial for plants when grown under pathogen pressure.

The level of fitness costs of induced defenses activated by exogenously applied hormones, pathogen infection or insect infestation is largely dependent on environmental factors such as day length, competition with other plants and nutrient availability (Vos *et al.*, 2013a). In a series of experiments testing the effects of BTH treatment on plant fitness under different nitrogen regimes, Dietrich *et al.* (2005) found that under high nitrogen conditions, plants could compensate for the BTH-induced reductions in rosette growth and seed production. Furthermore, investigating different Arabidopsis accessions and a range of *H. arabidopsidis* isolates, Salvaudon *et al.* (2008) found that the effect of infection with *H. arabidopsidis* could also lead to an increase in seed production in Arabidopsis accessions that had a low fecundity without infection. In the context of plant-herbivore interactions it has also been described that plants can sometimes compensate for the negative fitness effects of induced defenses (Trumble *et al.*, 1993) and several mechanisms have been suggested to play a role in this compensatory growth. For example, removal of leaf tissue can increase light levels to previously shaded leaves, thereby increasing photosynthetic capacity in those leaves. Furthermore, release of apical dominance and increased resource allocation from the root to the shoot can increase the growth rate of the shoot (Strauss & Agrawal, 1999).

In this study, we investigated how growth and seed production of Arabidopsis plants infected with the biotrophic pathogen *H. arabidopsidis*, is influenced by environmental factors and disease pressure. We show that under low nutrient availability and long-day conditions Arabidopsis plants showed increased fitness upon *H. arabidopsidis* infection and these effects seemed most pronounced when disease pressure was low.

RESULTS

Increased fitness of field-grown Arabidopsis plants upon infection with *H. arabidopsidis*

Fitness of Arabidopsis plants inoculated with *H. arabidopsidis* was compared to that of mock-treated plants when grown under field conditions in a common garden experiment. Rosette diameter and seed production were determined as indicators of plant fitness. Inoculation with *H. arabidopsidis* caused a significant repression in relative rosette growth during the first week after infection in comparison to non-inoculated plants (Figure 1). Surprisingly, plants compensated for this growth reduction by accomplishing a significant increase in relative rosette growth in the second week after inoculation with *H. arabidopsidis*. When overall relative rosette growth was calculated over the 2-week period that was measured in this experiment, no significant difference between *H. arabidopsidis*-inoculated plants and mock-treated plants was found. This indicates that within two weeks *H. arabidopsidis*-challenged plants had fully compensated the initial growth retardation.

Seed production was determined five weeks after inoculation, when most plants had finished producing new flowers and had started to senesce. Interestingly, *H. arabidopsidis*-infected plants produced 70% more seeds than healthy plants (Figure 1). Together these results show that under field conditions, infection of Arabidopsis with *H. arabidopsidis* can lead to enhanced plant fitness.

Low nutrient availability and long-day conditions positively affect plant fitness after *H. arabidopsidis* infection

To investigate in further detail which environmental conditions act positively on the *H. arabidopsidis*-mediated increase in plant fitness, we investigated rosette growth and seed production of *H. arabidopsidis*-infected plants grown under controlled growth chamber conditions in different soil types and under different nutrient availability regimes (Figure 2). Under short-day conditions (10-h day, 14-h night) and cultivation in SZ soil (the standard environment used in our laboratory), rosette growth and seed production of Arabidopsis were not significantly affected by *H. arabidopsidis* infection (Figure 3A). Also in the field soil no effect of *H. arabidopsidis* infection on plant fitness was found, except for a small enhancement in relative rosette growth in the third week and a positive trend toward increased overall rosette growth (Figure 3B). The difference

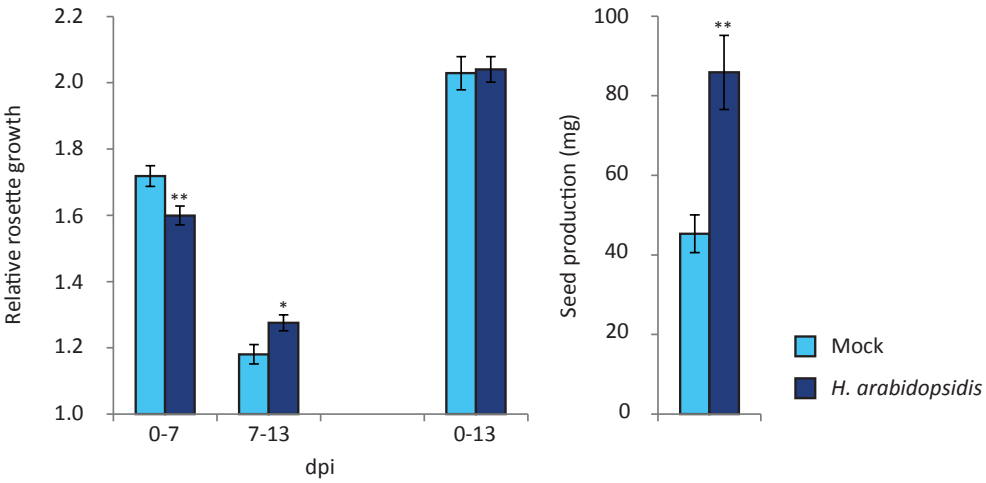


Figure 1: Relative rosette growth and seed production as fitness parameters in field-grown Arabidopsis infected with *H. arabidopsidis*.

Relative rosette growth and total seed production (mg) of mock-treated plants and plants inoculated with *H. arabidopsidis*. Shown are means of the relative increase in rosette diameter (fold-increase in rosette diameter over the indicated time period) and the overall relative rosette growth from time of inoculation until 2 weeks after inoculation, calculated for all individual plants. Seed production was measured by weighing the seeds of each plant. Asterisks indicate statistically significant differences between mock- and *H. arabidopsidis*-treated plants (Student's *t*-test; ** = $P<0.01$; * = $P<0.05$). Error bars represent SE, n=26-38 plants, dpi; days post inoculation.

with the significantly enhanced plant fitness that was observed in the common garden experiment (Figure 1) may be related to eradication of the naturally occurring microbial community, which was due to the required sterilization of the soil for this growth chamber experiment.

The role of nutrient availability in *H. arabidopsidis*-increased plant fitness was determined by cultivating Arabidopsis plants on river sand supplemented with different concentrations (5, 10 or 50%) of Hoagland nutrient solution. Under short-day conditions, all plants grown on sand showed a significant growth reduction in the first one or two weeks after inoculation, which was followed by a significant growth compensation in the third to fifth week (Figure 3C-E). This effect was irrespective of the amount of nutrients supplied. Overall, *H. arabidopsidis*-infected sand-grown plants showed a trend towards reduced rosette growth and reduced seed production, indicating that the repressive effect of *H. arabidopsidis* was greater than its beneficial effect on plant fitness. Thus, although under short-day growth chamber conditions some compensatory growth was observed on sand, the beneficial fitness effects of *H. arabidopsidis* on the plant were minimal and did not outweigh the costs associated with the infection.

Since the plants in the common garden experiment had experienced natural long-day conditions, a similar experiment as described above was performed under long-day conditions (16-h day, 8-h night). Overall, plants were better able to compensate their initial growth reduction after *H. arabidopsidis* infection under long-day conditions compared to short-day conditions (Figure 4). In SZ soil, there was a small growth reduction in the first week, followed by a significant growth induction in the second week after inoculation. Overall, there was no significant growth difference between mock-treated and infected plants and *H. arabidopsidis*-infected plants produced

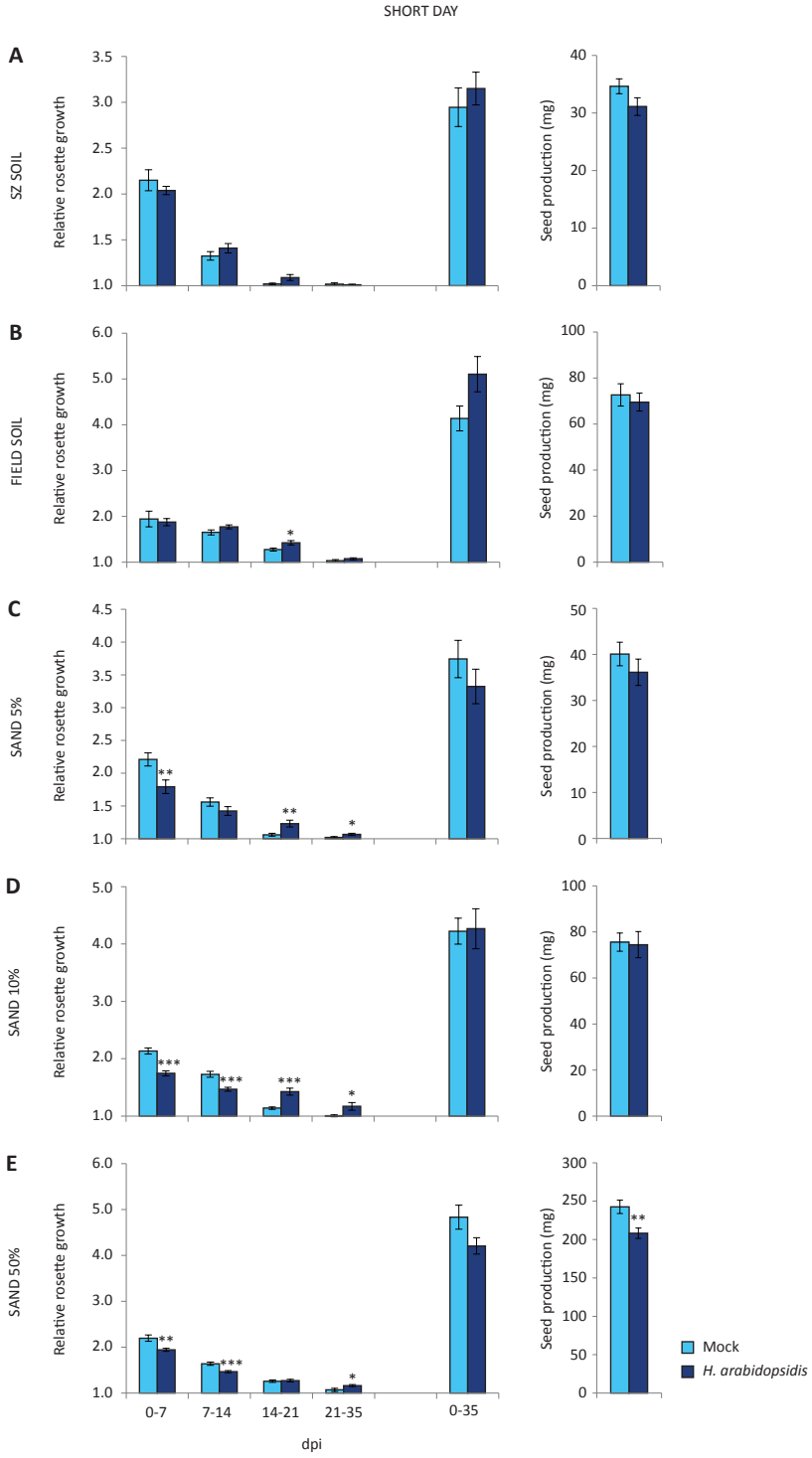
Figure 2: Impression of plants cultivated on the different soil types.
Arabidopsis plants were grown under controlled growth chamber conditions in different soil types. From left to right the pictures show a plant cultivated on SZ soil (the standard environment in our laboratory), field soil and river sand.

significantly more seeds (Figure 4A). Plants grown in field soil showed neither negative nor positive effects of *H. arabidopsidis* regarding rosette growth, but seed production was significantly increased in infected plants (Figure 4B). All sand-grown plants, supplied with different amounts of nutrients, showed a significant growth reduction in the first week after inoculation and subsequently, a significant growth compensation in the second and/or third week (Figure 4C-E). Plants grown on sand supplemented with 5% Hoagland solution showed a significant overall 35% increase in rosette growth and a significant ~30% increase in seed production after infection with *H. arabidopsidis* compared to mock-treated plants (Figure 4C). In plants supplied with 10 or 50% Hoagland solution, *H. arabidopsidis* infection did not significantly influence overall rosette growth and seed production (Figure 4D & E). Taken together, long-day conditions combined with low nutrient availability determined whether plants could compensate for the fitness costs related to *H. arabidopsidis* infection (Figure 3 & Figure 4).

Low nutrient availability and long-day conditions increase root:shoot ratio

It has been found that plants with higher root:shoot ratio were better able to compensate for the fitness costs imposed by herbivory (Strauss & Agrawal, 1999). Furthermore, low nutrient availability can enhance root:shoot ratio (Olff *et al.*, 1990). To investigate whether the ability of Arabidopsis to compensate for fitness costs after *H. arabidopsidis* infection is correlated with root:shoot ratio, we assayed plants grown on river sand supplemented with different amounts of nutrients, under short- and long-day conditions. Application of 5% versus 10% Hoagland solution increased the root:shoot ratio under short-day conditions and seemingly also under long-day conditions, although these latter results were obtained from different experiments and need to be considered with caution (Figure 5A). There was no difference in root:shoot ratio between short and long-day-grown plants supplemented with 10% Hoagland solution. Infection with *H. arabidopsidis* reduced root:shoot ratio when plants received 5% Hoagland solution, under both short- and long-day conditions. However, when plants were supplemented with 10% Hoagland solution, infection did not alter the root:shoot ratio (Figure 5A). These data suggest that plants grown under long-day conditions with low nutrient availability had the highest root:shoot ratio, which is in line with the conditions under which plants were best able to compensate after *H. arabidopsidis* infection.

To gain more insight in the effects of *H. arabidopsidis* infection on rosette and root growth, we examined the dry weight of the rosettes and the roots of plants grown on sand supplemented with 5% Hoagland solution under long-day conditions at 0, 7, 14 and 21 days after inoculation. The fold-increase in root dry weight was significantly lower in the first week after inoculation in comparison to mock-treated plants, but there were no differences in root dry weight gain in the subsequent weeks. Overall there was a

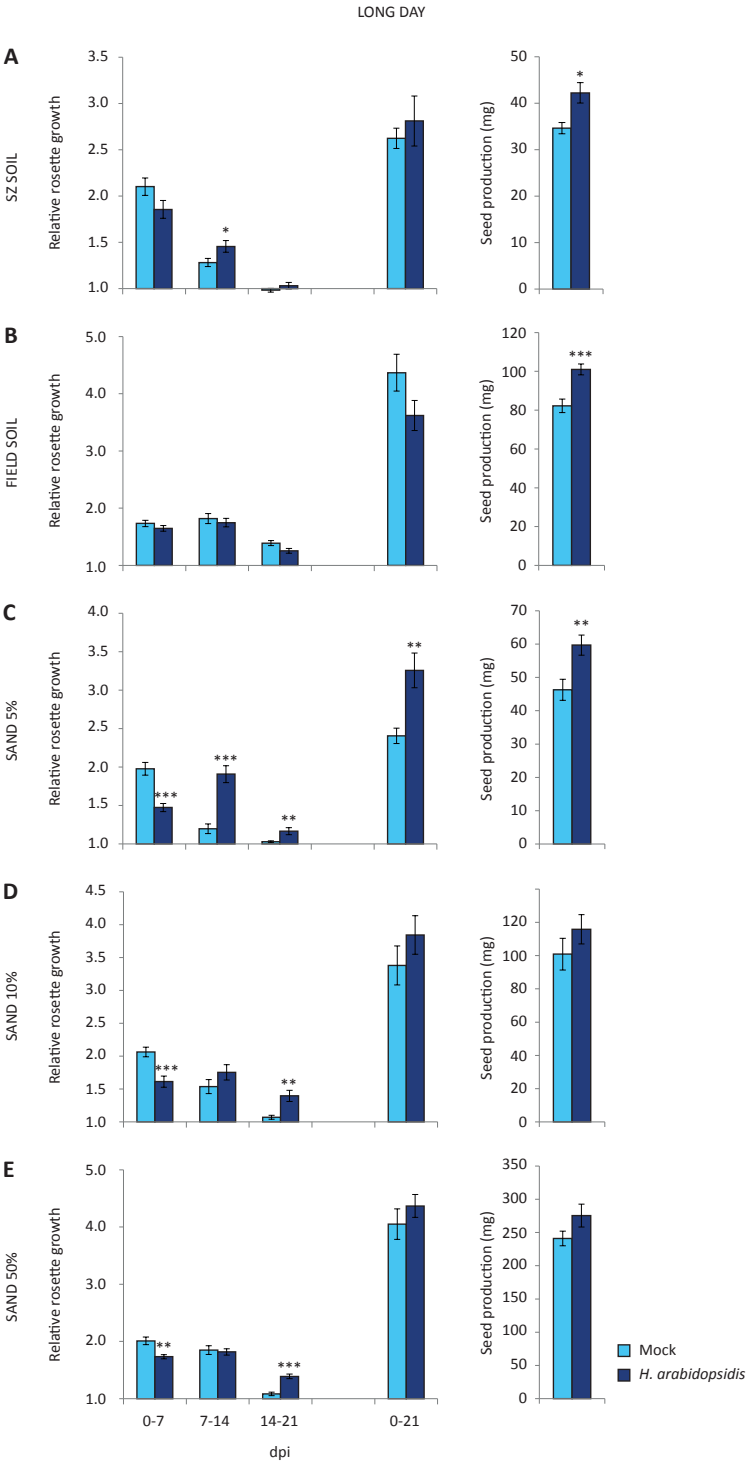

SHORT DAY
A
SZ SOIL
B
FIELD SOIL
C
SAND 5%
D
SAND 10%
E
SAND 50%
Relative rosette growth
Seed production (mg)
0-7
7-14
14-21
21-35
0-35
dpi
Mock
H. arabidopsidis

significantly lower fold-increase in dry weight of the roots of *H. arabidopsidis*-infected plants (Figure 5B). The fold-increase in dry weight of the rosettes was also significantly lower in the first week after infection with *H. arabidopsidis* than in mock-treated plants, which was followed by a significantly higher fold-increase in rosette dry weight in the second week. Overall there was no significant difference in fold-increase in dry weight of the rosettes at the end of the experiment (Figure 5B). Together, this indicates that the compensatory growth of *H. arabidopsidis*-infected plants grown under a combination of long-day conditions and low nutrient availability may be explained by an enhancement of rosette growth at the expense of root growth.

