# Making sense out of signaling during plant defense

**Interacties tussen signaal-transductieroutes tijdens de immuunrespons van planten**

(met een samenvatting in het Nederlands en Spaans)

## Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
woensdag 8 juli 2009 des middags te 14.30 uur

door

**Hector Antonio Leon-Reyes**

geboren op
25 februari 1976 in Quito-Ecuador

**Promotor:** Prof. dr. ir. Corné M.J. Pieterse

**Copromotor:** Dr. Tita Ritsema

**Promotiecommissie:** Prof. dr. Christiane Gatz
Prof. dr. Julia Bailey-Serres
Prof. dr. Johan Memelink
Prof. dr. Marcel Dicke

**ISBN:** 978-90-39350973
**Cover:** Hans van Pelt and Lorena Muñoz
**Lay-out:** Marjolein Kortbeek-Smithuis; Communications & Design, Faculty of Science, Universiteit Utrecht, The Netherlands

**Printed by:** Grafisch Bedrijf Ponsen & Looijen, Wageningen, The Netherlands

The research described in this thesis was financed by VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which is subsidized by the Netherlands Organization of Scientific Research.

## Contents

CHAPTER 1

# General introduction

Adapted from:
**Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S. and Van Wees, S.C.M.**
Networking by small-molecule hormones in plant immunity.
**Nature Chemical Biology** 5: 308-316 (2009)

## Phytohormones in plant defense

Plants live in complex environments in which they intimately interact with a broad range of microbial pathogens with different lifestyles and infection strategies. The evolutionary arms race between plants and their attackers provided plants with a highly sophisticated defense system that, like the animal innate immune system, recognizes pathogen molecules and responds by activating specific defenses that are directed against the invader encountered. Recent advances in plant immunity research provided exciting new insights into the underlying defense signaling network. Diverse small-molecule hormones play pivotal roles in the regulation of this network. Their signaling pathways cross-communicate in an antagonistic or synergistic manner, providing the plant with a powerful regulatory capacity to finely tailor its immune response. Pathogens, on the other hand, can manipulate the plant's defense signaling network for their own benefit by affecting phytohormone homeostasis to antagonize the induction of plant defenses.

Phytohormones are small molecules that are essential for the regulation of plant growth, development, reproduction, and survival. They act as signal molecules and occur in low concentrations. Changes in hormone concentration or sensitivity, such as triggered upon biotic and abiotic stress conditions, mediates a whole range of adaptive plant responses. Classic phytohormones are abscisic acid (ABA), auxins, cytokinins, ethylene (ET) and gibberellins, but small signal molecules such as brassinosteroids, jasmonates (JAs) and salicylic acid (SA) are recognized as phytohormones as well (Buchanan et al., 2000). The importance of SA, JAs, and ET as primary signals in the regulation of the plant's immune response is well established (Howe, 2004; Pozo et al., 2004; Van Loon et al., 2006a; Loake and Grant, 2007; Von Dahl and Baldwin, 2007). ABA (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008), auxins (Navarro et al., 2006; Wang et al., 2007), gibberellins (Navarro et al., 2008), cytokinins (Siemens et al., 2006; Walters and McRoberts, 2006), and brassinosteroids (Nakashita et al., 2003; Shan et al., 2008) emerged as players on the battle field as well (Fig. 1). This suggests that the regulation of plant growth, development and defense is interconnected in a complex network of cross-communicating hormone signaling pathways. The great regulatory potential of such a network may allow plants to quickly adapt to their biotic and abiotic environment and to utilize their resources in a cost-efficient manner. It is generally believed that hormone-regulated induced defense responses evolved to save energy under enemy-free conditions, since they only involve costs when defenses are activated upon pathogen or insect attack (Walters and Heil, 2007). These costs arise from the allocation of resources to defense and away from plant growth and development. Trade-offs between between plant growth rate and disease resistance have been well documented (Walters and Heil, 2007) and support the hypothesis that plant growth and defense are regulated by a network of interconnecting signaling pathways.

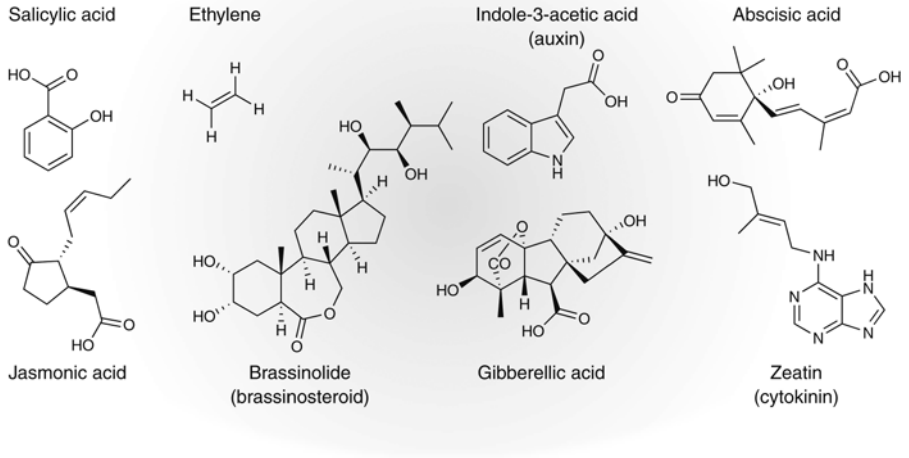



**Fig. 1.** **Phytohormones implicated in the plant immunity signaling network.**

Upon pathogen attack, the quantity, composition, and timing of the phytohormonal blend produced by the plant varies between plant species and depends greatly on the lifestyle and infection strategy of the invading attacker. This so-called signal signature results in the activation of a specific set of defense-related genes that eventually determines the nature and effectiveness of the immune response that is triggered by the attacker (De Vos et al., 2005). In recent years, molecular, genetic and genomic tools have been used to uncover the complexity of the hormone-regulated induced defense signaling network. Besides balancing the relative abundance of different hormones, intensive interplay between hormone signaling pathways emerged as an important regulatory mechanism by which the plant is able to tailor its immune response to the type of invader encountered. On the other hand, evidence is accumulating that pathogens can manipulate hormone-regulated signaling pathways to evade host immune responses. Here we review our current understanding of the roles of phytohormones in the plant's immune system, with a focus on cross-talk between defense hormone signaling pathways and its significance in plant-pathogen interactions.

## The plant immune system

In nature, plants are continuously threatened by a wide range of harmful pathogens and pests, including viruses, bacteria, fungi, oomycetes, nematodes, and insect herbivores. Each of these attackers exploits highly specialized features to establish a parasitic relationship with its host plant. According to their lifestyles, plant pathogens are generally divided into necrotrophs and biotrophs (Fig. 2; Glazebrook, 2005).

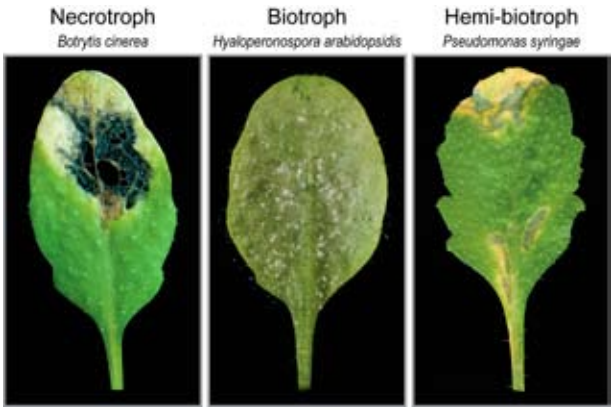



**Fig. 2. Disease symptoms on Arabidopsis leaves caused by the necrotrophic fungus *Botrytis cinerea,* the biotrophic oomycete *Hyaloperonospora arabidopsidis,* and the hemi-biotrophic bacterium *Pseudomonas syringae.* Photos: Hans van Pelt.**

Necrotrophs first destroy host cells, often through the production of phytotoxins, after which they feed on the contents. Biotrophs derive nutrients from living host tissues, commonly through specialized feeding structures (haustoria) that invaginate the host cell without disrupting it. Many plant pathogens display both lifestyles, depending on the stage of their life cycle, and are called hemi-biotrophs.

To defend themselves against all these different types of pathogens, plants possess an array of structural barriers and preformed antimicrobial compounds to prevent or attenuate invasion by potential attackers. Despite the diversity of these constitutive defenses, many microbes succeed in breaking through this pre-invasive layer of defense. However, a broad spectrum of inducible plant defenses can be recruited to limit further pathogen ingress. For this post-invasive line of defense, plants have evolved sophisticated strategies to perceive their attacker and to translate this perception into an effective immune response (Fig. 3; Jones and Dangl, 2006). First, the primary immune response recognizes common features of microbial pathogens, such as flagellin, chitin, glycoproteins, and lipopolysaccharides (Göhre and Robatzek, 2008; Nürnberger and Kemmerling, 2009). These microbial determinants are referred to as pathogen-associated molecular patterns (PAMPs; Chisholm et al., 2006; Jones and Dangl, 2006; Göhre and Robatzek, 2008). PAMPs activate pattern-recognition receptors (PRRs), which in turn initiate diverse downstream signaling events that ultimately result in the activation of a basal resistance that is called PAMP-triggered immunity (PTI; Chisholm et al., 2006; Jones and Dangl, 2006). During the co-evolutionary arms race between pathogens and their host plants, pathogens acquired effector molecules that are transported into the host cell to suppress PTI and promote virulence of the pathogen, resulting in effector-triggered susceptibility (ETS). In turn, plants acquired resistance (R) proteins that recognize these attacker-specific effectors, resulting in a secondary