Low disease pressure positively affects fitness compensation after *H. arabidopsidis* infection

Previously, it has been found that the magnitude of fitness costs associated with the activation of SA-dependent defense responses is dose-dependent (Cipollini, 2002; Van Hulten *et al.*, 2006). Therefore, we investigated whether the disease pressure, and thus the level of the associated SA-dependent defense responses induced by *H. arabidopsidis* infection, could influence the balance between costs and benefits for the plant. Different concentrations of *H. arabidopsidis* spore suspension (5, 50 and 250 spores/µl) were sprayed onto plants that were growing on river sand supplemented with 5% Hoagland solution under controlled long-day conditions. Figure 6A shows that the compensatory growth effect in this experiment was not as clear as in previous experiments. Rosette growth reduction in the first week was established by all *H. arabidopsidis* spore concentrations. However, compensation for this growth reduction in the second week was only found for the lowest spore concentration. Over the total three-week period, there was no difference in rosette growth between mock-treated plants and plants treated with 5 spores/µl. Treatment with 50 or 250 spores/µl resulted in significantly less growth compared to mock-treated plants. There were no significant differences in seed production between any of the treatments (Figure 6A), indicating that although plants treated with the lowest spore concentration compensated for

Figure 3: Effect of soil type on plant fitness of short-day grown plants infected with *H. arabidopsidis*.
Relative rosette growth and total seed production (mg) of mock-treated plants and plants inoculated with *H. arabidopsidis*. Plants were grown on SZ soil, autoclaved field soil or river sand supplemented with 5, 10 or 50% Hoagland solution under short-day conditions. Shown are means of the relative increase in rosette diameter (fold-increase in rosette diameter over the indicated time period) and the overall relative rosette growth from time of inoculation until 5 weeks after inoculation, calculated for all individual plants. Seed production was measured by weighing the seeds of each plant. Asterisks indicate statistically significant differences between mock- and *H. arabidopsidis*-treated plants (Student's *t*-test;*** = $P<0.001$; ** = $P<0.01$; * = $P<0.05$). Error bars represent SE, n=8-10 plants, dpi; days post inoculation.


LONG DAY
A
SZ SOIL
B
FIELD SOIL
C
SAND 5%
D
SAND 10%
E
SAND 50%
Relative rosette growth
Seed production (mg)
0-7
7-14
14-21
0-21
dpi
Mock
H. arabidopsidis

the initial reduction in rosette growth, fitness was not increased in these plants.The root:shoot ratio was also determined in this experiment. However, none of the spore concentrations significantly influenced the root:shoot ratio, although there was a trend towards lower root:shoot ratio in plants treated with 5 spores/µl (Figure 6B), comparable to the decrease in root:shoot ratio after *H. arabidopsidis* infection in the previous experiment (Figure 5A).

We also examined the dry weight of the roots and the rosettes at 0, 7 and 14 days after inoculation. The fold-increase in root dry weight of plants treated with 5 spores/µl was higher than in mock-treated plants in the first week after treatment, which was followed by a significant lower fold-increase in root dry weight in the second week. Plants treated with 5 spores/µl showed no significant difference regarding fold-increase in dry weight of the rosette, although there was a trend towards an increase in comparison to mock-treated plants (Figure 6C). Together, this gives an explanation for the trend towards lower root:shoot ratio in those plants. Plants treated with 50 or 250 spores/µl showed no significant effects on either rosette or root biomass, but overall there was a trend towards an increase by 50 spores/µl, while 250 spores/µl-treated plants tended to reduce their growth (Figure 6C). Taken together, plants treated with the highest spore concentration showed a negative effect on growth and were not able to compensate after *H. arabidopsidis* infection. Plants treated with the lowest spore concentration seemed to be best able to compensate for rosette growth inhibition after *H. arabidopsidis* infection. Comparable to the previous experiment, this was associated with a decrease in root:shoot ratio after infection in these plants, which may be explained by an enhancement of rosette growth at the expense of root growth.

DISCUSSION

Compensation of initial plant growth reduction inflicted by *H. arabidopsidis* infection

Growth compensation after an initial defense-related growth reduction has been recorded in plants after feeding by herbivores (Paige & Whitham, 1987; Trumble *et al.*,

Figure 4: Effect of soil type on plant fitness of long-day grown plants infected with *H. arabidopsidis*.
Relative rosette growth and total seed production (mg) of mock-treated plants and plants inoculated with *H. arabidopsidis*. Plants were grown on SZ soil, autoclaved field soil or river sand supplemented with 5, 10 or 50% Hoagland solution under long-day conditions. Shown are means of the relative increase in rosette diameter (fold-increase in rosette diameter over the indicated time period) and the overall relative rosette growth from time of inoculation until 3 weeks after inoculation, calculated for all individual plants. Seed production was measured by weighing the seeds of each plant. Asterisks indicate statistically significant differences between mock- and *H. arabidopsidis*-treated plants (Student's *t*-test;*** = $P<0.001$; ** = $P<0.01$; * = $P<0.05$). Error bars represent SE, n=8-10 plants, dpi; days post inoculation.

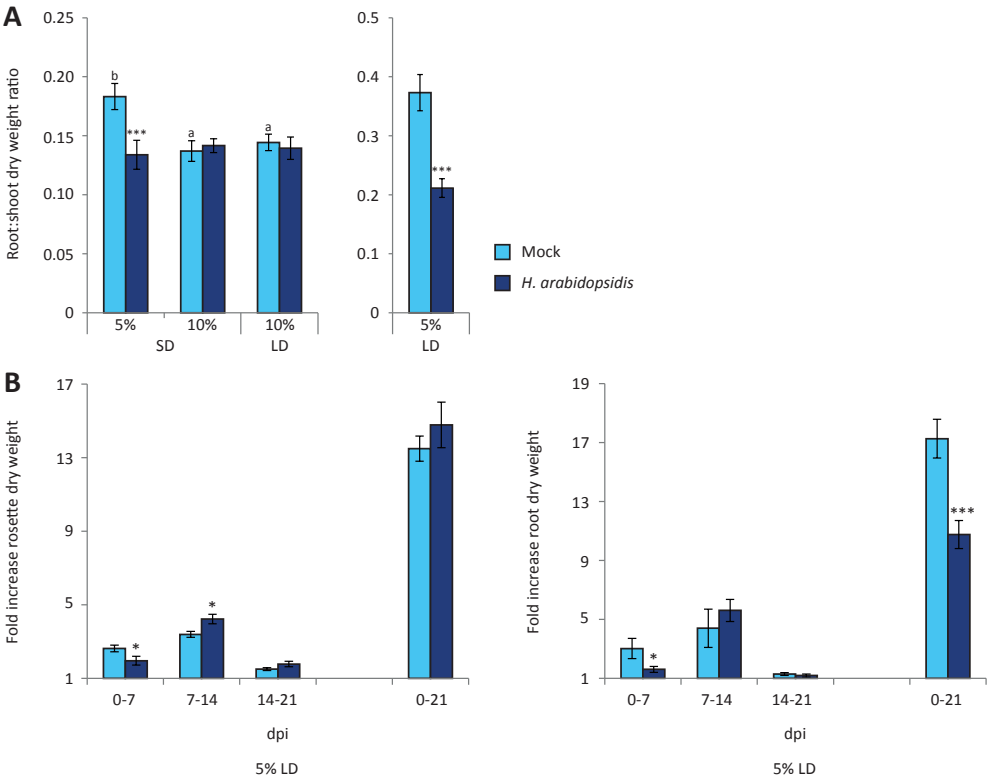


Figure 5: Effect of day length and nutrient availability on *H. arabidopsidis*-induced changes in root and shoot growth.

(A) Root:shoot ratio of mock-treated plants and plants inoculated with *H. arabidopsidis*. Plants were grown on river sand supplemented with 5 or 10% Hoagland solution under short-day (SD) or long-day (LD) conditions. Different letters indicate statistically significant differences between mock-treated plants cultivated under the different growing conditions. Asterisks indicate statistically significant differences between mock- and *H. arabidopsidis*-treated plants per growing condition (ANOVA, Tukey post-hoc test; *** = $P<0.001$). Error bars represent SE, $n=10$ plants.

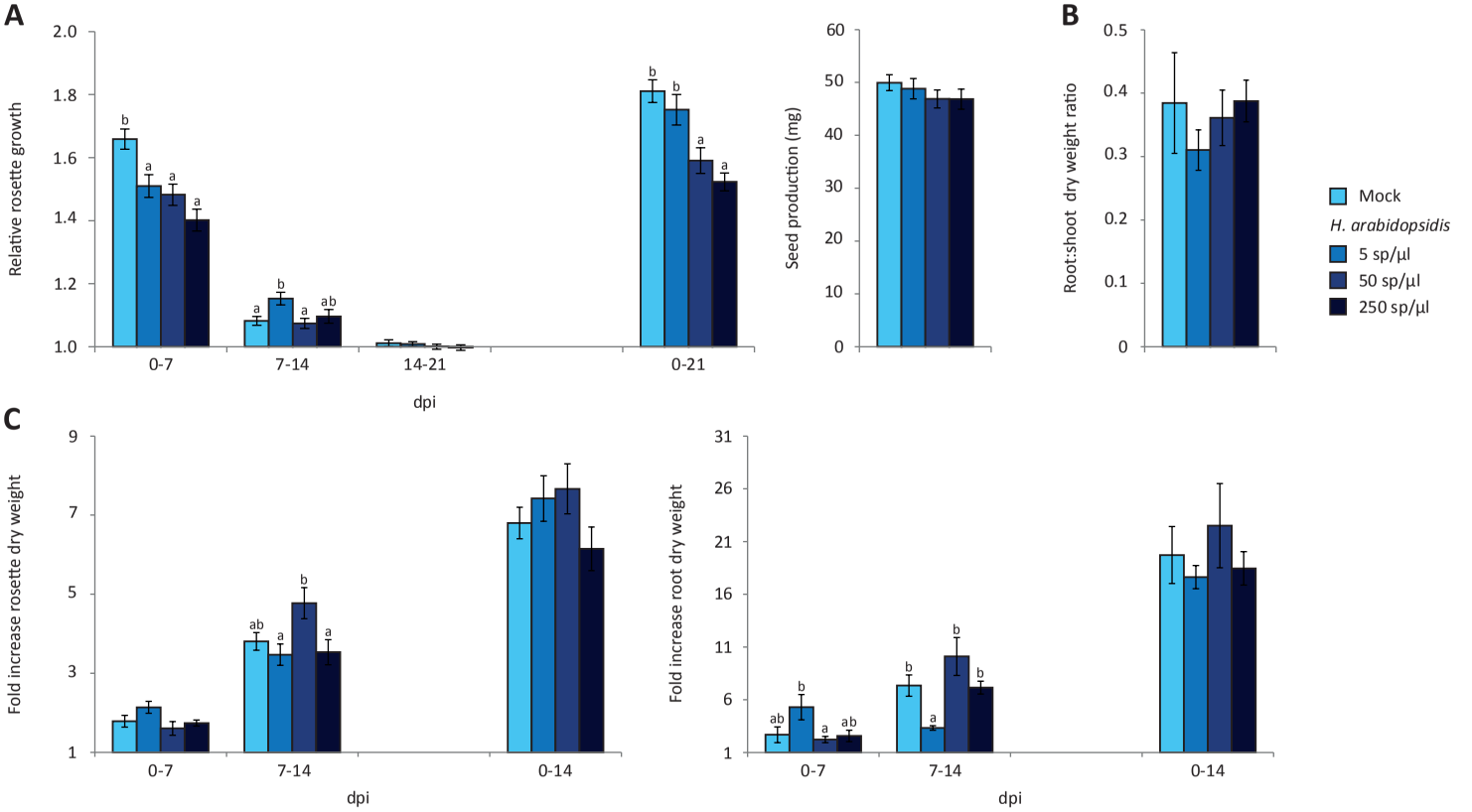
(B) Fold-increase in dry weight of rosettes and roots of mock-treated plants and plants inoculated with *H. arabidopsidis*. Plants were grown on river sand supplemented with 5 % Hoagland solution under long-day conditions. Shown are means of the relative increase in dry weight (fold-increase in dry weight over the indicated time period) and the overall fold-increase in dry weight from time of inoculation until 3 weeks after inoculation, calculated for all individual plants. Asterisks indicate statistically significant differences between mock- and *H. arabidopsidis*-treated plants (Student's *t*-test; *** = $P<0.001$; * = $P<0.05$). Error bars represent SE, $n=10$ plants, dpi; days post inoculation.

1993; Strauss & Agrawal, 1999; Agrawal, 2000) and after treatment with BTH (Dietrich *et al.*, 2005). We found that plants can also compensate in growth and seed production after infection with *H. arabidopsidis* (Figure 1). The growth reduction found in the first week after infection could be caused by allocation costs of the infection, since the plant is allocating resources to defense instead of to growth (Heil & Baldwin, 2002; Vos *et al.*, 2013a). The subsequent compensating effect was most pronounced under low nutrient availability, long-day conditions and low disease pressure (Figure 4 & Figure 6). We found a decrease in root growth after *H. arabidopsidis* infection (Figure 5B & Figure 6C), while rosette growth was not reduced or even enhanced (all Figures), suggesting that increased resource allocation from the root to the shoot might be a mechanism regulating compensatory growth after *H. arabidopsidis* infection.

Compensatory growth in plants attacked by herbivores is described as so-called 'by-product mutualism' (Agrawal, 2000), since the fitness benefit of the by-products of being consumed (for example, release of apical dominance) is greater than the cost of being eaten. As a biotrophic pathogen, *H. arabidopsidis* relies on a living host and an increase in plant biomass and seed production would expectedly lead to both enhanced spore production and greater amounts of host plants in the next generation. Therefore, the *H. arabidopsidis*-Arabidopsis interaction might involve a by-product mutualism, whereby *H. arabidopsidis* infection increases plant fitness, leading to larger and more host plants.

Effect of nutrient availability on compensatory growth of Arabidopsis

The compensatory effect on growth after BTH treatment was reported to be most pronounced when the plants were supplied with high nutrient levels (Dietrich *et al.*, 2005). On the contrary, we found that the compensatory fitness effects after infection with *H. arabidopsidis* were most extreme under low nutrient conditions (Figure 3 & Figure 4). Likewise, compensation after herbivory was reported to be larger at lower nutrient availability (Gertz & Bach, 1995). The latter may be related to an increase in root:shoot ratio that is generally caused by low levels of nutrients (Olff *et al.*, 1990), which in turn is associated with higher tolerance to herbivores (Strauss & Agrawal, 1999). To investigate if the root:shoot ratio also plays a role in the compensatory fitness effect related to *H. arabidopsidis* infection, we measured the root:shoot ratio of plants grown on river sand supplemented with 5 and 10 % Hoagland solution and cultivated under short- and long-day conditions. Lower nutrient availability indeed increased the root:shoot ratio (Figure 5A). Plants grown under a combination of long-day conditions and low nutrient availability had the highest root:shoot ratio (approximately 0.4 in two separate experiments; Figure 5A & Figure 6B), which is in line with the ability of those plants to compensate fitness costs after *H. arabidopsidis* infection. These results suggest that a relatively high root:shoot ratio in non-infected plants, could be


A
Relative rosette growth
2.0
1.8
1.6
1.4
1.2
1.0
b
a
a
a
b
b
a
ab
b
b
a
a
0-7
7-14
14-21
0-21
dpi
Seed production (mg)
60
50
40
30
20
10
0
B
Root:shoot dry weight ratio
0.5
0.4
0.3
0.2
0.1
0
Mock
H. arabidopsidis
5 sp/µl
50 sp/µl
250 sp/µl
C
Fold increase rosette dry weight
9
7
5
3
1
ab
a
b
a
0-7
7-14
0-14
dpi
Fold increase root dry weight
31
26
21
16
11
6
1
ab
b
a
ab
b
a
b
b
0-7
7-14
0-14
dpi

a good indicator of the ability of the plants to compensate growth reduction after *H. arabidopsidis* infection. However, more research is needed to support this hypothesis and investigate the mechanisms underpinning this growth control.

Effect of day length and disease pressure on fitness of *H. arabidopsidis*-infected Arabidopsis

Day length has been found to influence different plant traits like flowering, volatile emission, nutrient uptake and leaf thickness (Dorais *et al.*, 1996; Vänninen *et al.*, 2010). Furthermore, the resistance to different attacking organism has been reported to be influenced by day length. For example, resistance of strawberry plants to two-spotted spider mites, resistance of tomato plants to *Manduca sexta* caterpillars and resistance of potato plants to *Phytophthora infestans* was higher under long-day conditions compared to short-day conditions (Kennedy *et al.*, 1981; Patterson *et al.*, 1994; Lebecka & Sobkowiak, 2013). We found that plants were better able to compensate the fitness costs after *H. arabidopsidis* infection under long-day conditions compared to short-day conditions. Whether this enhanced compensation under long-day conditions is associated with enhanced tolerance or resistance to *H. arabidopsidis* is not known and could be investigated by assessing the activation of defense responses and the resistance level to *H. arabidopsidis* under the different day length conditions.

If the plant's ability to compensate growth after *H. arabidopsidis* infection is dependent on the disease pressure of the *H. arabidopsidis* infection, a low inoculation dose can be expected to result in better compensation. Plants were indeed demonstrated to be best able to compensate at the lowest disease pressure (Figure 6). In light of the by-product mutualism hypothesis, this makes sense since at higher disease pressure the costs associated with the infection are more likely to outweigh the benefits for the plant. However, whether the better compensation under long-day conditions is correlated with the better compensation under low disease pressure needs to be investigated.

Figure 6: Effect of different spore concentrations on plant fitness after inoculation with *H. arabidopsidis*.
Relative rosette growth and total seed production (mg) (A), root:shoot ratio (B) and fold-increase in dry weight of rosettes and roots (C) of mock-treated plants and plants inoculated with 5, 50 or 250 spores/µl of *H. arabidopsidis*. Plants were grown on river sand supplemented with 5% Hoagland solution under long-day conditions. Shown are means of the relative increase in rosette diameter, rosette dry weight or root dry weight (over the indicated time period) and the overall fold-increase from time of inoculation until 2 or 3 weeks after inoculation, calculated for all individual plants. Different letters indicate statistically significant differences between treatments at the indicated time point (ANOVA, Tukey post-hoc test, $P<0.05$). Error bars represent SE, n=7-20 plants, dpi; days post inoculation

Other environmental factors influencing plant fitness upon *H. arabidopsidis* infection
In dense stands of plants, such as agricultural monocultures, plants compete for light, water and nutrients. When grown in dense stands, the growth reduction in the first week could give *H. arabidopsidis*-infected plants an insurmountable backlog and they might not be able to compensate the initial growth reduction in the subsequent weeks. Testing *H. arabidopsidis* infection in a dense competition setup might give more insight in the possibility of plants to overcome the growth reduction of the first week in an agricultural realistic setting.

The plant fitness results obtained from the controlled conditions in the growth chambers resembled those from the variable conditions in the field experiment, but the 70% increase in seed production that was found in the field after infection with *H. arabidopsidis* was not reached in the growth chambers. This suggests that in the field, additional environmental factors also play a role in the plant's ability to compensate for the fitness costs upon *H. arabidopsidis* infection. For example, beneficial rhizobacteria and fungi that are naturally present in the field could have primed the plants for enhanced SA- and/or JA-dependent defense responses (Tjamos *et al.*, 2005; Van Wees *et al.*, 2008). Autoclaving the field soil, which was necessary to use it in our growth chambers, eradicated these soil microbes, which may have caused a reduction of the compensatory effect (Figure 1, Figure 3 & Figure 4). Additionally, also above-ground interactors and abiotic factors like temperature fluctuation, precipitation, and wind may have contributed to the enlarged compensatory fitness effect in the field.

Altogether, the results presented here show that interactions between plants and pathogens do not necessarily lead to negative fitness effects for the plants. Enhanced seed set and growth compensation of Arabidopsis infected with *H. arabidopsidis* was dependent on a combination of environmental factors like low nutrient availability, long-day conditions and low disease pressure. This combination of factors was associated with an increased root:shoot ratio in the control situation, which decreased upon infection with *H. arabidopsidis*, likely due to enhanced allocation of resources from the root to the shoot. Further exploring these beneficial fitness effects may give insight in the finely tuned intimate interactions between biotrophic microbes and their plant hosts and might provide useful leads for future crop protection.

MATERIAL & METHODS

Hyaloperonospora arabidopsidis inoculation

Hyaloperonospora arabidopsidis WACO9 was maintained on susceptible Arabidopsis *eds1* plants by weekly transfer to healthy 14-day-old seedlings as described (Koch & Slusarenko, 1990). Sporangia were obtained by washing diseased leaves in demineralized water. Debris was filtered out using Miracloth (Merck) and spores were resuspended in demineralized water to the final density (5, 50 or 250 spores/µl). Plants were inoculated by spraying the *H. arabidopsidis* spore suspension using a fine paint brush, after which the plants were kept at 100% relative humidity at 17°C for 7 days (growth chamber experiments) or 2 days (field experiment) to facilitate infection (Van Damme *et al.*, 2005). Subsequently, plants were transferred to other conditions as described in the sections below.

Rosette diameter and seed production

Rosette diameters were measured from pictures that had been taken at the indicated time points. Two opposite longitudinal measurements were taken of each rosette using ImageJ. On-picture rulers were used to convert measured pixels to realistic centimeters. To determine seed production, plants were watered every other day until they stopped producing new flowers. Inflorescences were harvested when all plants had finished flowering and the seeds were weighed on a microbalance with a 0.0001 g resolution.

Dry weight and root:shoot ratio

Roots and rosettes were harvested at the indicated time points and dried in a 60°C stove. Dry weight of the plants was determined on a microbalance with a 0.0001 g resolution when plants had fully dried. Root:shoot ratio was calculated using the dry weight of roots and rosettes.

Field experiment

Seeds of *Arabidopsis thaliana* accession Col-0 were imbibed in 0.1% agar at 4°C for 3 days. Subsequently, they were sown on non-autoclaved sand-potting soil mixture (5:12 v/v) in trays consisting of 200 cells (Teku Seedling Tray cat. No. JP3050/230), one seed per cell. Plants were kept in a greenhouse compartment with an 8-h day (24°C) and 16-h night (20°C) cycle at 100% relative humidity till germination. Subsequently, plants were cultivated in the same compartment at 70% relative humidity. Two-week-old seedlings were placed in an uncontrolled plastic semi-dome greenhouse to acclimatize for 5 days after which the seedlings with 4 true leaves were transplanted to a field site near Utrecht, the Netherlands (N 52° 5’23.88” E 5°10’21.70”). Plants were spaced 3.9 cm apart in 11 plots. To protect the plants from slugs, Escar-Co® pellets (Ecostyle)

were placed. Shade was provided by maze nettings placed at 1.5 m above the plants. Plants were watered regularly and not actively protected from insect herbivores and pathogens. After 10 days in the field plants were sprayed with water or *H. arabidopsidis* (50 spores/µl) using an artist paintbrush. To facilitate infection, plants were kept at 100% relative humidity for 48 h by enclosing the plants with see-through lids, after which the lids were removed. Rosette diameters were measured at 0, 7 and 13 days after treatment. Inflorescences were harvested when the plants were 2 months old and left to dry in a paper bag for another 3 months, after which the weight of the seeds was determined.

Soil types and plant cultivation

Seeds of *Arabidopsis thaliana* accession Col-0 were sown on river sand. Two weeks later, seedlings were transplanted into 60-ml pots. Pots were filled with a river sand-potting soil mixture (5:12 v/v) named SZ soil (Stek Zaai soil), field soil (obtained from the plots used for the field experiment) or river sand (Figure 2). All soil types had been autoclaved twice for 20 min with a 24-h interval. Plants were cultivated in a growth chamber with a 10-h day and 14-h night cycle at 70% relative humidity and 21°C. Plants were watered every other day and the plants cultivated on river sand grown plants received 50, 10 or 5% Hoagland solution (Hoagland & Arnon, 1938) containing 10, 2 or 1 µM sequestreen, respectively (CIBA-Geigy, Basel, Switzerland) once a week. Five-week-old plants were sprayed with water (mock) or *H. arabidopsidis* (50 spores/µl), after which they were kept at 100% relative humidity for 7 days. Subsequently, they were transferred to short-day conditions (10-h day and 14-h night cycle) or long-day conditions (16-h day and 8-h night cycle) at 70% relative humidity and 21°C.

ACKNOWLEDGEMENTS

This research was supported by VIDI grant no. 11281 of the Dutch Technology Foundation STW and VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which are part of the Netherlands Organization of Scientific Research (NWO), and ERC Advanced Investigator Grant no. 269072 of the European Research Council.

CHAPTER 6

Summarizing discussion

Hormone-induced plant defense responses

In complex natural environments, plants have to deal with a wide diversity of attackers, like microbial pathogens and herbivorous insects. Plants can ward off the majority of attackers with constitutive defenses, like rigid cell walls, thick cuticles, trichomes, thorns and toxic or repellent compounds (Osbourn, 1996). In addition, recognition of an attacking pathogen or insect results in the activation of specific plant defense signaling pathways and the expression of inducible defense responses (Pieterse *et al.*, 2012). It has been suggested that the inducible character of these defenses has evolved because of the significant fitness costs that are associated with the constitutive expression of defenses (Simms & Fritz, 1990; Heil & Baldwin, 2002). The production of plant hormones plays a crucial role in the activation of these induced defense responses (Pieterse *et al.*, 2009; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012).

The hormonal blend that is produced upon pathogen or insect attack is dependent on the lifestyle and invasion strategy of the attacker (De Vos *et al.*, 2005). Salicylic acid (SA) plays an important role in the activation of induced plant defenses against biotrophic pathogens. On the other hand, jasmonic acid (JA) is an important regulator in induced plant defense responses against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). Furthermore, abscisic acid (ABA) and ethylene (ET) have been found to play a modulating role on JA-dependent defense responses (Van Loon *et al.*, 2006b; Ton *et al.*, 2009). JA in combination with ABA results in activation of the MYC-branch of the JA-signaling pathway, which induces defense responses against herbivorous insects. The MYC-branch is regulated by the MYC-transcription factors MYC2, MYC3 and MYC4, leading to activation of the MYC-branch marker genes *VSP1* and *VSP2* (Figure 2 of Chapter 1; Chapter 2 & Chapter 3; Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Howe & Jander, 2008; Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011; Vos *et al.*, 2013b). On the other hand, JA together with ET results in activation of the ERF-branch, which is effective against necrotrophic pathogens. The ERF-branch is regulated by AP2/ERF-domain containing transcription factors ERF1 and ORA59, leading to activation of the ERF-branch marker gene *PDF1.2* (Figure 2 of Chapter 1; Chapter 2 & Chapter 3; Penninckx *et al.*, 1998; Glazebrook, 2005; Pré *et al.*, 2008).

Crosstalk between different hormone signaling pathways is hypothesized to allow the plant to activate effective over ineffective defenses in a cost-efficient manner (Pieterse *et al.*, 2012; Thaler *et al.*, 2012). Activation of the SA signaling pathway has been shown to have a strong suppressive effect on JA-dependent gene expression and defense responses against necrotrophic pathogens and herbivorous insects (Chapter 4; Van Wees *et al.*, 1999; Spoel *et al.*, 2003; Spoel *et al.*, 2007; Koornneef & Pieterse, 2008; Van der Does *et al.*, 2013; Caarls *et al.*, 2015). Furthermore, between the MYC- and the ERF-branch of the JA signaling pathway a mutually antagonistic relationship exists

(Chapter 2 & Chapter 4; Lorenzo *et al.*, 2004; Verhage *et al.*, 2011; Vos *et al.*, 2013b).

The main goal of this study was to investigate how activation of defense signaling pathways and the crosstalk between them influences plant defense and plant fitness. Therefore, we investigated the crosstalk between the MYC- and the ERF-branch of the JA signaling pathway and the modulating role of ABA and ET herein (Chapter 2). We also investigated the differential activation of the JA response pathway and the role of ABA signaling in undamaged systemic leaves of *Pieris rapae*-infested plants (Chapter 3). Furthermore, we tried to unravel whether hormonal crosstalk influences plant fitness under multi-attacker conditions (Chapter 4). Finally, we studied how environmental factors and disease pressure influence plant fitness after infection with the biotrophic pathogen *Hyaloperonospora arabidopsidis* (Chapter 5). In this chapter, the results presented in this thesis are discussed in the view of current knowledge on plant defense signaling. Moreover, the most important words of this thesis are summarized in Figure 1.

Molecular regulation of ABA-dependent defense responses during herbivory

ABA has been reported mostly to function in the regulation of developmental processes, such as seed germination, senescence, dormancy and in tolerance to abiotic stresses (Wasilewska *et al.*, 2008; Hauser *et al.*, 2011). However, there have also been reports describing ABA as an important modulator of plant defense responses (Chapter 2 & Chapter 3; Anderson *et al.*, 2004; Lorenzo *et al.*, 2004; Asselbergh *et al.*, 2008; Feng *et al.*, 2012; Sánchez-Vallet *et al.*, 2012; Vos *et al.*, 2013b). Limited water supply is an important trigger for ABA biosynthesis (Raghavendra *et al.*, 2010). Leaf wounding and herbivory are associated with leaf water loss (Aldea *et al.*, 2005; Consales *et al.*, 2012) and this abiotic stress may be the cause of the increased ABA levels that we observed upon herbivory by *P. rapae* in Arabidopsis (Chapter 2 & Chapter 3). Also in maize plants, increased ABA biosynthesis has been demonstrated upon belowground herbivory by the western corn rootworm *Diabrotica virgifera virgifera* (Erb *et al.*, 2009). Furthermore, it has previously been shown that caterpillar feeding induced significant changes in the expression of a set of ABA-responsive genes in Arabidopsis (Bodenhausen & Reymond, 2007; Appel *et al.*, 2014a). Together, these findings suggest that insect herbivory can lead to enhanced ABA signaling.

In Chapter 2 we show that, like the *MYC*-impaired *myc2* and *myc2,3,4* plants, the ABA biosynthesis mutant *aba2-1* was unable to activate the MYC-branch in response to feeding by *P. rapae*, indicating that ABA is essential to fully activate an appropriate defense response upon herbivory. Furthermore, exogenous application of ABA stimulated the MYC-branch marker genes *VSP1* and *VSP2* in Col-0 wild-type plants (Chapter 2 & Chapter 3). This ABA-induced activation of *VSP2* was absent in the *myc2* and *myc2,3,4* plants, indicating that ABA-regulated activation of the MYC-branch was dependent on the MYC transcription factors (Chapter 2).