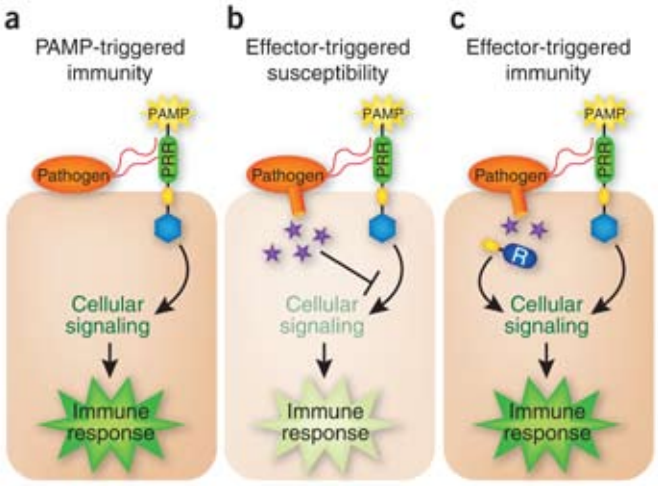



**Fig. 3. Simplified schematic representation of the plant immune system.**
**(a) Upon pathogen attack, pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signaling cascade that leads to PAMP-triggered immunity (PTI).**
**(b) Virulent pathogens have acquired effectors (purple stars) that suppress PTI, resulting in effector triggered susceptibility (ETS).**
**(c) In turn, plants have acquired resistance (R) proteins that recognize these attacker-specific effectors, resulting in a secondary immune response called effector-triggered immunity (ETI).**

immune response called effector-triggered immunity (ETI; Chisholm et al., 2006; Jones and Dangl, 2006). Ultimately, the final outcome of the battle depends on the balance between the ability of the pathogen to suppress the plant's immune system and the capacity of the plant to recognize the pathogen and to activate effectual defenses.

## Induced plant defense responses

Many early signaling components of PTI and ETI have been identified in recent years (Göhre and Robatzek, 2008; Schwessinger and Zipfel, 2008; Nürnberger and Kemmerling, 2009). Downstream of these early signaling events, plants respond by activating a large number of integrated defense responses to ward off the invader. The nature of the defense responses that are activated during PTI and ETI show substantial overlap (Tsuda et al., 2008). These defenses include cell wall fortification through the synthesis of callose and lignin; the production of antimicrobial secondary metabolites, such as phytoalexins; and the accumulation of pathogenesis-related (PR) proteins, such as chitinases and glucanases that degrade fungal and oomycete cell walls. Recognition of pathogen-specific effectors through the ETI system is particularly effective because it is followed by a burst of reactive oxygen species that culminates in a programmed hypersensitive cell death at the site of pathogen invasion, keeping the pathogen isolated from the rest of the plant and preventing

further damage (De Wit, 1997). Obviously, this hypersensitive response would favor growth of pathogens with a necrotrophic lifestyle as their virulence strategy relies on their capacity to kill host cells (Glazebrook, 2005). Therefore, the hypersensitive response is believed to be typically active against pathogens with a biotrophic lifestyle (Glazebrook, 2005). Immune responses that are active against necrotrophs are likely to be initiated in response to the action of pathogen-derived toxins or damage-associated molecular patterns (DAMPs), such as breakdown products of the plant cell wall that, upon release by the activity of pathogen-derived cell wall degrading enzymes, can stimulate plant defense responses (Schwessinger and Zipfel, 2008; Nürnberger and Kemmerling, 2009).

The regulation of the defense network that translates the pathogen-induced early signaling events into activation of effective defense responses depends profoundly on the action of phytohormones. Pathogen infection stimulates the plant to synthesize one or more hormonal signals depending on the type of attacker (De Vos et al., 2005). Compelling evidence for the key role of phytohormones in the plant's immune response comes from studies with the model plant species *Arabidopsis thaliana* (Arabidopsis) and *Nicotiana tabacum* (tobacco) in which various mutants and transgenics impaired in hormone biosynthesis, perception or signaling were demonstrated to display a severe alteration in the level of resistance to specific types of pathogens. From these studies it became evident that biotrophic pathogens are generally sensitive to defense responses that are regulated by SA, while pathogens with a necrotrophic lifestyle are commonly deterred by defenses that are controlled by JAs and ET (Thomma et al., 2001a; Glazebrook, 2005). In analogy to the defense response to necrotrophs, the wound response that is effective against insect herbivores is also regulated by the JA signaling pathway (Howe, 2004).

## Systemic immunity

Once plant defense responses are activated at the site of infection, a systemic defense response is often triggered in distal plant parts to protect these undamaged tissues against subsequent invasion by the pathogen. This long-lasting and broad-spectrum induced disease resistance is referred to as systemic acquired resistance (SAR; Fig. 4; Durrant and Dong, 2004) and is characterized by the coordinate activation of a specific set of *PR* genes, many of which encode for proteins with antimicrobial activity (Van Loon et al., 2006b). The onset of SAR can be triggered by PTI- and ETI-mediated pathogen recognition and is generally associated with increased levels of SA, locally at the site of infection and also systemically in distant tissues (Mishina and Zeier, 2007; Tsuda et al., 2008). Mutant and transgenic plants that are impaired in SA signaling are incapable of developing SAR and do not show *PR* gene activation upon pathogen infection (Durrant and Dong, 2004), indicating that SA is a necessary intermediate in the SAR signaling pathway. The regulatory

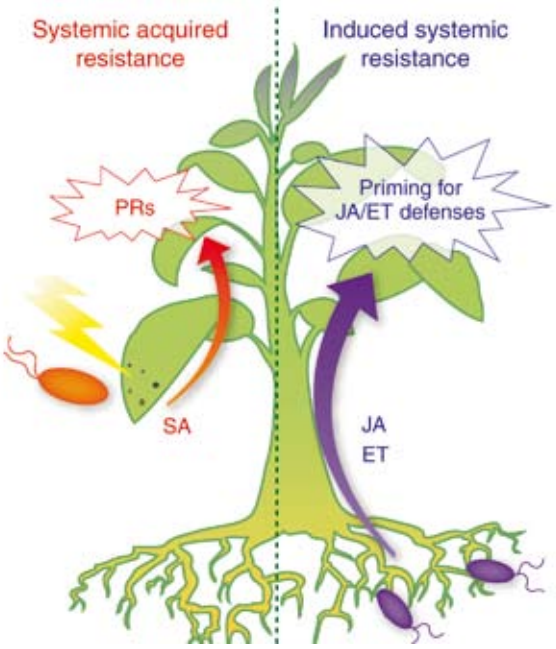



**Fig.4.** **Schematic representation of systemically induced immune responses. Systemic acquired resistance (SAR) is typically activated in healthy systemic tissues of locally infected plants. Upon pathogen infection, a mobile signal travels through the phloem to activate defense responses in distal tissues. Salicylic acid (SA) is an essential signal molecule for the onset of SAR, as it is required for the activation of a large set of genes that encode pathogenesis-related proteins (PRs) with antimicrobial properties. Induced systemic resistance (ISR) is typically activated upon colonization of plant roots by beneficial microorganisms. Like SAR, a long-distance signal travels through the phloem to activate systemic immunity in above-ground plant parts. ISR is commonly regulated by jasmonic acid (JA)- and ethylene (ET)-dependent signaling pathways and is typically not associated with the direct activation of PR genes. Instead, ISR-expressing plants are primed for accelerated JA- and ET-dependent gene expression, which becomes evident only after pathogen attack. Both SAR and ISR are effective against a broad spectrum of virulent plant pathogens.**

protein NPR1 (NONEXPRESSOR OF *PR* GENES1) emerged as an important transducer of the SA signal, which upon activation by SA acts as a transcriptional co-activator of *PR* gene expression (Dong, 2004). The nature of the systemically transported mobile signal that travels from the site of infection to establish SAR in distal tissues is one of the holy grails in plant defense signaling research. Recent studies, point to a role for methyl-SA, JAs, a plastid glycerolipid-based factor, and a lipid-transfer protein (Vlot et al., 2008).

Beneficial soil-borne microorganisms, such as mycorrhizal fungi and plant growth-promoting rhizobacteria, can induce a phenotypically similar form of systemic immunity called induced systemic resistance (ISR; Fig. 4; Van Loon et al., 1998; Pozo and Azcon-Aguilar, 2007). Like PAMPs of microbial pathogens, different beneficial microbe-associated molecular patterns (MAMPs) are recognized by the plant, which results in a mild, but effective activation of the immune response in systemic tissues (Bakker et al., 2007; Van der Ent et al., 2008; Van Wees et al., 2008).

In contrast to SA-dependent SAR, ISR triggered by beneficial microorganisms is often regulated by JA- and ET-dependent signaling pathways and is associated with priming for enhanced defense, rather than direct activation of defense (Conrath et al., 2006; Van Wees et al., 2008). While SAR is predominantly effective against biotrophic pathogens that are sensitive to SA-dependent defenses, ISR was shown to be effective against pathogens and insects that are sensitive to JA- and ET-dependent defenses (Ton et al., 2002; Van Oosten et al., 2008).

## Pathway cross-talk to fine-tune defense

In nature, plants often deal with simultaneous or subsequent invasion by multiple aggressors and beneficials, which can influence the primary induced defense response of the host plant (Stout et al., 2006; Poelman et al., 2008a). Activation of plant defense mechanisms is associated with ecological fitness costs (Walters and Heil, 2007). Hence, plants need regulatory mechanisms to effectively and efficiently adapt to changes in their complex environment. Cross-talk between hormonal signaling pathways provides the plant with such a powerful regulatory potential and allows the plant to finely tune its defense response to the invaders encountered (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Bostock, 2005; Pieterse and Dicke, 2007).