ABA treatment has been found to cause suppression of the ERF-branch genes *ORA59* and *PDF1.2* (Chapter 2; Anderson *et al.*, 2004). This suppressive effect of ABA was still detected in the *myc2* and *myc2,3,4* plants, indicating that ABA can suppress the ERF-branch independent of these three MYC-transcription factors (Chapter 2). We found that the suppression effect of ABA on *ORA59* and *PDF1.2* works via a similar mechanism as the suppression of SA on *ORA59* and *PDF1.2*, namely via inhibition of activation of the GCC-box promoter motif by JA (Chapter 2; Van der Does *et al.*, 2013). These results indicate that the GCC-box is sufficient for ABA-mediated suppression of JA-responsive gene expression.

Systemic leaves showed an increase in *MYC2* expression upon *P. rapae* feeding, but no increase in downstream *VSP1* expression (Chapter 3). Exogenous application of ABA resulted in enhanced expression of *VSP1* (Chapter 3), indicating that the systemic tissue is primed for activation of the MYC-branch. Furthermore, Abe *et al.* (2003) found that overexpression of *MYC2* in Arabidopsis primes the plants for enhanced sensitivity to ABA. Moreover, ABA biosynthesis upon *P. rapae* feeding appears to be dependent on the MYC transcription factors, as indicated by reduced ABA levels in *P. rapae*-infested *myc2* and *myc2,3,4* plants compared to Col-0 (Chapter 2). Taken together, these findings indicate that joint activation of ABA- and MYC-dependent signaling is necessary for full activation of herbivore-induced defense responses and repression of defenses against necrotrophic pathogens.

The effect of ABA-dependent defense responses on plant resistance

In systemic undamaged leaf tissue, there is likely no water loss (Aldea *et al.*, 2005) and the ABA levels are not upregulated (Chapter 3). This lack of ABA may have prevented the direct activation of costly defense responses, while instead the tissue became primed for activation of the MYC-branch (Chapter 3), which is a cost-efficient way of the plant to prepare itself for future attack (Van Hulten *et al.*, 2006; Vos *et al.*, 2013a). In accordance with this, secondary infestation with a *P. rapae* caterpillar significantly reduced caterpillar performance compared to caterpillars feeding from previously uninduced plants (Chapter 3). This effect was absent in the *aba2-1* and the *coi1-1* mutant plants, indicating that similar to local damaged leaves, joint activation of the JA and ABA pathways is required for the induction of herbivore-induced defense responses in undamaged systemic leaves.

In a series of no-choice and two-choice experiments, we found that enhancement of the ERF-branch upon caterpillar feeding, such as observed in *myc2* and *aba2-1* plants, resulted in strong caterpillar preference (Chapter 2), which is in line with previous results (Verhage *et al.*, 2011). However, the performance of the caterpillars was only minimally influenced by enhancement of the ERF-branch (Chapter 2). On the other hand, enhancement of the MYC-branch, such as occurs in *ein2-1* plants, did

not influence caterpillar preference, but had a strong negative effect on caterpillar performance. Furthermore, in a multi-attacker set-up with both *P. rapae* infestation and infection with the necrotrophic pathogen *Botrytis cinerea*, caterpillar feeding suppressed ERF-branch-controlled defense responses against *B. cinerea* (Chapter 2 & Chapter 4). This suppression of the ERF-branch rendered the plants more susceptible to *B. cinerea* (Chapter 4), but had no effect on caterpillar performance (Chapter 2). The plant's capacity to rewire its JA-regulated defense responses might be dependent on the damage intensity inflicted by the attackers (Mouttet *et al.*, 2013) and may contribute to maximizing the chance of survival.

Plant fitness under multi-species attack

Activation of inducible defense responses entails allocation costs and ecological costs (Herms & Mattson, 1992; Heil & Baldwin, 2002; Strauss *et al.*, 2002; Walters & Heil, 2007). Allocation costs occur when valuable resources are allocated to resistance instead of to growth and reproduction. Ecological costs occur when activation of defenses affects the interactions of the plant with other biotic and abiotic environmental factors, such as subsequent attackers or competing plants (Chapter 2 & Chapter 4; Heil, 2002; Cipollini *et al.*, 2003; Kessler & Halitschke, 2007; Poelman *et al.*, 2008; Traw & Bergelson, 2010; Vos *et al.*, 2013a). There is ample evidence of altered plant resistance to a subsequent attacker, when the plant is previously induced by a different attacker. For example, black bean aphids, A*phis fabae* displayed higher growth rate and fecundity on bean leaves infected with the necrotrophic pathogen *Botrytis fabae*, compared to uninfected leaves (Zebitz & Kehlenbeck, 1991). In Arabidopsis, infection with *Pseudomonas syringae* rendered the infected leaves more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Spoel *et al.*, 2007) and feeding by the generalist herbivore *Spodoptera exigua* lowered resistance to a virulent strain of *P. syringae* (Appel *et al.*, 2014b). In tobacco, *Manduca sexta* caterpillars consumed up to 2.5-times more leaf tissue from plants that had been inoculated with tobacco mosaic virus than from mock-treated plants (Preston *et al.*, 1999). Furthermore, in Chapter 4 we show that Arabidopsis plants induced with either the SA-pathway inducing *H. arabidopsidis* or the MYC-branch inducing *P. rapae* were more susceptible to the ERF-branch inducing necrotrophic pathogen *B. cinerea*.

Whether hormonal crosstalk, by prioritizing one set of defense responses over others, also contributes to plant fitness when plants are under multi-species attack has, to our knowledge, never been demonstrated. In Chapter 4, we show that infection with *B. cinerea* decreased rosette growth and seed production. However, this negative fitness effect was not influenced by pre-treatment with either *H. arabidopsidis* or *P. rapae*, despite the increased susceptibility to *B. cinerea* of these pre-treated plants. Furthermore, plants grown in competition did not show altered fitness when treated

simultaneously with SA and MeJA compared to plants treated with only one of the hormones. Together, this gives a first suggestion that crosstalk indeed could contribute to plant fitness under multi-species attack. Crosstalk mutants that are not affected in resistance need to be tested to come to a definite conclusion (Thaler *et al.,* 2012; Vos *et al.,* 2013). Furthermore, environmental conditions such as nutrient availability have been shown to impact the amount of fitness costs associated with the activation of defense responses (Cipollini *et al.*, 2003; Dietrich *et al.*, 2005), showing the importance of testing under realistic environmental conditions, since otherwise relevant costs might be missed.

Environmental factors influence plant fitness during infection with *H. arabidopsidis*

Growth compensation after activation of defense responses has previously been reported upon feeding by herbivores (Paige & Whitham, 1987; Strauss & Agrawal, 1999; Agrawal, 2000) and after treatment with benzothiadiazole (BTH), a chemical analogue of SA (Dietrich *et al.*, 2005). We found that plants can also compensate in growth and seed production after infection with *H. arabidopsidis* (Chapter 5). The growth reduction found in the first week after infection can be caused by allocation costs of the infection, since the plant is allocating resources to defense instead of to growth (Heil & Baldwin, 2002; Vos *et al.*, 2013a). The subsequent compensating effect was most pronounced under low nutrient availability and long-day conditions. Plants grown under these conditions had the highest root:shoot ratio in the control situation, which decreased upon infection with *H. arabidopsidis*. Rosette growth was not reduced or even enhanced after *H. arabidopsidis* infection, suggesting that increased resource allocation from the root to the shoot might be a mechanism regulating compensatory growth after *H. arabidopsidis* infection. Plants were demonstrated to be best able to compensate at low disease pressure, likely because at higher disease pressure the costs associated with the infection are more likely to outweigh the benefits for the plant.

Altogether, we show that interactions between plants and pathogens do not necessarily lead to negative fitness effects for the plants. As a biotrophic pathogen, *H. arabidopsidis* benefits from keeping its host alive and an increase in plant biomass and seed production would expectedly lead to both enhanced spore production and greater amounts of host plants in the next generation. Hence, it is tempting to speculate that the observed conditional plant fitness-promoting effect of *H. arabidopsidis* infection is a reflection of the plant-beneficial side of this pathogen, associated with its biotrophic lifestyle. Further exploration of these beneficial fitness effects may give insight in the finely tuned intimate interactions between biotrophic microbes and their plant hosts and might provide useful leads for future crop protection.

Tools for crop protection

In agriculture, crop losses due to pathogen infection or insect infestation, represents a total value of over €450 billion worldwide. However, allocation costs and ecological costs of induced plant defenses are a major problem for the implementation of enhanced resistance as a tool to improve crops (Walters & Heil, 2007). Plants often have to deal with simultaneous or subsequent attack by very different attackers. Hormonal crosstalk can make plants more susceptible to combinations of attackers. For example leaves infected with *H. arabidopsidis* or damaged by *P. rapae* feeding became more susceptible to *B. cinerea* infection (Chapter 4). As a result, hormonal crosstalk presents a challenge for translating fundamental knowledge into crop disease resistance traits. It has been suggested that crosstalk is a cost-saving strategy, and our results indicate that this might indeed be the case (Chapter 4). Therefore, if crosstalk could be (artificially) prevented, the simultaneous elevation of multiple defenses might protect crops to a multitude of attackers, but at the same time might have detrimental effects on yield.

Undamaged systemic leaves of *P. rapae*-infested plants did not show full activation of plant defenses, but instead became primed for subsequent attack by *P. rapae* (Chapter 3). Worrall *et al.* (2012) found that tomato plants grown from either β-aminobutyric acid (BABA-) or JA-treated seeds were increased resistant against biotrophic pathogens and insects or necrotrophic pathogens, respectively. Furthermore, the response was long-lasting, there was no crosstalk between the different forms of defense and there was also no growth reduction. The Arabidopsis accession Bur-0 is constitutively primed for enhanced defenses against both pathogens and insects (Ahmad *et al.*, 2011). Furthermore, induction of both SA- and JA/ET-dependent priming did not lead to crosstalk between these defense responses (Van Wees *et al.*, 2000). Together, this suggests that it might be a possibility to prime plants simultaneously for resistance against several types of attackers, while minimizing plant fitness costs (Van Hulten *et al.*, 2006). Therefore, it would be interesting to test if it is possible to treat plants with low amounts of chemicals to prime defenses against multiple attackers and determine whether plant fitness is unaltered.

However, priming has been studied mostly under tightly controlled growth chamber conditions with stabile temperature, light intensity, humidity, nutrient availability and without pathogen or insect pressure. In the field, plants interact with numerous naturally-occurring pathogens and insects, making it plausible that plants in the field are already primed (Pieterse & Dicke, 2007; Walters *et al.*, 2013). However, several researchers have investigated the priming phenomenon under field conditions and found evidence that multiple plants species can still show a priming response in a natural environment (Kessler *et al.*, 2006; Heil & Bueno, 2007; Choi *et al.*, 2014). Overall, understanding whether and how the functioning of the defense signaling pathways and the crosstalk between them influences plant defense and plant fitness is necessary for

successful application of defense traits in crops. Furthermore, it is important to test the molecular mechanisms and fitness effects revealed in this thesis in agricultural and natural environments to be able to apply this knowledge to improve crop protection.

Figure 1: Wordle of the thesis 'Unwiring jasmonic acid defense signaling – molecular regulation and ecological costs' by Irene Vos

Wordle (www.wordle.net) of all the text in this thesis, excluding in-text references, figure legends, material and methods sections, reference list, and frequently used words such as 'furthermore' and 'however'.

REFERENCES

Abe H, Urao T, Ito T, Seki M, Shinozaki K & Yamaguchi-Shinozaki K, 2003. Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* **15**, 63-78.

Abreu ME & Munné-Bosch S, 2009. Salicylic acid deficiency in *NahG* transgenic lines and *sid2* mutants increases seed yield in the annual plant *Arabidopsis thaliana*. *Journal of Experimental Botany* **60**, 1261-71.

Adams DO & Yang SF, 1979. Ethylene biosynthesis: identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. *Proceedings of the National Academy of Sciences, USA* **76**, 170-4.

Adie BAT, Pérez-Pérez J, Pérez-Pérez MM, Godoy M, Sánchez-Serrano JJ, Schmelz EA & Solano R, 2007. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *Plant Cell* **19**, 1665-81.

Agrawal AA, 2000. Overcompensation of plants in response to herbivory and the by-product benefits of mutualism. *Trends in Plant Science* **5**, 309-13.

Agrawal AA, Strauss SY & Stout MJ, 1999. Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* **53**, 1093-104.

Ahmad S, Van Hulten M, Martin J, Pieterse CMJ, Van Wees SCM & Ton J, 2011. Genetic dissection of basal defence responsiveness in accessions of *Arabidopsis thaliana*. *Plant, Cell and Environment* **34**, 1191-206.

Aldea M, Hamilton JG, Resti JP, Zangerl AR, Berenbaum MR & DeLucia EH, 2005. Indirect effects of insect herbivory on leaf gas exchange in soybean. *Plant, Cell and Environment* **28**, 402-11.

Alonso JM & Stepanova AN, 2004. The ethylene signaling pathway. *Science* **306**, 1513-5.

Alonso JM, Hirayama T, Roman G, Nourizadeh S & Ecker JR, 1999. EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* **284**, 2148-52.

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR & Kazan K, 2004. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *Plant Cell* **16**, 3460-79.

Appel HM & Cocroft RB, 2014. Plants respond to leaf vibrations caused by insect herbivore chewing. *Oecologia* **175**, 1257-66.

Appel HM, Fescemyer H, Ehlting J, Weston D, Rehrig E, Joshi T, Xu D, Bohlmann J & Schultz J, 2014a. Transcriptional responses of *Arabidopsis thaliana* to chewing and sucking insect herbivores. *Frontiers in Plant Science* **5**, 565.

Appel HM, Maqbool SB, Raina S, Jagadeeswaran G, Acharya BR, Hanley JC, Miller KP,

Hearnes L, Hearnes L, Raina R & Schultz JC, 2014b. Transcriptional and metabolic signatures of Arabidopsis responses to chewing damage by an insect herbivore and bacterial infection and the consequences of their interaction. *Frontiers in Plant Science* **5**, 441.

Asaduzzaman M & Asao T, 2012. Autotoxicity in beans and their allelochemicals. *Scientia Horticulturae* **134**, 26-31.

Asselbergh B, De Vleesschauwer D & Höfte M, 2008. Global switches and fine-tuning - ABA modulates plant pathogen defense. *Molecular Plant-Microbe Interactions* **21**, 709-19.

Baldwin IT, Halitschke R, Paschold A, Von Dahl CC & Preston CA, 2006. Volatile signaling in plant-plant interactions: "talking trees" in the genomics era. *Science* **311**, 812-5.

Baldwin IT, 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proceedings of the National Academy of Sciences, USA* **95**, 8113-8.

Baldwin IT & Callahan P, 1993. Autotoxicity and chemical defense: nicotine accumulation and carbon gain in solanaceous plants. *Oecologia* **94**, 534-41.

Ballaré CL, 2011. Jasmonate-induced defenses: a tale of intelligence, collaborators and rascals. *Trends in Plant Science* **16**, 249-57.

Barber NA, Adler LS, Theis N, Hazzard RV & Kiers ET, 2012. Herbivory reduces plant interactions with above- and belowground antagonists and mutualists. *Ecology* **93**, 1560-70.

Bechtold U, Lawson T, Mejia-Carranza J, Meyer RC, Brown IR, Altmann T, Ton J & Mullineaux PM, 2010. Constitutive salicylic acid defences do not compromise seed yield, drought tolerance and water productivity in the Arabidopsis accession C24. *Plant, Cell and Environment* **33**, 1959-73.

Beckers GJM, Jaskiewicz M, Liu Y, Underwood WR, He SY, Zhang S & Conrath U, 2009. Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. *Plant Cell* **21**, 944-53.

Berendsen RL, Pieterse CMJ & Bakker PAHM, 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science* **17**, 478-86.

Berrocal-Lobo M, Molina A & Solano R, 2002. Constitutive expression of *ETHYLENE-RESPONSE-FACTOR1* in Arabidopsis confers resistance to several necrotrophic fungi. *Plant Journal* **29**, 23-32.

Bi XB, Yang JX & Gao WW, 2010. Autotoxicity of phenolic compounds from the soil of American ginseng (*Panax quinquefolium* L.). *Allelopathy Journal* **25**, 115-21.

Bleecker AB & Kende H, 2000. Ethylene: a gaseous signal molecule in plant. *Annual Review of Cell and Developmental Biology* **16**, 1-18.

Bodenhausen N & Reymond P, 2007. Signaling pathways controlling induced resistance to insect herbivores in Arabidopsis. *Molecular Plant-Microbe Interactions* **20**, 1406-20.

Boller T & Felix G, 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology* **60**, 379-407.

Bostock RM, 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. *Annual Review of Phytopathology* **43**, 545-80.

Böttcher C & Pollmann S, 2009. Plant oxylipins: plant responses to 12-oxo-phytodienoic acid are governed by its specific structural and functional properties. *FEBS Journal* **276**, 4693-704.

Bowling SA, Guo A, Cao H, Gordon AS, Klessig DF & Dong X, 1994. A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. *Plant Cell* **6**, 1845-57.

Brooks DM, Bender CL & Kunkel BN, 2005. The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. *Molecular Plant Pathology* **6**, 629-39.

Brown JKM, 2002. Yield penalties of disease resistance in crops. *Current Opinion in Plant Biology* **5**, 339-44.

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux JP & Reymond P, 2010. Insect eggs suppress plant defence against chewing herbivores. *Plant Journal* **62**, 876-85.

Caarls L, Pieterse CMJ & Van Wees SCM, 2015. How salicylic acid takes transcriptional control over jasmonic acid signaling. *Frontiers in Plant Science* **6**, 170.

Canet JV, Dobón A, Ibáñez F, Perales L & Tornero P, 2010. Resistance and biomass in Arabidopsis: a new model for salicylic acid perception. *Plant Biotechnology Journal* **8**, 126-41.

Cerrudo I, Keller MM, Cargnel MD, Demkura PV, De Wit M, Patitucci MS, Pierik R, Pieterse CMJ & Ballaré CL, 2012. Low red/far-red ratios reduce Arabidopsis resistance to *Botrytis cinerea* and jasmonate responses via a COI1-JAZ10-dependent, salicylic acid-independent mechanism. *Plant Physiology* **158**, 2042-52.

Chen H, Wilkerson CG, Kuchar JA, Phinney BS & Howe GA, 2005. Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. *Proceedings of the National Academy of Sciences, USA* **102**, 19237-42.

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL & Solano R, 2007. The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**, 666-71.

Choi HK, Song GC, Yi HS & Ryu CM, 2014. Field evaluation of the bacterial volatile derivative 3-pentanol in priming for induced resistance in pepper. *Journal of Chemical Ecology* **40**, 882-92.

Cipollini D, Walters D & Voelckel C, 2014. Costs of resistance in plants: from theory to evidence. In: Voelckel C & Jander G, eds. *Annual plant reviews volume 47: insect-plant interactions.* Chichester, United Kingdom: John Wiley & Sons, Ltd, 263-307.

Cipollini D, 2010. Constitutive expression of methyl jasmonate-inducible responses delays reproduction and constrains fitness responses to nutrients in *Arabidopsis thaliana*. *Evolutionary Ecology* **24**, 59-68.

Cipollini D, Enright S, Traw MB & Bergelson J, 2004. Salicylic acid inhibits jasmonic acid-induced resistance of *Arabidopsis thaliana* to *Spodoptera exigua*. *Molecular Ecology* **13**, 1643-53.

Cipollini DF, 2002. Does competition magnify the fitness costs of induced responses in *Arabidopsis thaliana*? A manipulative approach. *Oecologia* **131**, 514-20.

Cipollini D, Purrington CB & Bergelson J, 2003. Costs of induced responses in plants. *Basic and Applied Ecology* **4**, 79-89.

Clarke SM, Cristescu SM, Miersch O, Harren FJM, Wasternack C & Mur LAJ, 2009. Jasmonates act with salicylic acid to confer basal thermotolerance in *Arabidopsis thaliana*. *New Phytologist* **182**, 175-87.

Coates ME & Beynon JL, 2010. *Hyaloperonospora arabidopsidis* as a pathogen model. *Annual Review of Phytopathology* **48**, 329-45.

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman MA, Pieterse CMJ, Poinssot B, Pozo MJ, Pugin A, Schaffrath U, Ton J, Wendehenne D, Zimmerli L & Mauch-Mani B, 2006. Priming: getting ready for battle. *Molecular Plant-Microbe Interactions* **19**, 1062-71.

Conrath U, Pieterse CMJ & Mauch-Mani B, 2002. Priming in plant-pathogen interactions. *Trends in Plant Science* **7**, 210-6.

Consales F, Schweizer F, Erb M, Gouhier-Darimont C, Bodenhausen N, Bruessow F, Sobhy I & Reymond P, 2012. Insect oral secretions suppress wound-induced responses in Arabidopsis. *Journal of Experimental Botany* **63**, 727-37.

Creelman RA & Mullet JE, 1997. Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Biology* **48**, 355-81.

Creelman RA, Tierney ML & Mullet JE, 1992. Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. *Proceedings of the National Academy of Sciences, USA* **89**, 4938-41.

Cui J, Bahrami AK, Pringle EG, Hernandez-Guzman G, Bender CL, Pierce NE & Ausubel FM, 2005. *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. *Proceedings of the National Academy of Sciences, USA* **102**, 1791-6.

Cui J, Jander G, Racki LR, Kim PD, Pierce NE & Ausubel FM, 2002. Signals involved in Arabidopsis resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. *Plant Physiology* **129**, 551-64.

Czechowski T, Stitt M, Altmann T, Udvardi MK & Scheible WR, 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology* **139**, 5-17.

Dangl JL, Dietrich RA & Richberg MH, 1996. Death don't have no mercy: cell death programs in plant-microbe interactions. *Plant Cell* **8**, 1793-807.

De Román M, Fernández I, Wyatt T, Sahrawy M, Heil M & Pozo MJ, 2011. Elicitation of foliar resistance mechanisms transiently impairs root association with arbuscular mycorrhizal fungi. *Journal of Ecology* **99**, 36-45.

De Vos M, Van Zaanen W, Koornneef A, Korzelius JP, Dicke M, Van Loon LC & Pieterse CMJ, 2006. Herbivore-induced resistance against microbial pathogens in Arabidopsis. *Plant Physiology* **142**, 352-63.

De Vos M, Van Oosten VR, Van Poecke RM, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Metraux JP, Van Loon LC, Dicke M & Pieterse CMJ, 2005. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. *Molecular Plant-Microbe Interactions* **18**, 923-37.

Dempsey DA & Klessig DF, 2012. SOS - too many signals for systemic acquired resistance? *Trends in Plant Science* **17**, 538-45.

Dempsey DA, Shah J & Klessig DF, 1999. Salicylic acid and disease resistance in plants. *Critical Reviews in Plant Sciences* **18**, 547-75.

Dicke M & Van Loon JJA, 2000. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. *Entomologia Experimentalis et Applicata* **97**, 237-49.

Dicke M, Sabelis MW, Takabayashi J, Bruin J & Posthumus MA, 1990. Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. *Journal of Chemical Ecology* **16**, 3091-118.

Dietrich R, Ploß K & Heil M, 2005. Growth responses and fitness costs after induction of pathogen resistance depend on environmental conditions. *Plant, Cell and Environment* **28**, 211-22.

Diezel C, von Dahl CC, Gaquerel E & Baldwin IT, 2009. Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiology* **150**, 1576-86.

Dinh ST, Baldwin IT & Galis I, 2013. The *HERBIVORE ELICITOR-REGULATED1* gene enhances abscisic acid levels and defenses against herbivores in *Nicotiana attenuata* plants. *Plant Physiology* **162**, 2106-24.

Doherty HM, Selvendran RR & Bowles DJ, 1988. The wound response of tomato plants can be inhibited by aspirin and related hydroxy-benzoic acids. *Physiological and Molecular Plant Pathology* **33**, 377-84.

Dong X, 2004. NPR1, all things considered. *Current Opinion in Plant Biology* **7**, 547-52.

Dorais M, Yelle S & Gosselin A, 1996. Influence of extended photoperiod on photosynthate partitioning and export in tomato and pepper plants. *New Zealand Journal of Crop and Horticultural Science* **24**, 29-37.

Durner J, Shah J & Klessig DF, 1997. Salicylic acid and disease resistance in plants. *Trends in Plant Science* **2**, 266-74.

Durrant WE & Dong X, 2004. Systemic acquired resistance. *Annual Review of Phytopathology* **42**, 185-209.

Ellis C & Turner JG, 2001. The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *Plant Cell* **13**, 1025-33.

El-Oirdi M, El-Rahman TA, Rigano L, El-Hadrami A, Rodriguez MC, Daayf F, Vojnov A & Bouarab K, 2011. *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *Plant Cell* **23**, 2405-21.

Erb M, Meldau S & Howe GA, 2012. Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science* **17**, 250-9.

Erb M, Köllner TG, Degenhardt J, Zwahlen C, Hibbard BE & Turlings TCJ, 2011. The role of abscisic acid and water stress in root herbivore-induced leaf resistance. *New Phytologist* **189**, 308-20.

Erb M, Flors V, Karlen D, De Lange E, Planchamp C, D'Alessandro M, Turlings TCJ & Ton J, 2009. Signal signature of aboveground-induced resistance upon belowground herbivory in maize. *Plant Journal* **59**, 292-302.

Felton GW & Tumlinson JH, 2008. Plant–insect dialogs: complex interactions at the plant–insect interface. *Current Opinion in Plant Biology* **11**, 457-63.

Feng DX, Tasset C, Hanemian M, Barlet X, Hu J, Trémousaygue D, Deslandes L & Marco Y, 2012. Biological control of bacterial wilt in *Arabidopsis thaliana* involves abscissic acid signalling. *New Phytologist* **194**, 1035-45.

Fernández-Calvo P, Chini A, Fernández-Barbero G, Chico JM, Gimenez-Ibanez S, Geerinck J, Eeckhout D, Schweizer F, Godoy M, Franco-Zorrilla JM, Pauwels L, Witters E, Puga MI, Paz-Ares J, Goossens A, Reymond P, De Jaeger G & Solano R, 2011. The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *Plant Cell* **23**, 701-15.

Ferrari S, Savatin DV, Sicilia F, Gramegna G, Cervone F & De Lorenzo G, 2013. Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Frontiers in Plant Science* **4**, 49.

Ferrari S, Plotnikova JM, De Lorenzo G & Ausubel FM, 2003. Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. *Plant Journal* **35**, 193-205.

Feys BJF, Benedetti CE, Penfold CN & Turner JG, 1994. Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* **6**, 751-9.

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C & Solano R, 2009. (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive

jasmonate. *Nature Chemical Biology* **5**, 344-50.

Frost CJ, Mescher MC, Carlson JE & De Moraes CM, 2008. Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiology* **146**, 818-24.

Gertz AK & Bach CE, 1995. Effects of light and nutrients on tomato plant compensation for herbivory by *Manduca sexta* (Lepidoptera: *Sphingidae*). *Great Lakes Entomologist* **27**, 217-22.

Glazebrook J, 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* **43**, 205-27.

Govrin EM & Levine A, 2002. Infection of Arabidopsis with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). *Plant Molecular Biology* **48**, 267-76.

Grant MR & Jones JDG, 2009. Hormone (dis)harmony moulds plant health and disease. *Science* **324**, 750-2.

Green TR & Ryan CA, 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science* **175**, 776-7.

Hammond-Kosack KE & Jones JDG, 1996. Resistance gene-dependent plant defense responses. *Plant Cell* **8**, 1773-91.

Hauser F, Waadt R & Schroeder JI, 2011. Evolution of abscisic acid synthesis and signaling mechanisms. *Current Biology* **21**, R346-55.

Heidel AJ & Dong X, 2006. Fitness benefits of systemic acquired resistance during *Hyaloperonospora parasitica* infection in *Arabidopsis thaliana*. *Genetics* **173**, 1621-8.

Heidel AJ, Clarke JD, Antonovics J & Dong X, 2004. Fitness costs of mutations affecting the systemic acquired resistance pathway in *Arabidopsis thaliana*. *Genetics* **168**, 2197-206.

Heil M, 2015. Extrafloral nectar at the plant-insect interface: a spotlight on chemical ecology, phenotypic plasticity, and food webs. *Annual Review of Entomology* **60**, 213-32.

Heil M, 2009. Damaged-self recognition in plant herbivore defence. *Trends in Plant Science* **14**, 356-63.

Heil M & Bueno JCS, 2007. Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. *Proceedings of the National Academy of Sciences, USA* **104**, 5467-72.

Heil M, Hilpert A, Krüger R & Linsenmair KE, 2004. Competition among visitors to extrafloral nectaries as a source of ecological costs of an indirect defence. *Journal of Tropical Ecology* **20**, 201-8.

Heil M, 2002. Ecological costs of induced resistance. *Current Opinion in Plant Biology* **5**, 345-50.

Heil M & Baldwin IT, 2002. Fitness costs of induced resistance: emerging experimental

support for a slippery concept. *Trends in Plant Science* **7**, 61-7.

Heil M, Hilpert A, Kaiser W & Linsenmair KE, 2000. Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? *Journal of Ecology* **88**, 645-54.

Herms DA & Mattson WJ, 1992. The dilemma of plants: to grow or defend. *Quarterly Review of Biology* **67**, 283-335.

Hoagland DR & Arnon DI, 1938. The water culture method for growing plants without soil. *Californian Agriculture Experimental Station Bulletin* **347**, 36-9.

Howe GA & Jander G, 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59**, 41-66.

Hua J, Sakai H, Nourizadeh S, Chen QG, Bleecker AB, Ecker JR & Meyerowitz EM, 1998. *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in arabidopsis. *Plant Cell* **10**, 1321-32.

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED & Schroeder JI, 2010. Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes and Development* **24**, 1695-708.

Jaskiewicz M, Conrath U & Peterhälnsel C, 2011. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Reports* **12**, 50-5.

Johnson PR & Ecker JR, 1998. The ethylene gas signal transduction pathway: a molecular perspective. *Annual Review of Genetics* **32**, 227-54.

Jones JDG & Dangl JL, 2006. The plant immune system. *Nature* **444**, 323-9.

Kazan K & Manners JM, 2013. MYC2: the master in action. *Molecular Plant* **6**, 686-703.

Kazan K & Manners JM, 2012. JAZ repressors and the orchestration of phytohormone crosstalk. *Trends in Plant Science* **17**, 22-31.

Kazan K & Manners JM, 2008. Jasmonate signaling: toward an integrated view. *Plant Physiology* **146**, 1459-68.

Kennedy GG, Yamamoto RT, Dimock MB, Williams WG & Bordner J, 1981. Effect of day length and light intensity on 2-tridecanone levels and resistance in *Lycopersicon hirsutum* f. *glabratum* to *Manduca sexta*. *Journal of Chemical Ecology* **7**, 707-16.

Kessler A & Halitschke R, 2007. Specificity and complexity: the impact of herbivore-induced plant responses on arthropod community structure. *Current Opinion in Plant Biology* **10**, 409-14.

Kessler A, Halitschke R, Diezel C & Baldwin IT, 2006. Priming of plant defense responses in nature by airborne signaling between *Artemisia tridentata* and *Nicotiana attenuata*. *Oecologia* **148**, 280-92.

Kessler A & Baldwin IT, 2002. Plant responses to insect herbivory: the emerging molecular analysis. *Annual Review of Plant Biology* **53**, 299-328.

Klessig DF & Malamy J, 1994. The salicylic acid signal in plants. *Plant Molecular Biology*

26, 1439-58.

Kloek AP, Verbsky ML, Sharma SB, Schoelz JE, Vogel J, Klessig DF & Kunkel BN, 2001. Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana coronatine-insensitive* (*coi1*) mutation occurs through two distinct mechanisms. *Plant Journal* **26**, 509-22.

Koch E & Slusarenko A, 1990. Arabidopsis is susceptible to infection by a downy mildew fungus. *Plant Cell* **2**, 437-45.

Koo AJK, Gao X, Daniel Jones A & Howe GA, 2009. A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. *Plant Journal* **59**, 974-86.

Koornneef A, Leon-Reyes A, Ritsema T, Verhage A, Den Otter FC, Van Loon LC & Pieterse CMJ, 2008. Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiology* **147**, 1358-68.