The importance of SA, JAs, and ET as dominant primary signals in local and systemic induced defense signaling has been well documented (Pozo et al., 2004; Van Loon et al., 2006a; Loake and Grant, 2007). In recent years, research on their biosynthesis pathways and the way they are perceived by other biomolecules significantly advanced our understanding of the signaling pathways that these small molecules regulate (a simplified schematic representation of the SA, JA, and ET signaling pathways is provided in Fig. 5) (Loake and Grant, 2007; Katsir et al., 2008; Kendrick and Chang, 2008). However, the way these signal molecules function in a complex network of interacting signaling pathways is less well studied. Early work in tomato (*Solanum lycopersicum*) demonstrated that SA and its acetylated derivative Aspirin are strong antagonists of the JA signaling pathway (Doherty et al., 1988), and that ET and JA signaling can act synergistically (Penninckx et al., 1998). The genomics era provided a wealth of new opportunities to investigate how the SA, JA, and ET signaling pathways are interconnected in the induced defense signaling network (Katagiri, 2004). Whole-genome expression profiling of a large set of Arabidopsis mutants affected in SA, JA, or ET signaling in response to infection by the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* (Glazebrook et al., 2003), confirmed that there is extensive cross-talk between the SA, JA, and ET response pathways and paved the way to model the network topology of the plant's immune response (Katagiri, 2004).

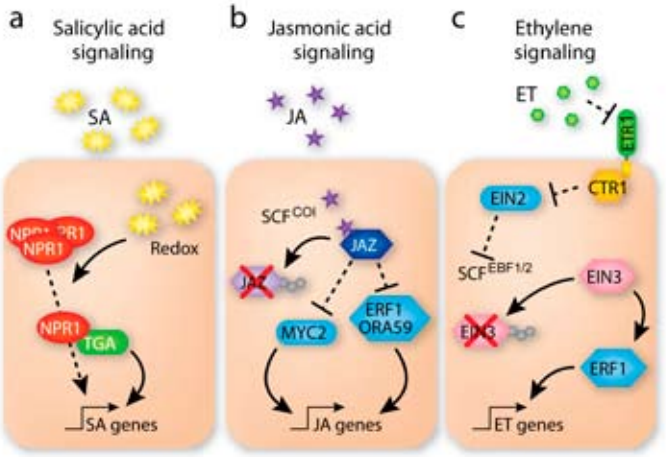



**Fig. 5. Simplified schematic representation of the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathways. a) SA mediates a change in the cellular redox potential, resulting in the reduction of the NPR1 oligomer to its active monomeric form. Monomeric NPR1 is then translocated into the nucleus where it functions as a transcriptional co-activator of SA-responsive genes, such as *PR-1*, by enhancing the binding of TGA transcription factors to SA-responsive promoter elements (Dong, 2004; Loake and Grant, 2007). b) in the JA-signaling cascade, the E3 ubiquitin ligase $SCF^{COI1}$ and Jasmonate ZIM-domain (JAZ) proteins form a complex that represses transcription of JA-responsive genes (Katsir et al., 2008). Upon accumulation of JA, JA-isoleucine (JA-Ile) binds to the F-box protein COI1 in the $SCF^{COI1}$ complex after which the JAZ proteins are ubiquitinated and subsequently degraded through the 26S proteasome. This results in the activation of JA-responsive genes through the action of transcription factors such as MYC2, ERF1, or ORA59 (Lorenzo and Solano, 2005; Pré et al., 2008). c) in the ET signaling cascade, the gaseous hormone ET is perceived by plasma membrane receptors such as ETR1 (Von Dahl and Baldwin, 2007; Kendrick and Chang, 2008). Genetically, these receptors are negative regulators of the ET response, because in the absence of ET they maintain the negative regulatory role of CTR1, which represses the positive regulator EIN2. Upon perception of ET, the repression of ET signaling by CTR1 is relieved, allowing downstream signaling through EIN2. Subsequently, critical positive regulators of ET-responsive gene expression, such as EIN3, become active because the E3 ubiquitin ligase $SCF^{EBF1/2}$-dependent 26S proteasome degradation of these proteins becomes inhibited. EIN3-like transcription factors activate transcription factors such as ERF1, resulting in the expression of downstream ET-responsive genes.**

One of the best studied examples of defense-related signal cross-talk is the antagonistic interaction between the SA and the JA response pathway. Pharmacological experiments with Arabidopsis revealed that JA-responsive marker genes, such as *PDF1.2* and *VSP2,* are highly sensitive to suppression by exogenous application of SA, whereas the SA-responsive marker gene *PR-1* can be suppressed by JA signaling (Fig. 6) (Spoel et al., 2003; Koornneef et al., 2008b). SA-mediated suppression of JA-responsive gene expression was observed in a large number of Arabidopsis accessions collected from very different geographic origins, highlighting the potential significance of this phenomenon in the regulation of induced plant defenses in nature (Koornneef et al., 2008b). Although many reports describe an antagonistic interaction between SA- and JA-dependent signaling, synergistic interactions have been described as well (Schenk et al., 2000; Van Wees et al., 2000; Mur et al., 2006). For example in Arabidopsis, treatment with low concentrations of JA and SA resulted in a synergistic effect on the JA- and SA-responsive genes

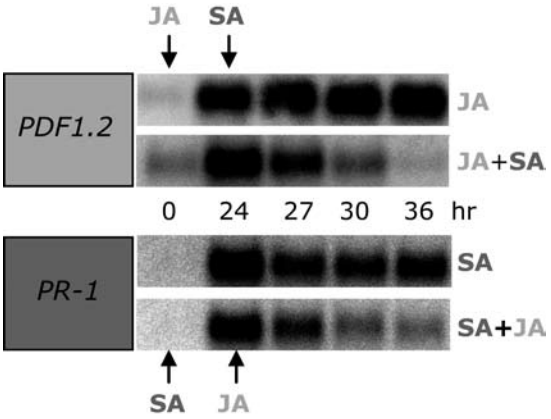



**Fig. 6. Mutual antagonism between SA and JA signaling in Arabidopsis. Induction of the JA-responsive gene *PDF1.2* is suppressed by SA (top panel), while the SA-responsive gene *PR-1* is suppressed by JA (bottom panel).**

*PDF1.2* and *PR-1,* respectively. However, at higher concentrations the effects were antagonistic, demonstrating that the outcome of the SA-JA interaction is dependent upon the relative concentration of each hormone (Thaler et al., 2002a; Mur et al., 2006). Kinetics of SA-JA biosynthesis and signaling during the interaction of a plant with its attacker(s) are could be highly instrumental in tailoring the defense response to the attacker encountered. Additionally, SA and JAs can be readily modified into derivatives e.g. methylation, adenylation or conjugation with altered biological activity, which adds yet another layer of regulation.

Many examples of trade-offs between SA-dependent resistance against biotrophic pathogens and JA-dependent defense against necrotrophic pathogens and insect herbivory have been documented (Bostock, 2005; Stout et al., 2006). For example, Moran (1998) demonstrated that pathogen-induced SAR in cucumber was associated with reduced resistance against feeding by spotted cucumber beetles (*Diabrotica undecimpunctata howardi)* and enhanced reproduction of melon aphids (*Aphis gossypii*). Moreover, activation of the SA pathway by *P. syringae* suppressed JA signaling and rendered infected leaves more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Spoel et al., 2007).

## Signaling nodes in the SA-JA-ET network

To date, many examples of positive and negative cross-talk between SA, JA, and ET signaling have been reported and are documented in a series of informative reviews (Lorenzo and Solano, 2005; Robert-Seilaniantz et al., 2007; Kazan and Manners, 2008; Koornneef and Pieterse, 2008; López et al., 2008; Spoel and Dong, 2008). A number of key signaling nodes emerged from these studies and will be highlighted

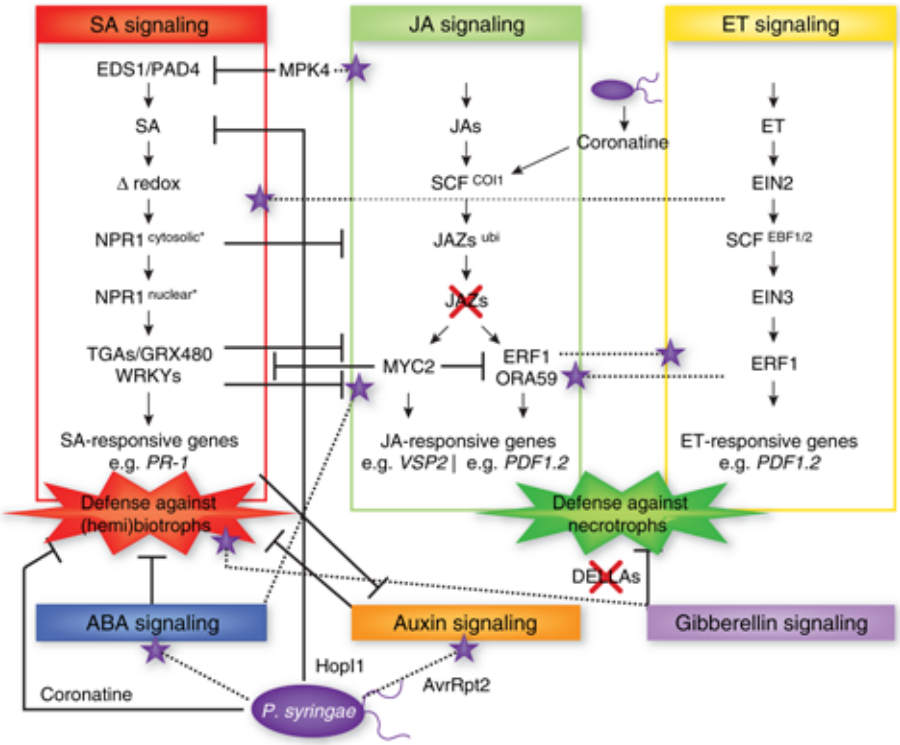



**Fig. 7. Networking by phytohormones in the plant immune response. Cross-communication between hormone signaling pathways provides the plant with a large regulatory capacity to tailor its defense response to different types of attackers. On the other hand, pathogens such as *Pseudomonas syringae* produce effector proteins (e.g. coronatine, HopI1 and AvrRpt2) that manipulate the signaling network to suppress host immune responses and promote virulence. The SA, JA and ET signaling pathways represent the backbone of the defense signaling network, with other hormonal signaling pathways feeding into it. Only those signal transduction components that are relevant to this review are shown. ⊥ = negative effect; ⋆ = positive effect.**

in the following sections according to their role in SA-JA, JA-ET, or ET-SA cross-talk (Fig. 7).