Koornneef A & Pieterse CMJ, 2008. Cross talk in defense signaling. *Plant Physiology* **146**, 839-44.

Koornneef M, Jorna ML, Brinkhorst-Van der Swan DLC & Karssen CM, 1982. The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) heynh. *Theoretical and Applied Genetics* **61**, 385-93.

Koricheva J, Gange AC & Jones T, 2009. Effects of mycorrhizal fungi on insect herbivores: a meta-analysis. *Ecology* **90**, 2088-97.

Landgraf R, Schaarschmidt S & Hause B, 2012. Repeated leaf wounding alters the colonization of *Medicago truncatula* roots by beneficial and pathogenic microorganisms. *Plant, Cell and Environment* **35**, 1344-57.

Lawrence PK & Koundal KR, 2002. Plant protease inhibitors in control of phytophagous insects. *Electronic Journal of Biotechnology* **5**, 121-43.

Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T & Ryals J, 1996. Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant Journal* **10**, 71-82.

Lazebnik J, Frago E, Dicke M & Van Loon JJA, 2014. Phytohormone mediation of interactions between herbivores and plant pathogens. *Journal of Chemical Ecology* **40**, 730-41.

Lebecka R & Sobkowiak S, 2013. Host-pathogen interaction between *Phytophthora infestans* and *Solanum tuberosum* following exposure to short and long daylight hours. *Acta Physiologiae Plantarum* **35**, 1131-9.

Lebeda A & Cohen Y, 2011. Cucurbit downy mildew (*Pseudoperonospora cubensis*)-biology, ecology, epidemiology, host-pathogen interaction and control. *European Journal of Plant Pathology* **129**, 157-92.

LeNoble ME, Spollen WG & Sharp RE, 2004. Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. *Journal of Experimental Botany* **55**, 237-45.

Leon-Reyes A, Du Y, Koornneef A, Proietti S, Körbes AP, Memelink J, Pieterse CMJ & Ritsema T, 2010a. Ethylene signaling renders the jasmonate response of Arabidopsis insensitive to future suppression by salicylic acid. *Molecular Plant-Microbe Interactions* **23**, 187-97.

Leon-Reyes A, Van der Does D, De Lange ES, Delker C, Wasternack C, Van Wees SCM, Ritsema T & Pieterse CMJ, 2010b. Salicylate-mediated suppression of jasmonate-responsive gene expression in Arabidopsis is targeted downstream of the jasmonate biosynthesis pathway. *Planta* **232**, 1423-32.

Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, Millenaar FF, Welschen RAM, Ritsema T & Pieterse CMJ, 2009. Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. *Plant Physiology* **149**, 1797-809.

Livak KJ & Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402-8.

López-Ráez JA, Kohlen W, Charnikhova T, Mulder P, Undas AK, Sergeant MJ, Verstappen F, Bugg TDH, Thompson AJ, Ruyter-Spira C & Bouwmeester H, 2010. Does abscisic acid affect strigolactone biosynthesis? *New Phytologist* **187**, 343-54.

Lorenzo O & Solano R, 2005. Molecular players regulating the jasmonate signalling network. *Current Opinion in Plant Biology* **8**, 532-40.

Lorenzo O, Chico JM, Sánchez-Serrano JJ & Solano R, 2004. *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *Plant Cell* **16**, 1938-50.

Lorenzo O, Piqueras R, Sánchez-Serrano JJ & Solano R, 2003. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* **15**, 165-78.

Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, DeVera ME, Liang X, Tör M & Billiar T, 2007. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunological Reviews* **220**, 60-81.

Luna E, Bruce TJA, Roberts MR, Flors V & Ton J, 2012. Next-generation systemic acquired resistance. *Plant Physiology* **158**, 844-53.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A & Grill E, 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**, 1064-8.

Mabood F & Smith D, 2007. The role of salicylates in rhizobium-legume symbiosis and abiotic stresses in higher plants. In: Hayat S & Ahmad A, eds. *Salicylic acid: a plant hormone.* Dordrecht, the Netherlands: Springer, 151-62.

Malamy J, Carr JP, Klessig DF & Raskin I, 1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**, 1002-4.

Métraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W & Inverardi B, 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* **250**, 1004-6.

Millenaar FF, Cox MCH, De Jong Van Berkel YEM, Welschen RAM, Pierik R, Voesenek LAJC & Peeters AJM, 2005. Ethylene-induced differential growth of petioles in Arabidopsis. Analyzing natural variation, response kinetics, and regulation. *Plant Physiology* **137**, 998-1008.

Mishina TE & Zeier J, 2007. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *Plant Journal* **50**, 500-13.

Mithöfer A & Boland W, 2012. Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology* **63**, 431-50.

Mithöfer A & Boland W, 2008. Recognition of herbivory-associated molecular patterns. *Plant Physiology* **146**, 825-31.

Mithöfer A, Wanner G & Boland W, 2005. Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiology* **137**, 1160-8.

Mittler R & Lam E, 1996. Sacrifice in the face of foes: pathogen-induced programmed cell death in plants. *Trends in Microbiology* **4**, 10-5.

Moore JW, Loake GJ & Spoel SH, 2011. Transcription dynamics in plant immunity. *Plant Cell* **23**, 2809-20.

Mou Z, Fan W & Dong X, 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113**, 935-44.

Mousavi SAR, Chauvin A, Pascaud F, Kellenberger S & Farmer EE, 2013. *GLUTAMATE RECEPTOR-LIKE* genes mediate leaf-to-leaf wound signalling. *Nature* **500**, 422-6.

Mouttet R, Kaplan I, Bearez P, Amiens-Desneux E & Desneux N, 2013. Spatiotemporal patterns of induced resistance and susceptibility linking diverse plant parasites. *Oecologia* **173**, 1379-86.

Müller R, De Vos M, Sun JY, Sønderby IE, Halkier BA, Wittstock U & Jander G, 2010. Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *Journal of Chemical Ecology* **36**, 905-13.

Mur LAJ, Kenton P, Atzorn R, Miersch O & Wasternack C, 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology* **140**, 249-62.

Mur LAJ, Naylor G, Warner SA, Sugars JM, White RF & Draper J, 1996. Salicylic acid

potentiates defence gene expression in tissue exhibiting acquired resistance to pathogen attack. *Plant Journal* **9**, 559-71.

Nambara E & Marion-Poll A, 2005. Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56**, 165-85.

Ndamukong I, Abdallat AA, Thurow C, Fode B, Zander M, Weigel R & Gatz C, 2007. SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive *PDF1.2* transcription. *The Plant Journal* **50**, 128-39.

Nickstadt A, Thomma BPHJ, Feussner I, Kangasjärvi J, Zeier J, Loeffler C, Scheel D & Berger S, 2004. The jasmonate-insensitive mutant *jin1* shows increased resistance to biotrophic as well as necrotrophic pathogens. *Molecular Plant Pathology* **5**, 425-34.

Niu Y, Figueroa P & Browse J, 2011. Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. *Journal of Experimental Botany* **62**, 2143-54.

Olff H, Andel JV & Bakker JP, 1990. Biomass and shoot/root allocation of five species from a grassland succession series at different combinations of light and nutrient supply. *Functional Ecology* **4**, 193-200.

Oñate-Sánchez L & Vicente-Carbajosa J, 2008. DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including seeds and siliques. *BMC Research Notes* **1**, 93.

Osbourn AE, 1996. Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell* **8**, 1821-31.

Paige KN & Whitham TG, 1987. Overcompensation in response to mammalian herbivory: the advantage of being eaten. *American Naturalist* **129**, 407-16.

Pangesti N, Weldegergis BT, Langendorf B, Van Loon JJA, Dicke M & Pineda A, 2015. Rhizobacterial colonization of roots modulates plant volatile emission and enhances the attraction of a parasitoid wasp to host-infested plants. *Oecologia*, doi: 10.1007/s00442-015-3277-7.

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TFF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF & Cutler SR, 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**, 1068-71.

Patterson CG, Archbold DD, Rodriguez JG & Hamilton-Kemp TR, 1994. Daylength and resistance of strawberry foliage to the twospotted spider mite. *HortScience* **29**, 1329-31.

Pel MJC & Pieterse CMJ, 2013. Microbial recognition and evasion of host immunity. *Journal of Experimental Botany* **64**, 1237-48.

Penninckx IAMA, Thomma BPHJ, Buchala A, Métraux JP & Broekaert WF, 1998. Concomitant activation of jasmonate and ethylene response pathways is required

for induction of a plant defensin gene in Arabidopsis. *Plant Cell* **10**, 2103-14.

Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Métraux JP, Manners JM & Broekaert WF, 1996. Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *Plant Cell* **8**, 2309-23.

Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM & Bakker PAHM, 2014. Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology* **52**, 347-75.

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A & Van Wees SCM, 2012. Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology* **28**, 489-521.

Pieterse CMJ, Leon-Reyes A, Van der Ent S & Van Wees SCM, 2009. Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* **5**, 308-16.

Pieterse CMJ & Dicke M, 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. *Trends in Plant Science* **12**, 564-9.

Pieterse CMJ, Van Wees SCM, Van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ & Van Loon LC, 1998. A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell* **10**, 1571-80.

Pineda A, Soler R, Weldegergis BT, Shimwela MM, Van Loon JJA & Dicke M, 2013. Non-pathogenic rhizobacteria interfere with the attraction of parasitoids to aphid-induced plant volatiles via jasmonic acid signalling. *Plant, Cell and Environment* **36**, 393-404.

Pineda A, Zheng SJ, Van Loon JJA, Pieterse CMJ & Dicke M, 2010. Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends in Plant Science* **15**, 507-14.

Poelman EH, Van Loon JJA & Dicke M, 2008. Consequences of variation in plant defense for biodiversity at higher trophic levels. *Trends in Plant Science* **13**, 534-41.

Pozo MJ, Van der Ent S, Van Loon LC & Pieterse CMJ, 2008. Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytologist* **180**, 511-23.

Pozo MJ & Azcón-Aguilar C, 2007. Unraveling mycorrhiza-induced resistance. *Current Opinion in Plant Biology* **10**, 393-8.

Pré M, Atallah M, Champion A, De Vos M, Pieterse CMJ & Memelink J, 2008. The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant Physiology* **147**, 1347-57.

Preston CA, Lewandowski C, Enyedi AJ & Baldwin IT, 1999. Tobacco mosaic virus inoculation inhibits wound-induced jasmonic acid-mediated responses within but not between plants. *Planta* **209**, 87-95.

Purrington CB, 2000. Costs of resistance. *Current Opinion in Plant Biology* **3**, 305-8.

Raghavendra AS, Gonugunta VK, Christmann A & Grill E, 2010. ABA perception and signalling. *Trends in Plant Science* **15**, 395-401.

Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, Agrawal AA, Felton GW & Jander G, 2012. Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant Physiology* **158**, 854-63.

Raupach GS & Kloepper JW, 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* **88**, 1158-64.

Redman AM, Cipollini Jr. DF & Schultz JC, 2001. Fitness costs of jasmonic acid-induced defense in tomato, *Lycopersicon esculentum*. *Oecologia* **126**, 380-5.

Reymond P & Farmer EE, 1998. Jasmonate and salicylate as global signals for defense gene expression. *Current Opinion in Plant Biology* **1**, 404-11.

Ritsema T, Van Zanten M, Leon-Reyes A, Voesenek LACJ, Millenaar FF, Pieterse CMJ & Peeters AJM, 2010. Kinome profiling reveals an interaction between jasmonate, salicylate and light control of hyponastic petiole growth in *Arabidopsis thaliana*. *PLoS ONE* **5**, e14255.

Rivas-San Vicente M & Plasencia J, 2011. Salicylic acid beyond defence: its role in plant growth and development. *Journal of Experimental Botany* **62**, 3321-38.

Robert-Seilaniantz A, Grant M & Jones JDG, 2011. Hormone crosstalk in plant disease and defense: more than just JASMONATE-SALICYLATE antagonism. *Annual Review of Phytopathology* **49**, 317-43.

Ross AF, 1961. Systemic acquired resistance induced by localized virus infections in plants. *Virology* **14**, 340-58.

Rudrappa T, Czymmek KJ, Paré PW & Bais HP, 2008. Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiology* **148**, 1547-56.

Rushton PJ, Somssich IE, Ringler P & Shen QJ, 2010. WRKY transcription factors. *Trends in Plant Science* **15**, 247-58.

Salvaudon L, Héraudet V & Shykoff JA, 2008. *Arabidopsis thaliana* and the Robin Hood parasite: a chivalrous oomycete that steals fitness from fecund hosts and benefits the poorest one? *Biology Letters* **4**, 526-9.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989, *Molecular cloning: a laboratory manual,* 2nd edition edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sánchez-Vallet A, López G, Ramos B, Delgado-Cerezo M, Riviere MP, Llorente F, Fernández PV, Miedes E, Estevez JM, Grant M & Molina A, 2012. Disruption of abscisic acid signaling constitutively activates Arabidopsis resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. *Plant Physiology* **160**, 2109-24.

Sato T, Fujikake H, Ohtake N, Sueyoshi K, Takahashi T, Sato A & Ohyama T, 2002. Effect of exogenous salicylic acid supply on nodule formation of hypernodulating mutant

and wild type of soybean. *Soil Science and Plant Nutrition* **48**, 413-20.

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC & Manners JM, 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proceedings of the National Academy of Sciences, USA* **97**, 11655-60.

Schmittgen TD & Livak KJ, 2008. Analyzing real-time PCR data by the comparative C T method. *Nature Protocols* **3**, 1101-8.

Schweizer F, Fernández-Calvo P, Zander M, Diez-Diaz M, Fonseca S, Glauser G, Lewsey MG, Ecker JR, Solano R & Reymond P, 2013. Arabidopsis basic helix-loop-helix transcription factors MYC2, MYC3, and MYC4 regulate glucosinolate biosynthesis, insect performance, and feeding behavior. *Plant Cell* **25**, 3117-32.

Segarra G, Van der Ent S, Trillas I & Pieterse CMJ, 2009. MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. *Plant Biology* **11**, 90-6.

Shah J & Zeier J, 2013. Long-distance communication and signal amplification in systemic acquired resistance. *Frontiers in Plant Science* **4**, 30.

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J, He SY, Rizo J, Howe GA & Zheng N, 2010. Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. *Nature* **468**, 400-7.

Simms EL & Fritz RS, 1990. The ecology and evolution of host-plant resistance to insects. *Trends in Ecology and Evolution* **5**, 356-60.

Slaughter A, Daniel X, Flors V, Luna E, Hohn B & Mauch-Mani B, 2012. Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. *Plant Physiology* **158**, 835-43.

Soler R, Erb M & Kaplan I, 2013. Long distance root–shoot signalling in plant–insect community interactions. *Trends in Plant Science* **18**, 149-56.

Song S, Huang H, Gao H, Wang J, Wu D, Liu X, Yang S, Zhai Q, Li C, Qi T & Xie D, 2014. Interaction between MYC2 and ETHYLENE INSENSITIVE3 modulates antagonism between jasmonate and ethylene signaling in Arabidopsis. *Plant Cell* **26**, 263-79.

Spoel SH, Johnson JS & Dong X, 2007. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proceedings of the National Academy of Sciences, USA* **104**, 18842-7.

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Métraux JP, Brown R, Kazan K, Van Loon LC, Dong X & Pieterse CMJ, 2003. NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* **15**, 760-70.

Staswick PE & Tiryaki I, 2004. The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. *Plant Cell* **16**, 2117-27.

Stepanova AN & Alonso JM, 2009. Ethylene signaling and response: where different regulatory modules meet. *Current Opinion in Plant Biology* **12**, 548-55.

Stintzi A & Browse J, 2000. The Arabidopsis male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. *Proceedings of the National Academy of Sciences, USA* **97**, 10625-30.

Stout MJ, Thaler JS & Thomma BPHJ, 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. *Annual Review of Entomology* **51**, 663-89.

Strauss SY, Rudgers JA, Lau JA & Irwin RE, 2002. Direct and ecological costs of resistance to herbivory. *Trends in Ecology and Evolution* **17**, 278-85.

Strauss SY & Agrawal AA, 1999. The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology and Evolution* **14**, 179-85.

Sun JQ, Jiang HL & Li CY, 2011. Systemin/jasmonate-mediated systemic defense signaling in tomato. *Molecular Plant* **4**, 607-15.