### *SA-JA*

Several molecular players in SA-JA cross-talk have been identified in recent years, including the mitogen-activated protein kinase MPK4 (MPK4; Petersen et al., 2000), the lipase-like proteins EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) and PAD4 (PHYTOALEXIN-DEFICIENT4 ) (Brodersen et al., 2006), the defense regulatory protein NPR1 (Spoel et al., 2003) the fatty acid desaturase SSI2 (SUPPRESSOR OF SA INSENSITIVITY2) (Kachroo et al., 2001), the glutaredoxin GRX480 (Ndamukong et al., 2007), and WRKY transcription factor proteins such as WRKY70 (Li et al., 2004). Mutation or ectopic expression of the corresponding genes have opposite effects on SA and JA signaling and resistance against biotrophs and necrotrophs, respectively, indicating that these proteins are important regulators

of SA-JA cross-talk (Koornneef and Pieterse, 2008). The majority of the identified cross-talk regulators play pivotal roles in SA signal transduction in which NPR1 plays a central role. NPR1 acts downstream of EDS1 and PAD4 in the SA signaling pathway (Brodersen et al., 2006). In addition, NPR1 regulates the SA-mediated expression of *GRX480* and *WRKY70*, which encode proteins that suppress JA-dependent gene expression (Li et al., 2004; Ndamukong et al., 2007). By contrast, SSI2 was identified in a screen for suppressors of the mutant *npr1-5* phenotype (Kachroo et al., 2001) and, thus, exerts an NPR1-independent role in the regulation of SA-JA cross-talk. Mutant *ssi2* plants are defective in stearoyl-ACP desaturase, resulting in altered fatty acid content. Mutations that restored the lowered 18:1 fatty acid levels rescued the *ssi2* mutant phenotype, suggesting a role for fatty acid signaling in SA-JA cross-talk (Kachroo et al., 2001; Kachroo et al., 2003).

Changes in the cellular redox state play a major role in the transduction of the SA signal (Mou et al., 2003; Dong, 2004). The NPR1 protein is an important transducer of SA-induced redox changes. Besides functioning as a crucial transcriptional co-activator of SA-responsive *PR* genes (Dong, 2004), NPR1 is also a key regulator in SA-mediated suppression of JA signaling (Spoel et al., 2003; Pieterse and Van Loon, 2004). SA-induced redox changes activate NPR1 by reducing inactive NPR1 oligomers to active monomers (Dong, 2004). Active NPR1 monomers are translocated to the nucleus where they interact with TGA transcription factors that activate SA-responsive genes (Mou et al., 2003; Dong, 2004). Interestingly, nuclear localization of SA-activated NPR1 is not required for the suppression of JA-responsive genes, indicating that the antagonistic effect of SA on JA signaling is modulated through a function of NPR1 in the cytosol (Spoel et al., 2003). In rice (*Oryza sativa*), a similar cytosolic function of NPR1 in SA-JA cross-talk was reported (Yuan et al., 2007).

Glazebrook and co- workers provided additional evidence for a differential role of cytosolic and nuclear NPR1 in the regulation of SA- and JA/ET-dependent signaling, respectively. They showed that two different alleles of the Arabidopsis *npr1* mutant *(npr1-1* and *npr1-3*) behaved differently in terms of transcriptome changes upon infection by *P. syringae* (Glazebrook et al., 2003). The *npr1-1* mutant, which has a mutation in a crucial ankyrin-repeat domain, was affected in the expression of SA- as well as JA/ET-dependent genes. However, the *npr1-3* mutant, which produces a truncated cytoplasmatically localized NPR1 protein (Dong, 2004), was only affected in SA-dependent gene expression, suggesting that the cytoplasmatic function of NPR1 plays a role in the control of JA/ET-dependent responses. NPR1 has been implicated in several other JA/ET-dependent defense responses, including beneficial rhizobacteria-mediated ISR (Van Loon et al., 1998; Van Wees et al., 2008) and JA/ET-dependent resistance against the soil-borne fungus *Verticillium longisporum* (Johansson et al., 2006). However, the molecular mechanisms by which NPR1 exerts its role these JA/ET-dependent defenses remains to be elucidated.

### *JA-ET*

In many cases the interaction between JA and ET signaling is a synergistic one. A classic example is the regulation of the Arabidopsis plant defensin gene *PDF1.2*, which requires concomitant activation of the JA and the ET response pathway (Penninckx et al., 1998). ERFs are members of the large plant-specific APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily of transcription factors. Two members of this superfamily, ERF1 and ORA59, emerged as principal integrators of the JA and ET signaling pathways (Lorenzo et al., 2003; Pré et al., 2008). The expression of both *ERF1* and *ORA59* is induced by JA and ET and can be activated synergistically by both hormones. In addition, overexpression of the transcription factor genes *ERF1* or *ORA59* in the JA-insensitive mutant *coi1* or *ERF1* in the ET-insensitive mutant *ein2* constitutively activated the *PDF1.2* gene, indicating that these transcription factors are important nodes of convergence of JA and ET signaling.

Like ERF1 and ORA59, the basic helix-loop-helix leucine zipper transcription factor MYC2 (originally called JIN1 for JASMONATE INSENSITIVE1) has been demonstrated to play an important role in the regulation of JA-responsive genes (Lorenzo and Solano, 2005). Upon induction of the JA pathway, MYC2 differentially regulates two distinct classes of JA-responsive genes. MYC2 functions as a positive regulator of JA-responsive genes such as *VSP2* and *LOX2*, whereas it acts as a negative regulator of JA/ET-responsive genes such as *PDF1.2* that are activated by ERFs (Lorenzo et al., 2004). Hence, when the JA response is activated in combination with ET, the ERF-branch of the JA response is activated, while the MYC2-branch of the JA response is activated when ET is absent. ABA was shown to play a role in favoring the MYC2-dependent branch of the JA response (Anderson et al., 2004). Such a differential JA response was clearly apparent when the whole-genome expression profile of Arabidopsis was analyzed after infection with the necrotroph *A. brassicicola,* which induces the production of both JA and ET, or infestation with herbivorous Western flower thrips (*Frankliniella occidentalis*), which stimulates the biosynthesis of JAs but not that of ET (De Vos et al., 2005). Hence, the interplay between the ERFs and MYC2 may allow the plant to activate the set of JA-responsive genes that is required for optimal defense against the attacker encountered. While JA signaling mutants are generally more susceptible to necrotrophic pathogens such as *Botrytis cinerea* (Thomma et al., 2001a; Glazebrook, 2005), the Arabidopsis mutant *jin1/myc2* showed enhanced resistance against this pathogen (Nickstadt et al., 2004), Since the inhibitory effect of MYC2 on the ERF-branch of the JA response is relieved in the *jin1/myc2* mutant, the enhanced resistance against *B. cinerea* may be caused by a potentiated expression of ERF-dependent defenses in this mutant. Interestingly, MYC2 is also implicated in mediating the suppression of SA-dependent defenses by coronatine, a phytotoxic virulence factor of *P. syringae* that mimics the action of Jas (Nickstadt et al., 2004;

Laurie-Berry et al., 2006) (see section "Decoy strategies of the attacker"), which makes this transcription factor an important node in the SA-JA-ET signaling network.

### *ET-SA*

ET has been demonstrated to be an important modulator of the plant's defense response to pathogen and insect attack (Van Loon et al., 2006a; Von Dahl and Baldwin, 2007). For instance, from a study with ET-insensitive (Tetr) tobacco plants, it was concluded that ET is essential for the onset of SA-dependent SAR that is triggered upon infection by tobacco mosaic virus (Verberne et al., 2003). Moreover, ET was shown to enhance the response of Arabidopsis to SA, resulting in a potentiated expression of the SA-responsive marker gene *PR-1* (Lawton et al., 1994; De Vos et al., 2006). This synergistic effect of ET on SA-induced *PR-1* expression was blocked in the ET-insensitive mutant *ein2* (De Vos et al., 2006), indicating that the modulation of the SA-pathway by ET is EIN2 dependent and, thus, functions through the ET signaling pathway. Further evidence for SA-ET cross-talk came from the network topology study of Glazebrook and coworkers in which the global expression profiles of *P. syringae*-infected Arabidopsis wild-type and signaling-defective mutant plants were analyzed (Glazebrook et al., 2003). This study showed extensive cross-talk between the SA and ET signaling pathways, as evidenced by the fact that the expression of many SA-responsive genes was significantly affected in the *ein2* mutant background.

## Hormone pathways connected to the SA-JA-ET backbone

Whilst the SA, JA and ET response pathways serve as the backbone of the induced defense signaling network, studies in Arabidopsis demonstrated that other hormone response pathways feed into it (Fig. 7). ABA is a hormone commonly associated with plant development and abiotic stress, but its role in biotic stress becomes increasingly evident (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008). ABA is connected to the SA-JA-ET network as it was shown to attenuate JA/ET-dependent gene expression (Anderson et al., 2004) and to affect JA biosynthesis and resistance against JA-inducing necrotrophic pathogens (Adie et al., 2007b; Flors et al., 2008). Moreover, ABA was demonstrated to antagonize the onset of SA-dependent defenses and SAR (Mohr and Cahill, 2007; Yasuda et al., 2008). Interestingly, NaCl-activated abiotic stress had a similar suppressive effect on the SA-dependent SAR in Arabidopsis (Yasuda et al., 2008). Conversely, activation of SAR suppressed the expression of ABA-related genes, indicating that ABA serves as an important regulator that functions at the crossroad of abiotic and biotic stress responses.