Taki N, Sasaki-Sekimoto Y, Obayashi T, Kikuta A, Kobayashi K, Ainai T, Yagi K, Sakurai N, Suzuki H, Masuda T, Takamiya KI, Shibata D, Kobayashi Y & Ohta H, 2005. 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. *Plant Physiology* **139**, 1268-83.

Thaler JS, Humphrey PT & Whiteman NK, 2012. Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science* **17**, 260-70.

Thaler JS & Bostock RM, 2004. Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects. *Ecology* **85**, 48-58.

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA & Browse J, 2007. JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. *Nature* **448**, 661-5.

Thines M, Voglmayr H & Göker M, 2008. Taxonomy and phylogeny of the downy mildews (*Peronosporaceae*). In: Lamour K & Kamoun S, eds. *Oomycete genetics and genomics: diversity, interactions, and research tools*. Hoboken, United States of America: John Wiley & Sons, Inc., 47-75.

Tjamos SE, Flemetakis E, Paplomatas EJ & Katinakis P, 2005. Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. *Molecular Plant-Microbe Interactions* **18**, 555-61.

Ton J, Flors V & Mauch-Mani B, 2009. The multifaceted role of ABA in disease resistance. *Trends in Plant Science* **14**, 310-7.

Ton J, D'Alessandro M, Jourdie V, Jakab G, Karlen D, Held M, Mauch-Mani B & Turlings TCJ, 2007. Priming by airborne signals boosts direct and indirect resistance in maize. *Plant Journal* **49**, 16-26.

Ton J, Jakab G, Toquin V, Flors V, Iavicoli A, Maeder MN, Métraux JP & Mauch-Mani B, 2005. Dissecting the ß-Aminobutyric acid–induced priming phenomenon in Arabidopsis. *Plant Cell* **17**, 987-99.

Ton J & Mauch-Mani B, 2004. β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *Plant Journal* **38**, 119-30.

Ton J, Van Pelt JA, Van Loon LC & Pieterse CMJ, 2002. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis. *Molecular Plant-Microbe Interactions* **15**, 27-34.

Traw MB & Bergelson J, 2010. Plant immune system incompatibility and the distribution of enemies in natural hybrid zones. *Current Opinion in Plant Biology* **13**, 466-71.

Trumble JT, Kolodny-Hirsch DM & Ting IP, 1993. Plant compensation for arthropod herbivory. *Annual Review of Entomology* **38**, 93-119.

Tytgat TOG, Verhoeven KJF, Jansen JJ, Raaijmakers CE, Bakx-Schotman T, McIntyre LM, Van der Putten WH, Biere A & Van Dam NM, 2013. Plants know where it hurts: root and shoot jasmonic acid induction elicit differential responses in *Brassica oleracea*. *PLoS ONE* **8**, e65502.

Umezawa T, Sugiyama N, Mizoguchi M, Hayashi S, Myouga F, Yamaguchi-Shinozaki K, Ishihama Y, Hirayama T & Shinozaki K, 2009. Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **106**, 17588-93.

Van Dam NM & Baldwin IT, 2001. Competition mediates costs of jasmonate-induced defences, nitrogen acquisition and transgenerational plasticity in *Nicotiana attenuata*. *Functional Ecology* **15**, 406-15.

Van Damme M, Andel A, Huibers RP, Panstruga R, Weisbeek PJ & Van den Ackerveken G, 2005. Identification of Arabidopsis loci required for susceptibility to the downy mildew pathogen *Hyaloperonospora parasitica*. *Molecular Plant-Microbe Interactions* **18**, 583-92.

Van der Does D, Leon-Reyes A, Koornneef A, Van Verk MC, Rodenburg N, Pauwels L, Goossens A, Körbes AP, Memelink J, Ritsema T, Van Wees SCM & Pieterse CMJ, 2013. Salicylic acid suppresses jasmonic acid signaling downstream of SCF^{COI1}-JAZ by targeting GCC promoter motifs via transcription factor ORA59. *Plant Cell* **25**, 744-61.

Van der Ent S, Van Hulten M, Pozo MJ, Czechowski T, Udvardi MK, Pieterse CMJ & Ton J, 2009a. Priming of plant innate immunity by rhizobacteria and β-aminobutyric acid: differences and similarities in regulation. *New Phytologist* **183**, 419-31.

Van der Ent S, Van Wees SCM & Pieterse CMJ, 2009b. Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. *Phytochemistry* **70**, 1581-8.

Van der Ent S, Verhagen BWM, Van Doorn R, Bakker D, Verlaan MG, Pel MJC, Joosten RG, Proveniers MCG, Van Loon LC, Ton J & Pieterse CMJ, 2008. *MYB72* is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis.

Plant Physiology **146**, 1293-304.

Van Hulten M, Ton J, Pieterse CMJ & Van Wees SCM, 2010. Plant defense signaling from the underground primes aboveground defenses to confer enhanced resistance in a cost-efficient manner. In: Baluska F & Ninkovic V, eds. *Plant communication from an ecological perspective; signaling and communication in plants.* Berlin, Germany: Springer, 43-60.

Van Hulten M, Pelser M, Van Loon LC, Pieterse CMJ & Ton J, 2006. Costs and benefits of priming for defense in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **103**, 5602-7.

Van Kan JAL, Van 't Klooster JW, Wagemakers CAM, Dees DCT & Van der Vlugt-Bergmans CJB, 1997. Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. *Molecular Plant-Microbe Interactions* **10**, 30-8.

Van Loon LC, Rep M & Pieterse CMJ, 2006a. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology* **44**, 135-62.

Van Loon LC, Geraats BPJ & Linthorst HJM, 2006b. Ethylene as a modulator of disease resistance in plants. *Trends in Plant Science* **11**, 184-91.

Van Oosten VR, Bodenhausen N, Reymond P, Van Pelt JA, Van Loon LC, Dicke M & Pieterse CMJ, 2008. Differential effectiveness of microbially induced resistance against herbivorous insects in Arabidopsis. *Molecular Plant-Microbe Interactions* **21**, 919-30.

Van Wees SCM, Van Pelt JA, Bakker PAHM & Pieterse CMJ, 2013. Bioassays for assessing jasmonate-dependent defenses triggered by pathogens, herbivorous insects, or beneficial rhizobacteria. In: Goossens A & Pauwels L, eds. *Jasmonate signalling, methods in molecular biology.* Berlin, Germany: Springer-Humana Press, 35-49.

Van Wees SCM, Van der Ent S & Pieterse CMJ, 2008. Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology* **11**, 443-8.

Van Wees SCM, De Swart EAM, Van Pelt JA, Van Loon LC & Pieterse CMJ, 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **97**, 8711-6.

Van Wees SCM, Luijendijk M, Smoorenburg I, Van Loon LC & Pieterse CMJ, 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. *Plant Molecular Biology* **41**, 537-49.

Vänninen I, Pinto DM, Nissinen AI, Johansen NS & Shipp L, 2010. In the light of new greenhouse technologies: 1. Plant-mediated effects of artificial lighting on arthropods and tritrophic interactions. *Annals of Applied Biology* **157**, 393-414.

Verhage A, Vlaardingerbroek I, Raaijmakers C, Van Dam NM, Dicke M, Van Wees SCM & Pieterse CMJ, 2011. Rewiring of the jasmonate signaling pathway in Arabidopsis during insect herbivory. *Frontiers in Plant Science* **2**, 47.

Verhage A, Van Wees SCM & Pieterse CMJ, 2010. Plant immunity: it's the hormones talking, but what do they say? *Plant Physiology* **154**, 536-40.

Verhagen BWM, Glazebrook J, Zhu T, Chang HS, Van Loon LC & Pieterse CMJ, 2004. The transcriptome of rhizobacteria-induced systemic resistance in Arabidopsis. *Molecular Plant-Microbe Interactions* **17**, 895-908.

Vidhyasekaran P, 2015. Abscisic acid signaling system in plant innate immunity. In: Vidhyasekaran P, ed. *Plant hormone signaling systems in plant innate immunity.* Dordrecht, the Netherlands: Springer, 245-309.

Vlot AC, Dempsey DA & Klessig DF, 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology* **47**, 177-206.

Voesenek LACJ, Harren FJM, Bögemann GM, Blom CWPM & Reuss J, 1990. Ethylene production and petiole growth in rumex plants induced by soil waterlogging: the application of a continuous flow system and a laser driven intracavity photoacoustic detection system. *Plant Physiology* **94**, 1071-7.

Von Dahl CC & Baldwin IT, 2007. Deciphering the role of ethylene in plant-herbivore interactions. *Journal of Plant Growth Regulation* **26**, 201-9.

Vos IA, Pieterse CMJ & Van Wees SCM, 2013a. Costs and benefits of hormone-regulated plant defences. *Plant Pathology* **62**, 43-55.

Vos IA, Verhage A, Schuurink RC, Watt LG, Pieterse CMJ & Van Wees SCM, 2013b. Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. *Frontiers in Plant Science* **4**, 539.

Walling LL, 2008. Avoiding effective defenses: strategies employed by phloem-feeding insects. *Plant Physiology* **146**, 859-66.

Walters DR, Ratsep J & Havis ND, 2013. Controlling crop diseases using induced resistance: challenges for the future. *Journal of Experimental Botany* **64**, 1263-80.

Walters DR, Paterson L, Walsh DJ & Havis ND, 2008. Priming for plant defense in barley provides benefits only under high disease pressure. *Physiological and Molecular Plant Pathology* **73**, 95-100.

Walters DR & Heil M, 2007. Costs and trade-offs associated with induced resistance. *Physiological and Molecular Plant Pathology* **71**, 3-17.

Wasilewska A, Vlad F, Sirichandra C, Redko Y, Jammes F, Valon C, Frei Dit Frey N & Leung J, 2008. An update on abscisic acid signaling in plants and more…. *Molecular Plant* **1**, 198-217.

Wasternack C & Hause B, 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Annals of Botany* **111**, 1021-58.

White RF, 1979. Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* **99**, 410-2.

Wildermuth MC, Dewdney J, Wu G & Ausubel FM, 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414**, 562-5.

Windram O, Madhou P, Mchattie S, Hill C, Hickman R, Cooke E, Jenkins DJ, Penfold CA, Baxter L, Breeze E, Kiddle SJ, Rhodes J, Atwell S, Kliebenstein DJ, Kim Y, Stegle O, Borgwardt K, Zhang C, Tabrett A, Legaie R, Moore J, Finkenstadt B, Wild DL, Mead A, Rand D, Beynon J, Ott S, Buchanan-Wollaston V & Denby KJ, 2012. Arabidopsis defense against *Botrytis cinerea*: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. *Plant Cell* **24**, 3530-57.

Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T, Gershenzon J & Vogel H, 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proceedings of the National Academy of Sciences, USA* **101**, 4859-64.

Worrall D, Holroyd GH, Moore JP, Glowacz M, Croft P, Taylor JE, Paul ND & Roberts MR, 2012. Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. *New Phytologist* **193**, 770-8.

Xie D, Feys BF, James S, Nieto-Rostro M & Turner JG, 1998. COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science* **280**, 1091-4.

Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, Wang Z & Xie D, 2009. The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. *Plant Cell* **21**, 2220-36.

Yang DL, Yao J, Mei CS, Tong XH, Zeng LJ, Li Q, Xiao LT, Sun TP, Li J, Deng XW, Lee CM, Thomashow MF, Yang Y, He Z & He SY, 2012. Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proceedings of the National Academy of Sciences, USA* **109**, 1192-200.

Zamioudis C, Hanson J & Pieterse CMJ, 2014. ß-Glucosidase BGLU42 is a MYB72-dependent key regulator of rhizobacteria-induced systemic resistance and modulates iron deficiency responses in Arabidopsis roots. *Journal of Physiology* **204**, 368-79.

Zamioudis C, Mastranesti P, Dhonukshe P, Blilou I & Pieterse CMJ, 2013. Unraveling root developmental programs initiated by beneficial *Pseudomonas* bacteria. *Plant Physiology* **62**, 304-18.

Zamioudis C & Pieterse CMJ, 2012. Modulation of host immunity by beneficial microbes. *Molecular Plant-Microbe Interactions* **25**, 139-50.

Zander M, Thurow C & Gatz C, 2014. TGA transcription factors activate the salicylic acid-suppressible branch of the ethylene-induced defense program by regulating *ORA59* expression. *Plant Physiology* **165**, 1671-83.

Zander M, Chen S, Imkampe J, Thurow C & Gatz C, 2012. Repression of the *Arabidopsis*

thaliana jasmonic acid/ethylene-induced defense pathway by TGA-interacting glutaredoxins depends on their C-terminal ALWL motif. *Molecular Plant* **5**, 831-40.

Zarate SI, Kempema LA & Walling LL, 2007. Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. *Plant Physiology* **143**, 866-75.

Zarei A, Körbes AP, Younessi P, Montiel G, Champion A & Memelink J, 2011. Two GCC boxes and AP2/ERF-domain transcription factor ORA59 in jasmonate/ethylene-mediated activation of the *PDF1.2* promoter in Arabidopsis. *Plant Molecular Biology* **75**, 321-31.

Zebitz CPW & Kehlenbeck H, 1991. Performance of *Aphis fabae* on chocolate spot disease-infected faba bean plants. *Phytoparasitica* **19**, 113-9.

Zehnder GW, Murphy JF, Sikora EJ & Kloepper JW, 2001. Application of rhizobacteria for induced resistance. *European Journal of Plant Pathology* **107**, 39-50.

Zeilmaker T, Ludwig NR, Elberse J, Seidl MF, Berke L, Van Doorn A, Schuurink RC, Snel B & Van den Ackerveken G, 2015. Downy mildew resistant 6 and DMR6-like oxygenase 1 are partially redundant but distinct suppressors of immunity in Arabidopsis. *Plant Journal* **81**, 210-22.

Zheng XY, Spivey NW, Zeng W, Liu PP, Fu ZQ, Klessig DF, He SY & Dong X, 2012. Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host and Microbe* **11**, 587-96.

SAMENVATTING

Infectie met pathogenen of vraatschade door insecten zorgt jaarlijks wereldwijd voor een verlies van meer dan €450 miljard aan landbouwgewassen. Planten hebben te maken met een enorme variëteit aan pathogenen en insecten die de plant kunnen aanvallen. Om zich hier tegen te weren kunnen planten een afweerrespons activeren die werkzaam is tegen deze aanvallers. Dit doen planten door hormonen te produceren. De samenstelling en hoeveelheid van de geproduceerde plantenhormonen zijn afhankelijk van het type aanvaller. Salicylzuur, jasmonzuur, abscisinezuur en ethyleen zijn de belangrijkste hormonen voor het activeren van de afweer van planten. Als een plant zijn afweersysteem activeert wordt kostbare energie van de plant gebruikt voor afweer en is deze niet meer beschikbaar voor groei en zaadproductie. Doordat de verschillende hormoon-signaleringsroutes elkaar positief of negatief kunnen beïnvloeden, kan de plant de afweerrespons zo goed mogelijk afstemmen op de aanvaller van dat moment en de afweerreactie op een zo efficiënt mogelijke wijze aanzetten. De interacties tussen de verschillende hormoon-signaleringsroutes wordt crosstalk genoemd.

Salicylzuur speelt een belangrijke rol in het activeren van afweer tegen biotrofe pathogenen (een biotroof pathogeen kan alleen leven op levend plantenmateriaal). Activatie van de salicylzuur signaleringsroute leidt uiteindelijk tot expressie van het gen *PR1*, een markergen voor activatie van de salicylzuur route. De jasmonzuur signaleringsroute bestaat uit twee verschillende takken. De MYC-tak reguleert de afweer tegen herbivore insecten. Deze tak wordt gereguleerd samen met abscisinezuur. Als de MYC-tak wordt geactiveerd zorgen de transcriptiefactoren MYC2, MYC3 en MYC4 voor expressie van de genen *VSP1* en *VSP2*, belangrijke markergenen voor activatie van de MYC-tak. De ERF-tak reguleert de afweer tegen necrotrofe pathogenen (een necrotroof pathogeen leeft van dood plantenmateriaal). Deze tak wordt gereguleerd samen met ethyleen. Activatie van de ERF-tak zorgt via de transcriptiefactoren ORA59 en ERF1, voor expressie van het gen *PDF1.2* , een belangrijk markergen voor activatie van de ERF-tak.