Auxins play a role in virtually every stage of plant development (Benjamins and Scheres, 2008). The auxin response pathway is connected to the SA-JA-ET signaling network in different ways. For instance, auxin has been demonstrated to affect JA biosynthesis (Nagpal et al., 2005) and the expression of genes involved in JA production (Liu and Wang, 2006). Secondly, auxin signaling was shown to promote disease susceptibility to *P. syringae* (Navarro et al., 2006; Chen et al., 2007), a process that can be counteracted by SA (Wang et al., 2007). Whole-genome expression profiling revealed that SA interferes with auxin responses by global repression of auxin-related genes, including the auxin receptor gene *TIR1*. The inhibitory effect of SA on auxin responses stimulated effective defenses against the (hemi)biotrophic pathogens *H. arabidopsidis* and *P. syringae,* resulting in heightened resistance to these pathogens (Wang et al., 2007). Hence, the antagonistic effect of SA on auxin signaling seems to be an intrinsic part of SA-dependent resistance against biotrophs.

Recently, gibberellins were shown to hook up to the SA-JA-ET network as well. Gibberellins are hormones that control plant growth by regulating the degradation of growth-repressing DELLA proteins. Navarro and coworkers demonstrated that DELLA proteins promote susceptibility to biotrophic pathogens and resistance to necrotrophic pathogens by modulating the relative strength of the SA and JA signaling pathways (Navarro et al., 2008). Hence, it was postulated that by regulating the stability of DELLA proteins, gibberellins are able to modulate the SA-JA-ET network and affect the final outcome of the immune response.

Cytokinins often work in concert with auxins in processes such as cell division and differentiation of plant tissues. They are linked to the response of plants to biotrophic pathogens that alter the host's physiology (Walters and McRoberts, 2006), such as *Plasmodiophora brassicae* that causes aberrant root growth (club roots) in *Brassica* species (Siemens et al., 2006). However, little is known about their connection with the SA-JA-ET network.

Brassinosteroids play a key role in cell expansion and division, differentiation, and reproductive development. When applied exogenously, they are able to induce a broad-spectrum disease resistance (Nakashita et al., 2003). Brassinosteroids are perceived by the receptor BRI1, which interacts with the receptor-like kinase BAK1 to initiate an intracellular signaling cascade that regulates growth-related processes (Belkhadir and Chory, 2006). Interestingly, BAK1 also interacts with receptors that recognize PAMPs, such as bacterial flagellin, resulting in the initiation of innate immunity (Chinchilla et al., 2007; Heese et al., 2007). Pathogen-specific effectors of *P. syringae* have been shown to interfere with this process by binding to BAK1 themselves and consequently impede the host immune response (Shan et al., 2008). However, the role of BAK1 in the innate immune response seems to be independent of the function of BAK1 in brassinosteroid signaling (Kemmerling et

al., 2007). Hence, a connection between brassinosteroid signaling and the SA-JA-ET network remains to be established.

## Decoy strategies of the attacker

Interplay between hormonal signaling pathways may provide the plant with a powerful regulatory potential, it is also a potential target for plant attackers to manipulate the plant defense signaling network for their own benefit (Pieterse and Dicke, 2007; Robert-Seilaniantz et al., 2007). A classic example of a pathogen that ingeniously makes use of the plant's hormonal signaling network is the Crown Gall disease-causing pathogen *Agrobacterium tumefaciens*, which is often used for the production of genetically modified transgenic plants (Tzfira and Citovsky, 2006). *Agrobacterium* genetically transforms its host plant by transferring a piece of the tumor-inducing (Ti) plasmid into the host's genome. The transferred T-DNA carries genes that encode for the production of auxins and cytokinins, resulting in uncontrolled host cell proliferation from which the pathogen can benefit. That pathogens can produce phytohormone-like compound as part of their colonization strategy has been well documented (Mauch-Mani and Mauch, 2005; Robert-Seilaniantz et al., 2007). For instance, the necrotrophic pathogen *B. cinerea* is known to produce ET (Cristescu et al., 2002), whereas numerous bacteria have been shown to produce auxin, possibly to promote virulence (Spaepen et al., 2007). An interesting example is the name giver of the phytohormone gibberellin, the necrotrophic fungus *Gibberella fujikuroi* (Robert-Seilaniantz et al., 2007). This fungus was demonstrated to produce significant amounts of gibberellin, possibly to disable JA-dependent necrotroph resistance through gibberellin-mediated destabilization of DELLA proteins that play a role in controlling the balance between SA- and JA-dependent defenses (Navarro et al., 2008).

A well-studied example of a pathogen that suppresses the host's innate immune response by manipulating phytohormone homeostasis is the bacterial pathogen *P. syringae* (Nomura et al., 2005). *P. syringae* is able to inject different virulence effector proteins into the host cell via the type III protein secretion system. Several of these virulence factors have been demonstrated to suppress host defense responses (Fig. 7). For instance, the type III effector AvrRpt2 was shown to promote bacterial virulence in a compatible Arabidopsis host by altering the host's auxin physiology, resulting in enhanced disease (Chen et al., 2007). In addition, type III effectors of *P. syringae* were shown to activate the ABA signaling pathway as a major virulence strategy, resulting in the suppression of host immunity and enhanced disease (de Torres-Zabala et al., 2007). As a counter measure, plants activate SA-dependent defenses that are effective against this pathogen and are able to neutralize some of the immune suppressive effects, such as the effector-induced auxin response (Wang et al., 2007). However, the pathogen is in turn able to suppress SA-dependent

defenses. For instance through the action of the virulence factor HopI1, which is able to suppress SA accumulation in the chloroplast (Jelenska et al., 2007). In addition, *P. syringae* produces the phytotoxin coronatine, which functions as a JA mimic and suppresses SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Brooks et al., 2005; Uppalapati et al., 2007). Interestingly, the degree of interplay between the SA- and JA-dependent defenses differs among Arabidopsis accessions (Traw et al., 2003), which suggests intraspecific variability in how these defense pathways cross-communicate. In an ecological context, this natural variation in pathway crosstalk might be instrumental in avoiding the decoy strategies that are employed by the plant's enemies.

## Outline of the thesis

To elucidate molecular mechanisms involved in plant immunity, Arabidopsis has been demonstrated to be an excellent model species. In many Arabidopsis-pathogen interactions, the roles of small-molecule hormones in the regulation of plant immunity have been demonstrated and the underlying mechanisms uncovered. Since plant defense mechanisms have evolved during the co-evolutionary arms race between plants and their attackers and come with costs in addition to benefits, insights into their significance for plant fitness should ideally come from ecological studies. On the other hand, there is a clear need to understand how complex high-dimensional signal interactions are translated into a definite coordinated defense response that is tailored to the type of pathogen that the plant is encountering. The main goal of the research described in this thesis was to unravel the molecular mechanism underlying cross-communication between the defense-related signaling pathways that are regulated by the phytohormones SA, JA and ET.

In Chapter 2, we investigated the kinetics of the interplay between the SA and JA pathways. We used the JA-inducing necrotrophs *A. brassicicola* and *B. cinerea*, the SA-inducing biotroph *H. arabidopsidis,* and the JA-inducing insect herbivores *F. occidentalis* and *Pieris rapae*, to show that SA-mediated suppression of JA-responsive genes can be triggered biologically as well as chemically. Furthermore, the robustness of the SA-JA cross-talk phenomenon was shown by its conservation among 18 different Arabidopsis accessions. The longevity and sensitivity of SA-JA cross-talk was investigated by time course analyses, as well as dose-response assays. JA-responsive gene expression was readily suppressed by SA for several days, even when triggered by very low doses of SA. Time interval studies revealed that SA has a window of opportunity to suppress MeJA-responsive gene expression, and that this time interval correlates with the SA-induced redox change in the plant tissue. Thus, redox modulation is likely to play a central role in the regulatory mechanism underlying SA-JA cross-talk.

In chapter 3, we determined the role of ET in SA-JA cross-talk. In Arabidopsis, NPR1 was demonstrated to be required for SA-mediated suppression of JA-dependent defenses. Since ET is known to enhance SA/NPR1-dependent defense responses, we investigated the role of ET in the SA-JA signal interaction. Pharmacological experiments showed that ET potentiated SA/NPR1-dependent *PR-1* transcription, while it rendered the antagonistic effect of SA on MeJA-induced *PDF1.2* and *VSP2* expression NPR1 independent. This overriding effect of ET on NPR1 function in SA-JA cross-talk was absent in the *npr1-1/ein2-1* double mutant, demonstrating that it is mediated via ET signaling. Abiotic and biotic induction of the ET response similarly abolished the NPR1 dependency of the SA-JA signal interaction. Hence, we concluded in this chapter that the interaction between ET and NPR1 plays an important modulating role in the fine tuning of the defense signaling network that is activated upon pathogen and insect attack.

In Chapter 4, we aimed to identify novel signaling components involved in the regulation of SA-JA cross-talk. We tested 45 well-characterized hormone- and defense-related Arabidopsis mutants for their ability to display SA-JA cross-talk. Mutant *cev1*, which constitutively expresses JA and ET responses, was identified as a novel SA-JA cross-talk mutant. Pharmacological assays, mutant analysis, and studies with the ET-signaling inhibitor 1-methylcyclopropene (1-MCP) revealed that ET-mediated potentiation of the JA response plays an important role in neutralizing the antagonistic effect of SA on JA signaling. The AP2/ERF transcription factor ORA59 was identified as an important regulator of this process. From this study we concluded that strong induction of the JA and ET pathways, such as triggered after infection by necrotrophic pathogens, renders the plant insensitive for future SA-mediated suppression of JA-dependent defenses, which may prioritize the JA/ET-pathway over the SA-pathway during multi-attacker interactions.

In chapter 5, we investigated the hypothesis that SA may suppress JA signaling by down regulating the JA biosynthesis pathway. Pharmacological experiments showed that SA strongly antagonizes JA-responsive expression of the JA biosynthesis genes *LOX2, AOS*, *AOC3,* and *OPR3*. However, pharmacological experiments with JA biosynthesis mutants revealed that induction of the JA pathway by exogenous application of MeJA can be suppressed to wild-type levels. We therefore concluded that SA targets the JA signaling pathway at a position downstream of the JA biosynthesis pathway.

In chapter 6, we systematically searched for SA targets in the JA signaling pathway down stream of JA biosynthesis. In this study, we demonstrated that SA does not affect the stability of the of JA ZIM-domain proteins (JAZs), which act as JA signaling repressors. We also provide evidence that the $SCF^{COI1}$ complex, which targets JAZ proteins for degradation in the proteosome, is not affected by SA. Activation of JA-responsive gene expression in the JA-insensitive mutant *coi1-1* through ectopic expression of the AP2/ERF transcription factor ERF1, revealed

that SA-mediated suppression of JA signaling acts down stream of the $SCF^{COI1}$/JAZ complex and that SA is likely to exert its antagonistic action at the transcriptional level. *In silico* promoter analysis of JA-responsive genes that are suppressed by SA in a whole-genome expression profiling study, showed that the 1-kb promoter regions of the SA-suppressed, MeJA-inducible genes are significantly enriched for the GCC-box motif (A)GCCGCC. Transgenic lines expressing the *GUS* reporter gene under control of four copies of the GCC-box were still responsive to SA-mediated suppression of MeJA-induced activation of the promoter. Hence, we conclude that SA exerts its antagonistic action down stream of the $SCF^{COI1}$/JAZ complex and targets GCC-box containing promoters to suppress JA-responsive gene expression.

In Chapter 7, the results presented in this thesis are discussed in view of the current status of research in plant defense signaling.

CHAPTER 2

# Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation

**Annemart Koornneef, Antonio Leon-Reyes, Tita Ritsema, Adriaan Verhage, Floor C. Den Otter, L.C. Van Loon and Corné M.J. Pieterse**

Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, the Netherlands

**Plant Physiology** 147: 1358-1368 (2008)

**Abstract**

Cross-talk between salicylic acid (SA) and jasmonic acid (JA) signaling pathways plays an important role in the regulation and fine-tuning of induced defenses that are activated upon pathogen or insect attack. Pharmacological experiments revealed that transcription of JA-responsive marker genes, such as *PDF1.2* and *VSP2*, is highly sensitive to suppression by SA. This antagonistic effect of SA on JA signaling was also observed when the JA pathway was biologically activated by necrotrophic pathogens or insect herbivores, and when the SA pathway was triggered by a biotrophic pathogen. Furthermore, all 18 *Arabidopsis thaliana* accessions tested displayed SA-mediated suppression of JA-responsive gene expression, highlighting the potential significance of this phenomenon in induced plant defenses in nature. During plant-attacker interactions, the kinetics of SA and JA signaling are highly dynamic. Mimicking this dynamic response by applying SA and methyl jasmonate (MeJA) at different concentrations and time intervals revealed that *PDF1.2* transcription is readily suppressed when the SA response was activated at or after the onset of the JA response, and that this SA-JA antagonism is long-lasting. However, when SA was applied more than 30 h prior to the onset of the JA response, the suppressive effect of SA was completely absent. The window of opportunity of SA to suppress MeJA-induced *PDF1.2* transcription coincided with a transient increase in glutathione levels. The glutathione biosynthesis inhibitor L-buthionine-sulfoximine (BSO) strongly reduced *PDF1.2* suppression by SA, suggesting that SA-mediated redox modulation plays an important role in the SA-mediated attenuation of the JA signaling pathway.

## Introduction

In nature, plants interact with a wide range of microbial pathogens and herbivorous insects. During the evolutionary arms race between plants and their attackers, primary and secondary immune responses evolved to recognize common or highly specialized features of microbial pathogens (Chisholm et al., 2006; Jones and Dangl, 2006), resulting in sophisticated mechanisms of defense. Although the arms race between plants and herbivorous insects has been intensively debated (Musser et al., 2002; Schoonhoven et al., 2005), knowledge of the underlying mechanisms is relatively limited. In the past years, various genomics approaches exponentially expanded our understanding of the molecular mechanisms by which plants tailor their defense response to pathogen and insect attack (Glazebrook et al., 2003; Tao et al., 2003; Eulgem et al., 2004; Reymond et al., 2004; De Vos et al., 2005; Kempema et al., 2007). The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) emerged as key players in the regulation of the signaling networks involved

(Howe, 2004; Pozo et al., 2004; Grant and Lamb, 2006b; Van Loon et al., 2006a; Von Dahl and Baldwin, 2007). Other plant hormones, such as abscisic acid (Mauch-Mani and Mauch, 2005), brassinosteroids (Nakashita et al., 2003), and auxins (Navarro et al., 2006; Wang et al., 2007), have been reported to play a role in the plant immune response as well, but their significance is less well understood. SA-, JA-, and ET-dependent pathways regulate defense responses that are differentially effective against specific types of attackers. Pathogens with a biotrophic lifestyle are generally more sensitive to SA-dependent responses, whereas necrotrophic pathogens and herbivorous insects are commonly deterred by JA/ET-dependent defenses (Thomma et al., 2001a; Kessler and Baldwin, 2002; Glazebrook, 2005).

There is ample evidence that SA and JA signaling pathways are mutually antagonistic (Pieterse et al., 2001; Kunkel and Brooks, 2002; Glazebrook et al., 2003; Rojo et al., 2003; Bostock, 2005; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008). This pathway cross-talk is thought to provide the plant with a powerful regulatory potential that helps deciding which defensive strategy to follow, depending on the type of attacker encountered (Reymond and Farmer, 1998). Yet, it appears that attackers have also evolved ways to manipulate plants for their own benefit by suppressing induced defenses via modulation of the plant signaling network. A nice example is the response of Arabidopsis to silverleaf whitefly (*Bemisia tabaci*) nymphs. The nymphs of this phloem-feeding insect may sabotage effectual JA-dependent host defenses by activating the antagonistic SA signaling pathway (Zarate et al., 2007). Pathogens suppress host defenses as well, by using virulence factors that antagonize the plant immune response (Nomura et al., 2005). One of these virulence factors is the *Pseudomonas syringae* phytotoxin coronatine, which functions as a jasmonate analog. During the interaction with susceptible Arabidopsis plants, coronatine suppresses SA-dependent defenses, thereby promoting susceptibility to this pathogen (Zhao et al., 2003; Brooks et al., 2005; Cui et al., 2005; Laurie-Berry et al., 2006).

Several key regulatory proteins involved in SA-JA cross-talk have been identified in Arabidopsis. For instance, the transcription factor WRKY70 was shown to act as an activator of SA-responsive genes and a repressor of JA-inducible genes, thereby functioning as a molecular switch between both pathways (Li et al., 2004). Previously, we demonstrated that the defense regulatory protein NPR1 is required for SA-JA cross-talk (Spoel et al., 2003). Induction of the SA response, either by pathogen infection or by exogenous application of SA, strongly suppressed JA-responsive genes, such as *PDF1.2, LOX2,* and *VSP2*. However, in mutant *npr1-1* plants, this SA-mediated suppression of JA-responsive gene expression was completely abolished. Nuclear localization of NPR1, which is essential for SA-mediated defense gene expression (Kinkema et al., 2000), was not required for the suppression of JA-responsive genes, indicating that the antagonistic effect of SA on JA signaling is modulated through a function of NPR1 in the cytosol (Spoel et al.,

2003). Recently, overexpression of the SA-regulated glutaredoxin GRX480 was found to antagonize JA-responsive *PDF1.2* transcription (Ndamukong et al., 2007), suggesting a role for redox regulation in SA-mediated suppression of JA-responsive gene expression.

While genetic approaches are ideal for identifying key players of pathway cross-talk, they do not provide full insight into the actual functioning of this regulatory mechanism in response to pathogen and insect attack. Previously, we monitored changes in the signal signature and transcriptome of Arabidopsis upon attack by various microbial pathogens and herbivorous insects (De Vos et al., 2005). Clearly, timing, magnitude, and composition of the blend of signals produced play a primary role in orchestrating the induced defense response (De Vos et al., 2005). However, additional layers of regulation, such as pathway cross-talk, are needed to fine-tune the final outcome of the resistance reaction (Thaler et al., 2002a; De Vos et al., 2006; Mur et al., 2006; Pieterse and Dicke, 2007). Here, we demonstrate that biological or chemical induction of the SA response strongly suppresses the expression of the JA-responsive genes *PDF1.2* and *VSP2,* such as triggered upon treatment with methyl jasmonate (MeJA) or attack by the JA-inducing necrotrophs *Alternaria brassicicola* and *Botrytis cinerea,* or the insect herbivores *Frankliniella occidentalis* and *Pieris rapae.* Using a pharmacological approach to dissect the kinetics and mechanisms underlying SA-JA cross-talk, we demonstrate that the SA-mediated antagonistic effect on JA-responsive gene expression is conserved among Arabidopsis accessions and that the kinetics of SA and JA signaling play an important role in the outcome of the SA-JA interaction. Furthermore, we provide evidence that the antagonistic effect of SA on JA-responsive gene transcription is linked to SA-induced changes in glutathione levels, suggesting that the antagonistic effect of SA on JA signaling is modulated by redox changes.