Het belangrijkste doel van het onderzoek dat in dit proefschrift wordt beschreven is om te onderzoeken hoe activatie van de afweer gerelateerde hormoon-signaleringsroutes en de crosstalk tussen de routes de afweer, groei en zaadproductie van planten beïnvloedt.

In hoofdstuk 2 laten we zien dat na vraat door rupsen van het kleine koolwitje (*Pieris rapae*) jasmonzuur en abscisinezuur worden aangemaakt in Arabidopsis planten. We hebben de regulatie van de activatie van de afweer na vraat van *P. rapae* verder onderzocht door gebruik te maken van verschillende mutanten, zoals een mutant die niet meer in staat is abscisinezuur te produceren en een mutant waarin de MYC

transcriptiefactoren niet meer werkzaam zijn. Hiermee laten we zien dat abscisinezuur onmisbaar is voor activatie van de MYC-tak, maar ook dat abscisinezuur de ERF-tak heel sterk kan onderdrukken. Ethyleen wordt niet geproduceerd door de plant na vraat door *P. rapae*, maar wel na infectie met de necrotrofe schimmel *Botrytis cinerea*. Als we planten eerst infecteren met *B. cinerea*, wordt de ERF-tak aangeschakeld. Als de planten daarna worden aangevreten door *P. rapae* kunnen de planten de afweerrespons omschakelen en alsnog de MYC-tak activeren. Alles bij elkaar laat dit zien dat abscisinezuur en ethyleen een belangrijke rol spelen in de regulatie van de activatie van de MYC- en de ERF-tak om zo de precieze afweerrespons van de plant te bepalen en de overlevingskans van de plant te vergroten.

Uit het onderzoek in hoofdstuk 3 blijkt hoe de onbeschadigde bladeren van een door *P. rapae* aangevreten Arabidopsis plant reageren. Vergelijkbaar met in de aangevreten bladeren, is er productie van jasmonzuur en activatie van de transcriptiefactor *MYC2* in de onbeschadigde bladeren. Er is echter geen productie van abscisinezuur en geen activatie van het markergen *VSP1* in de onbeschadigde bladeren. Als we de onbeschadigde bladeren vervolgens behandelen met abscisinezuur of als de onbeschadigde bladeren worden aangevreten door een tweede *P. rapae* rups, zorgt dit voor een sterk verhoogde activatie van *MYC2* en *VSP1*. De rupsen groeien ook minder goed op planten die al een keer zijn aangevreten dan op planten die dat niet zijn. Deze resultaten laten zien dat in de onbeschadigde bladeren van een door rupsen aangevallen plant de afweerrespons nog niet volledig geactiveerd is, maar dat deze extra snel en extra sterk geactiveerd kan worden op het moment dat dit nodig is. Dit fenomeen waarbij de afweerrespons nog niet volledig is geactiveerd, maar als het ware in de startblokken staat, noemen we priming. Omdat de afweerrespons nog niet volledig wordt geactiveerd bij priming, is dit een afweermechanisme waar relatief weinig kostbare energie van de plant voor nodig is. In een omgeving waar de kans op een aanval door een herbivoor of een pathogeen hoog is, wegen de voordelen van priming (snellere en hogere activatie van het afweersysteem) vaak op tegen de kosten (het in de startblokken zetten van de afweerrespons).

De crosstalk tussen de verschillende hormoon-signaleringsroutes wordt vaak geïnterpreteerd als een kosten besparende strategie, omdat er geen energie verloren gaat aan het activeren van onnodige afweerresponsen. Dit geavanceerde regulerende systeem kan er echter wel voor zorgen dat er ecologische kosten ontstaan, omdat activatie van de afweerrespons tegen één bepaald pathogeen de afweer tegen een ander pathogeen kan onderdrukken. Sommige pathogenen kunnen de plantenhormonen zelfs namaken. Zo kunnen ze de benodigde afweerrespons onderdrukken en de crosstalk in hun eigen voordeel gebruiken. In Hoofdstuk 4 hebben we onderzocht of crosstalk inderdaad een kosten besparende strategie is. We hebben planten geïnfecteerd met meerdere aanvallers en gekeken naar afweer, groei en zaadproductie van Arabidopsis

planten. Als planten eerst hun afweerrespons tegen *P. rapae* rupsen of tegen de biotrofe oömyceet *Hyaloperonospera arabidopsidis* activeren, zijn ze daarna vatbaarder voor de necrotrofe schimmel *B. cinerea*. Ondanks deze verhoogde vatbaarheid voor *B. cinerea*, zijn er geen extra negatieve effecten op groei en zaadproductie in vergelijking met planten die maar door één aanvaller zijn belaagd. Dit is een eerste indicatie dat crosstalk inderdaad een kosten besparende strategie is.

De kosten van het activeren van afweer kunnen worden beïnvloed door omgevingsfactoren, zoals aantal lichturen in een dag, competitie met andere planten en aanwezigheid van voedingsstoffen. In Hoofdstuk 5 hebben we onderzocht welke en op welke wijze omgevingsfactoren bepalen wat de kosten zijn van infectie van Arabidopsis met de biotrofe oömyceet *H. arabidopsidis*. In alle geïnfecteerde planten was de groei in de eerste week na infectie verminderd ten opzichte van niet geïnfecteerde planten. Indien de planten waren opgegroeid met weinig voedingsstoffen en onder lange dag omstandigheden (14 uur licht per dag), konden de planten in de tweede week na infectie deze groei achterstand weer inhalen. Over de hele groeiperiode genomen, groeiden de planten zelfs meer en produceerden ze meer zaad na infectie met *H. arabidopsidis* dan planten die niet waren geïnfecteerd. Dit effect was het duidelijkst als de planten waren geïnfecteerd met een lage dosis *H. arabidopsidis*. Deze resultaten laten zien dat onder bepaalde omstandigheden er helemaal geen kosten zijn van infectie met *H. arabidopsidis* en dat infectie zelf een positief effect kan hebben op de groei en zaadproductie van de plant. Omdat *H. arabidopsidis* een biotroof pathogeen is, is het voor dit pathogeen voordelig als de plant in leven blijft. Het is daarom verleidelijk om te speculeren dat het positieve effect op de groei en zaadproductie van de plant geassocieerd is met de biotrofe levensstijl van *H. arabidopsidis*. Het onderzoeken van deze positieve interacties tussen planten en pathogenen kan bruikbare informatie opleveren voor de bescherming en verbetering van landbouwgewassen.

Kosten van afweer zijn een groot probleem voor het verhogen van de afweer van planten in de landbouw, omdat implementatie van afweer in landbouwgewassen kan resulteren in negatieve effecten op de groei en zaadproductie van de gewassen. De balans tussen afweer en groei en zaadproductie is daarom van cruciaal belang voor boeren en veredelaars. Met de resultaten die in dit proefschrift worden beschreven hebben we geprobeerd om moleculaire kennis te combineren met de ecologische relevantie van afweermechanismen om zo inzicht te krijgen in hoe en waarom planten verschillende afweersignalen integreren om op een zo kosten-efficiënt mogelijke manier om te gaan met de verschillende organismen waar ze mee te maken krijgen in een natuurlijke omgeving. Deze kennis is onmisbaar om uiteindelijk afweermechanismen te kunnen toepassen om landbouwgewassen te verbeteren.

DANKWOORD

Hatsa! Dat is klaar. Ik ben blij dat er nu weer ruimte in m'n hoofd is om over andere dingen na te denken dan over Arabidopsis, JA, ABA en *Pieris*. Nu ik ben aangekomen aan dit laatste stuk van mijn proefschrift merk ik dat ik het lastig vind om dit stuk te schrijven. Ik heb enorm genoten van mijn promotietijd en dan zijn er natuurlijk een heleboel mensen die ik wil bedanken voor wat ze voor me gedaan hebben tijdens mijn promotietijd, of gewoon omdat ze er zijn! Ik hoop dat ik niemand vergeet.

Als eerste natuurlijk Saskia. Ik vond het ontzettend fijn dat jij mijn begeleider was de afgelopen vier jaar! Jouw kritische blik op mijn teksten was zeer nuttig. Ondanks dat ik wel eens heb gezucht als je voor de zoveelste keer nog een keer mijn tekst wilde doorlezen, werd het er uiteindelijk altijd beter van! Zo heb jij vast wel een gezucht als ik weer vroeg weg ging om te gaan korfballen, maar ik heb je wel zo ver gekregen dat je afgelopen jaar een stukje van de zaalfinale hebt gekeken en ik quote even je Whatsapp daarover 'Korfbal is best wel leuk!!!' Sas, ik hoop dat jij er, net zoals ik, geen spijt van hebt dat je me hebt aangenomen na mijn presentatie over neushoorns. We hebben heel veel gelachen samen en dat maakt dat mijn promotietijd echt een hele leuke tijd was!!

Corné, ik vond het heel fijn om onder jouw begeleiding mijn promotieonderzoek uit te voeren. De relaxte, open sfeer, waardoor ik altijd bij jou binnen kan lopen maakte dat ik heel verbaasd was dat op een congres een PhD uit Norwich jou niks durfde te vragen omdat jij DE Corné Pieterse was. DE Corné Pieterse kan je namelijk altijd alles vragen! Ik heb de afgelopen jaren (te?) hard gelachen om je ongepaste opmerkingen, je beledigingen (met name als je ietwat ontactisch een spreker aankondigde op een congres) en je verhalen tijdens de koffie. Jij maakt dat PMI een gezellige vrolijke en vooral inspirerende groep is waarin het heel fijn werken is!

All PMI members from the past and the present, Hans, Anja, Roeland, Merel, Iván, Nora, Chiel, Erqin, Yeling, Colette, Silvia, Eline, Dieuwertje, Ainhoa, Christos, Marcel, Richard, Lotte, Peter, Paul, Giannis, Silvia, Marciel, Ke, Dmitry, Tieme, Guido, Alexandra, Joyce, Miek, Pim, Dongping, Joël, Manon, Tom, Stan and Joost I'd like to thank you for all the amazing PMI moments. Of course, especially my roomies, Lotte, Richard and Marcel, thank you for the interesting talks, letting off steam, restaurant recommendations, cat stories, Christmas dinner, dog stories, wedding, coffee moments, but most of all, a lot of laughs! Giannis, no water? Just wine! Roeland, mag ik nu m'n trui terug?

I would also like to thank my three amazing master students. Merel, Lewis and Liselotte I think you all find a lot of your work in this thesis, thanks!

Naast Arabidopsis, JA, ABA en *Pieris* was ook korfbal een rode draad voor, tijdens en hopelijk ook nog lang na mijn promotietijd. Ivo, Rutger, Thomas, Mitchel, Paul, David, Anders, Arno, Roald, Arnold, Eric, Andreas, Patricia, Sanne, Suzan, Sterre, Lienke, Laura, Karen, Jorine, Naomi, Stephanie, Anita en alle andere mensen waar ik de afgelopen jaren mee heb samengespeeld, bedankt voor alle fijne momenten. Die hebben me zeker geholpen, vooral tijdens het afronden van mijn proefschrift! En verder natuurlijk Martijn & Maaike, Martijn & Wendy, Roy, Johan & Suzanne, Kim & Sebastiaan, Matthijs & Judy, Jantine & John, Karel & Franka; weekendjes weg, keihard lachen tijdens het bedenken en doorvertellen van roddels, vroeg opstaan voor het familietoernooi, gezellige feestjes, dank daarvoor! Roy bedankt voor je hulp met de layout van dit boekje! Tot slot, Teja & John, Contardo & Bianca, Huib & Jans, en alle andere HKC'ers, jullie maken HKC tot de superfijne vereniging die het is!

Renate & John, Corinne, Tom & Tess, Peter & Pim, Reginald en Harry, bedankt voor alle mooie, leuke, vrolijke, enthousiaste, lachen, gieren, brullen, zonnige, wijn, bier, baco, limonadesiroop, ezelen, regenwormen, treinen, eindeloos veel andere spelletjes, Tox, Berkenplas, halverwege-SCHIER en natuurlijk SCHIER momenten! Wanneer gaan we weer?

Marieke, bedankt voor alle etentjes, borrels, feestjes, wandelingen in de zon, biertjes, spontane dinsdagavond stapavond, en heel veel gezelligheid! We gaan zeker nog een keer de 4-daagse lopen samen!

En dan mijn familie. Jullie waren altijd geïnteresseerd in wat ik nou allemaal aan het doen was. Opa, Oma, Mária, Leen, Jessica, Marty, Kevin en Amy en alle andere familie bedankt daarvoor! Jess, je bent de allertofste schoonzus! Laten we snel weer sushi gaan eten, wijntjes gaan drinken, Trivia spelen en kattenkwaad van de katten vergelijken :D.

Luuk, zoals je zelf zei: waar jij was, daar was ik en waar ik was, daar was jij. Koen, m'n grote 'kleine' broer, bergwandelingen, familietoernooien en alle andere momenten. Ik ben supertrots en blij dat jullie twee straks als mijn paranimfen naast me staan!

Pap en mam jullie zijn er altijd, altijd een fijne plek om thuis te komen voor gezelligheid, lekker eten en drinken of een spelletje. Jullie heben altijd het volste vertrouwen in mij. Bedankt voor alles wat jullie voor mij hebben gedaan! Pap, ik ben nu eindelijk (bijna) net zo slim als jij :D.

Als laatste, John. Jij hebt zeker de afgelopen maanden het meeste last gehad van het afronden van mijn proefschrift. Nu heb ik weer alle tijd voor jou! Bedankt dat je de aller aller aller liefste bent! Ik hou van je.

CURRICULUM VITAE

Irene Vos werd geboren op 7 februari 1987 te Renkum. In 2005 behaalde zij haar VWO diploma aan de Christelijke Scholengemeenschap Het Streek te Ede en startte zij met haar studie Biologie aan de Universiteit Utrecht. In 2008 behaalde zij haar BSc diploma waarna zij aan de master opleiding Environmental Biology begon, wederom aan de Universiteit Utrecht. Tijdens deze master werden twee onderzoeksstages uitgevoerd. De eerste stage vond plaats bij de leerstoelgroep Plant Ecofysiologie aan de Universiteit Utrecht. Onder begeleiding van Diederik Keuskamp werd gewerkt aan het project getiteld: “The role of brassinosteroids and auxin in low blue light induced hypocotyl elongation”. De tweede stage werd uitgevoerd in het Hluhluwe-iMfolozi Park, Kwazulu Natal, Rebuplic of South-Africa. Onder begeleiding van dr. Joris Cromsigt, Centre for Ecological and Evolutionary Synthesis, Oslo University, Norway, werd gewerkt aan het project genaamd: “Spatial heterogeneity of resources – variation in space and time in the distribution and persistence of grazing lawns”. Onder begeleiding van Prof. dr. ir. Corné Pieterse werd de masterscriptie geschreven met de titel “The influence of abiotic factors on indirect plant defense in rice”. In 2010 studeerde zij af en in 2011 begon zij met haar promotieonderzoek bij de leerstoelgroep Plant-Microbe Interacties. Onder begeleiding van Prof. dr. ir. Corné Pieterse en Dr. Saskia van Wees werd het onderzoek uitgevoerd dat is beschreven in dit proefschrift.

LIST OF PUBLICATIONS

Vos IA, Moritz L, Pieterse CMJ & Van Wees SCM. Impact of hormonal crosstalk on resistance and fitness of plants under multi-attacker conditions. *Submitted.*

Vos IA, Verhage A, Watt LG, Vlaardingerbroek I, Schuurink RC, Pieterse CMJ, Van Wees SCM. Abscisic acid is essential for differential regulation of jasmonic acid-dependent defenses during Arabidopsis-insect interactions. *In preparation for submission*.

Vos IA, Pieterse CMJ & Van Wees SCM, 2013. Costs and benefits of hormone-regulated plant defences. *Plant Pathology* 62, 43-55.

Vos IA, Verhage A, Schuurink RC, Watt LG, Pieterse CMJ & Van Wees SCM, 2013. Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. *Frontiers in Plant Science* 4, 539.

Keuskamp DH, Sasidharan R, **Vos IA**, Peeters AJM, Voesenek LACJ & Pierik R, 2011. Blue-light-mediated shade avoidance requires combined auxin and brassinosteroid action in Arabidopsis seedlings. *Plant Journal* 67, 208-17.