## Results

### SA suppresses JA responses triggered by necrotrophic pathogens and herbivorous insects

In Arabidopsis, pharmacological experiments revealed that SA can antagonize the expression of JA-responsive genes, such as *PDF1.2* and *VSP2* (Spoel et al., 2003). To investigate the potential significance of this signal interaction in the defense response of plants to multiple attackers, we tested the effect of SA on the JA response as triggered by necrotrophic pathogens and herbivorous insects. To this end, the JA response was biologically activated by inoculating wild-type Col-0 plants with the necrotrophic fungi *Alternaria brassicicola* or *Botrytis cinerea*, and by infesting Col-0 plants with cell-content-feeding Western flower thrips (*Frankliniella occidentalis*) or tissue-chewing caterpillars of the small cabbage white (*Pieris rapae*) (Thomma et

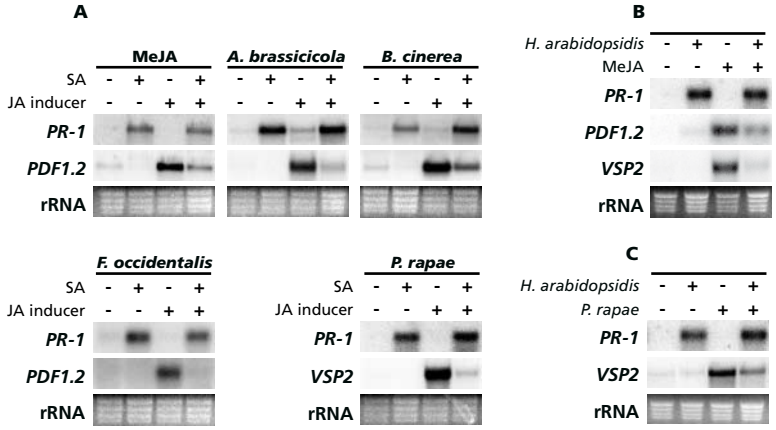



**Figure 1. Biological induction of SA and JA signaling pathways results in SA-JA cross-talk.**
**A, Exogenous application of 1 mM SA suppresses the expression of the JA-responsive marker genes *PDF1.2.* and *VSP2,* triggered by MeJA, the necrotrophic pathogens *A. brassicicola* and *B. cinerea,* and the insect herbivores *F. occidentalis* and *P. rapae.***
**B, Infection with the SA-inducing biotrophic pathogen *H. arabidopsidis* antagonizes MeJA-induced expression of *PDF1.2* and *VSP2*.**
**C, *H. arabidopsidis* suppresses *P. rapae*-induced expression of the JA-responsive gene *VSP2*.**
**For northern blot analysis, leaf tissue was harvested 24 h after the second treatment. Ribosomal RNA (rRNA) was used to check for equal loading of RNA samples.**

al., 1998; De Vos et al., 2005). After one day, non-induced and induced plants were treated with 1 mM SA and leaves were harvested 24 h later to analyze the expression levels of the SA-responsive marker gene *PR-1* and the JA-responsive marker gene *PDF1.2*. Because *P. rapae* specifically suppresses the expression of *PDF1.2* (De Vos, 2006), we used *VSP2* as a JA-responsive marker in the Arabidopsis-*P. rapae* interaction. Fig. 1A shows that the necrotrophic pathogens and the herbivorous insects activated the JA-responsive marker genes to similar levels as did the chemical agent MeJA. In combination with SA, the JA-responsive genes were consistently suppressed, indicating that exogenously applied SA is able to antagonize the JA response as induced by a broad range of attackers.

To investigate whether biological activation of the SA pathway would similarly antagonize JA signaling, Col-0 plants were inoculated with the SA-inducing biotrophic pathogen *Hyaloperonospora arabidopsidis*. At 3 days after inoculation, *PR-1* transcripts continuously accumulated to high levels (Fig. 1B), confirming that the SA signaling pathway was activated. Subsequently, *H. arabidopsidis*-inoculated plants were treated with 0.1 mM MeJA. The transcription of MeJA-induced *PDF1.2* and *VSP2* genes was found to be suppressed in *H. arabidopsidis*-inoculated plants, compared to non-inoculated plants (Fig. 1B). When *P. rapae* larvae were allowed to feed on *H. arabidopsidis*-infected Col-0 plants, the expression of *VSP2* was strongly reduced in comparison to caterpillar-infested plants that were not inoculated with the pathogen (Fig. 1C). Together, these results indicate that pathogen-induced SA

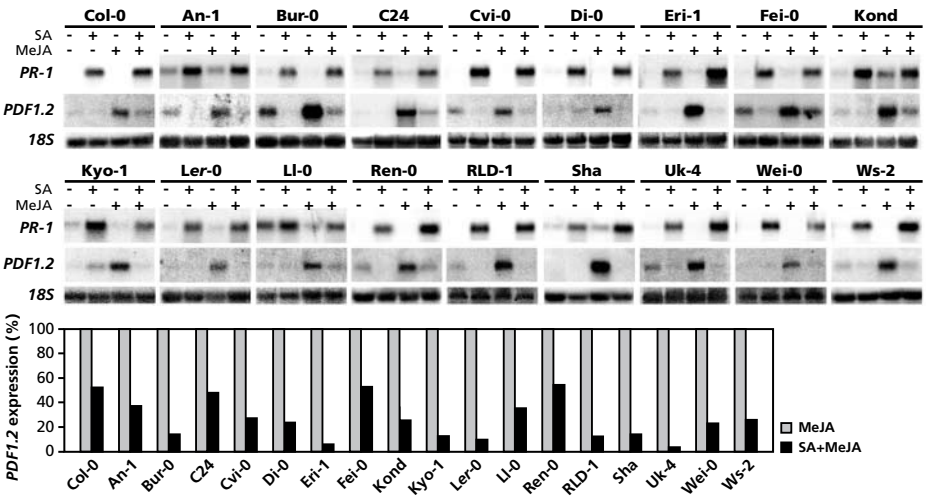



**Figure 2. SA-JA cross-talk is conserved among Arabidopsis accessions.**
**Northern blot analysis of *PR-1* and *PDF1.2* gene expression in 18 Arabidopsis accessions after treatment with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 24 h after chemical treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager. *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

negatively affects JA signaling and that during multitrophic interactions, the SA pathway can be prioritized over the JA pathway.

### SA-JA signal interaction is conserved among Arabidopsis accessions

Naturally occurring variation in Arabidopsis accessions can be exploited to study the biological relevance and genetics of specific plant traits, such as resistance to pathogens and pests (Koornneef et al., 2004). To investigate whether Arabidopsis displays natural variation for SA-JA signal interaction, we analyzed the antagonistic effect of SA on MeJA-induced *PDF1.2* transcription in 18 Arabidopsis accessions collected from very different geographical origins. All accessions were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. One day later, the expression of SA-responsive *PR-1* and JA-responsive *PDF1.2* was assessed (Fig. 2). The single treatments with SA or MeJA clearly activated their corresponding marker genes *PR-1* and *PDF1.2*, although the basal *PR-1* and *PDF1.2* transcript levels varied among the accessions. In the SA/MeJA combination treatments, SA-induced *PR-1* expression was not affected by MeJA in the majority of the accessions. Conversely, all accessions displayed a strong SA-mediated downregulation of both MeJA-induced and basal levels of *PDF1.2* transcription. Hence, although several studies have reported on a differential responsiveness of Arabidopsis accessions to the plant hormones SA and (Me)JA (Rao et al., 2000; Kliebenstein et al., 2002; Van

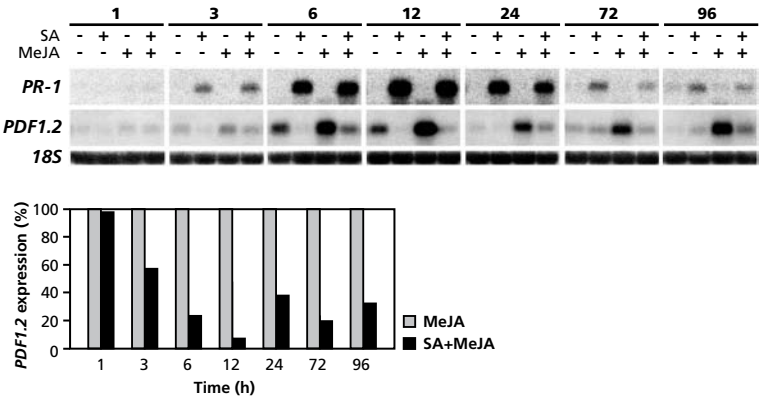



**Figure 3.** **Very low doses of SA antagonize *PDF1.2* transcription.**
**Northern blot analysis of *PR-1* and *PDF1.2* gene expression in Col-0 plants treated with 1000, 100, 10, 1, or 0.1 μM SA, with or without 0.1 mM MeJA. Leaf tissue was harvested 24 h after chemical treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager. *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

Leeuwen et al., 2007), our study demonstrates that the SA-mediated antagonism on JA-responsive gene expression is conserved among Arabidopsis accessions.

### *PDF1.2* transcription is antagonized by low doses of SA

To investigate the dosage effect of SA on SA-JA cross-talk, SA was applied to Col-0 plants as a foliar drench in concentrations ranging from 1000 to 0.1 μM, either alone or in combination with 0.1 mM MeJA. After 1 day, leaf tissue was harvested and *PR-1* and *PDF1.2* expression was assessed. SA concentrations below 100 μM had no effect on *PR-1* transcription, but still antagonized MeJA-induced expression of *PDF1.2* (Fig. 3). In fact, MeJA-induced *PDF1.2* transcription was suppressed by concentrations of SA as low as 0.1 μM, although the effect was less pronounced than the suppression observed by 1000 μM SA. A higher dose of MeJA (1 mM) could not overrule the suppressive effect of SA on *PDF1.2* expression (data not shown). These results highlight the robustness and sensitivity of the antagonistic effect of SA on JA-responsive genes, such as *PDF1.2*.

### SA triggers a fast and long-lasting antagonistic effect on MeJA-induced *PDF1.2*

In response to pathogen or insect attack, Arabidopsis reacts by producing an attacker-specific signal signature (De Vos et al., 2005). The kinetics of the defense signal production play an important role in shaping the final outcome of the induced defense response (Reymond and Farmer, 1998). To investigate the effectiveness of SA-JA signal interaction in view of the dynamic changes in defense signal production, we monitored the time frame during which SA is able to effectively suppress

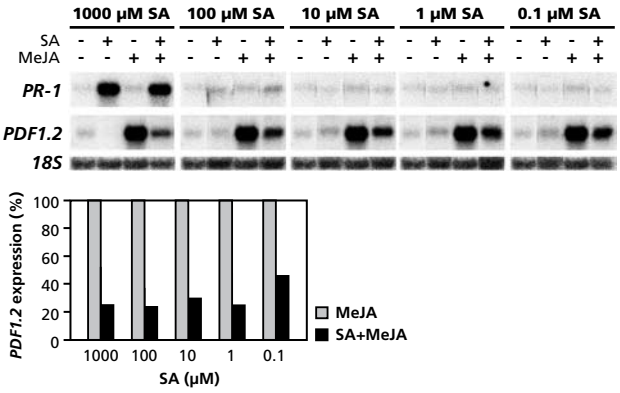



**Figure 4.** **SA exerts a fast and long-lasting antagonistic effect on *PDF1.2* transcription.**
**Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in Col-0 plants treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 1, 3, 6, 12, 24, 72, and 96 h after chemical treatment. To check for equal RNA loading a probe for *18S* rRNA was used. Signal intensities were quantified using a Phosphor imager. *PDF1.2* levels in the single MeJA treatments were set to 100%.**

*PDF1.2* transcription. Col-0 plants were treated with SA, MeJA, or a combination of both chemicals and the expression of *PR-1* and *PDF1.2* was assessed at several time points after induction. Fig. 4 shows that in the single treatments *PR-1* and *PDF1.2* transcripts were detectable 3 h after chemical application. The basal level of *PDF1.2* expression was relatively high at 6 and 12 h after treatment, which could be due to sampling at later time points during the day. In the combination treatment, again no effect of MeJA on SA-induced *PR-1* was observed. However, SA readily antagonized MeJA-induced transcription of *PDF1.2*. The suppression of *PDF1.2* by SA was clearly visible up to 4 days after chemical treatment, even though by that time SA-induced *PR-1* expression had decreased to almost undetectable levels. It can thus be concluded that the antagonistic effect of SA on JA-responsive gene expression is induced rapidly, and lasts up to several days after induction of the SA signal.

### Longevity of SA-JA signal interaction

To investigate the longevity of the SA-mediated antagonistic effect on MeJA-induced *PDF1.2* transcription, SA and MeJA were either applied simultaneously or with an interval of 3 days. Subsequently, leaf tissue was harvested 1 day after application of the last chemical for northern blot analysis of *PR-1* and *PDF1.2* expression. Simultaneous treatment with SA and MeJA resulted in a typical suppression of MeJA-induced *PDF1.2* expression by SA (Fig. 5A, left panel). When SA was applied 3 days after MeJA, a similar SA-mediated suppression of *PDF1.2* was evident (Fig. 5A, middle panel). Note that in the middle panel in Fig. 5A MeJA-induced transcript levels of *PDF1.2* are lower than in the other two panels, because RNA was isolated 4 days instead of 1 day after the MeJA treatment. However,

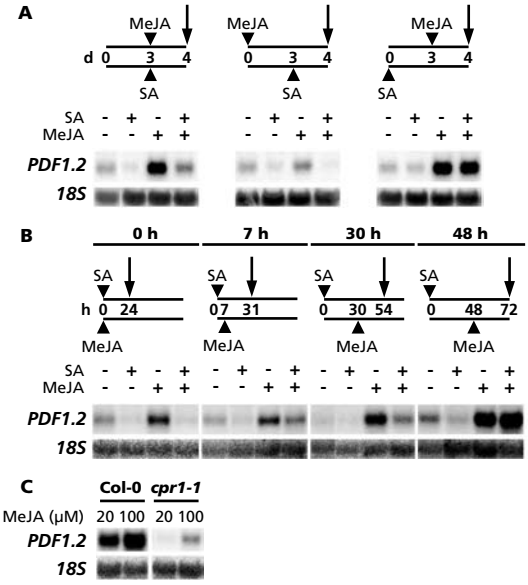



**Figure 5.** Longevity of SA-JA cross-talk.
Northern blot analysis of *PDF1.2* gene expression in Col-0 plants treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. In the combination treatments, SA and MeJA were applied in different orders and with different time intervals. Leaf tissue was harvested (indicated by an arrow) 24 h after application of the last chemical. To check for equal loading, a probe for *18S* rRNA was used.
A, Effect of SA on MeJA-induced *PDF1.2* transcription when SA was applied simultaneously with (left panel), 3 days after (middle panel), or 3 days before MeJA (right panel).
B, Effect of SA on MeJA-induced *PDF1.2* transcription when SA was supplied 0, 7, 30, or 48 h before MeJA.
C, Effect of constitutive expression of the SA response on *PDF1.2* transcription. *PDF1.2* mRNA levels were determined in Col-0 and *cpr1-1* plants 24 h after treatment with 20 or 100 μM MeJA.

when SA was applied 3 days prior to the MeJA treatment, the antagonistic effect on *PDF1.2* expression could no longer be observed (Fig. 5A, right panel). These results indicate that SA is capable of suppressing JA-responsive gene expression when it is produced simultaneously with, or after the onset of the JA response. However, when SA is applied prior to activation of the JA pathway, the antagonistic effect of SA on JA signaling is only effective within a certain time frame after induction of the SA signal.

To investigate the window of opportunity of SA to suppress MeJA-induced expression of *PDF1.2*, we applied SA at several time points before MeJA. In all cases, Col-0 leaf tissue was harvested 1 day after the MeJA treatment for northern blot analysis of *PDF1.2* gene expression. The antagonistic effect of SA on MeJA-induced *PDF1.2* expression was evident when SA was applied simultaneously with MeJA, or up to 30 h before the MeJA treatment (Fig. 5B). However, when the time interval between the SA and MeJA treatments was extended to 48 h, the SA-mediated suppression of MeJA-induced *PDF1.2* was no longer observed. It can thus be concluded that the antagonistic effect of SA on JA signaling is transient

and that the suppressive effect is lost between 30 and 48 h after induction of the SA signal.

If the antagonistic effect of SA on JA signaling is only apparent during a certain time frame after induction of the SA signal, then constant activation of the SA-dependent signaling pathway should result in continuous downregulation of JA-responsive genes such as *PDF1.2*. We tested this hypothesis by comparing *PDF1.2* expression in wild-type Col-0 and mutant *cpr1-1* plants after application of 20 and 100 μM MeJA. The *cpr1-1* mutant has elevated endogenous levels of SA and shows constitutive *PR-1* expression (Bowling et al., 1994). Fig. 5C shows that *PDF1.2* expression was induced by both concentrations of MeJA in wild-type Col-0. However, in mutant *cpr1-1*, the effect of the MeJA treatment on the level of *PDF1.2* expression was strongly reduced. These results indicate that a continuous activation of the SA response is associated with a constitutive suppression of JA-responsive gene expression.

### SA-mediated suppression of JA signaling coincides with a cellular increase in glutathione levels

Changes in the cellular redox state play a major role in SA signal transduction (Després et al., 2003; Mou et al., 2003). SA-mediated redox changes activate the regulatory protein NPR1 by monomerization of inactive NPR1 oligomers, which results in the induction of SA-responsive genes such as *PR-1* (Mou et al., 2003; Dong, 2004). SA-activated NPR1 is also essential in mediating the antagonism between SA- and JA-dependent signaling (Spoel et al., 2003). Therefore, we hypothesized that the transient nature of the antagonistic effect of SA on JA signaling might be associated with changes in the cellular redox state. As a marker of the redox potential, we monitored the level of glutathione in Arabidopsis leaves upon application of SA (Fig. 6A and 6B). Glutathione is a low-molecular weight antioxidant that functions as a major determinant of cellular redox homeostasis (Noctor and Foyer, 1998; Hellens et al., 2000; Schafer and Buettner, 2001). Both the concentration of the total glutathione pool and the ratio between reduced (GSH) and oxidized (GSSG) glutathione can influence the redox potential of the cell (Schafer and Buettner, 2001). Basal glutathione levels fluctuated between 158 and 280 nmol $g^{-1}$ fresh weight during the course of the experiment, which is in accordance to previously published data (Karpinski et al., 1997; Mou et al., 2003). In addition, glutathione levels were influenced diurnally, showing a general increase during daylight conditions, followed by a decrease during night-time (Fig. 6A), as described previously (Bielawski and Joy, 1986; Koike and Patterson, 1988; Schupp and Rennenberg, 1988; Noctor et al., 1997). Pathogen attack and application of SA or one of its functional analogs have been shown to trigger an increase in total glutathione content (Fodor et al., 1997; Vanacker et al., 2001; Mou et al., 2003; Mateo et al., 2006). Similarly, SA treatment resulted in a transient increase in the

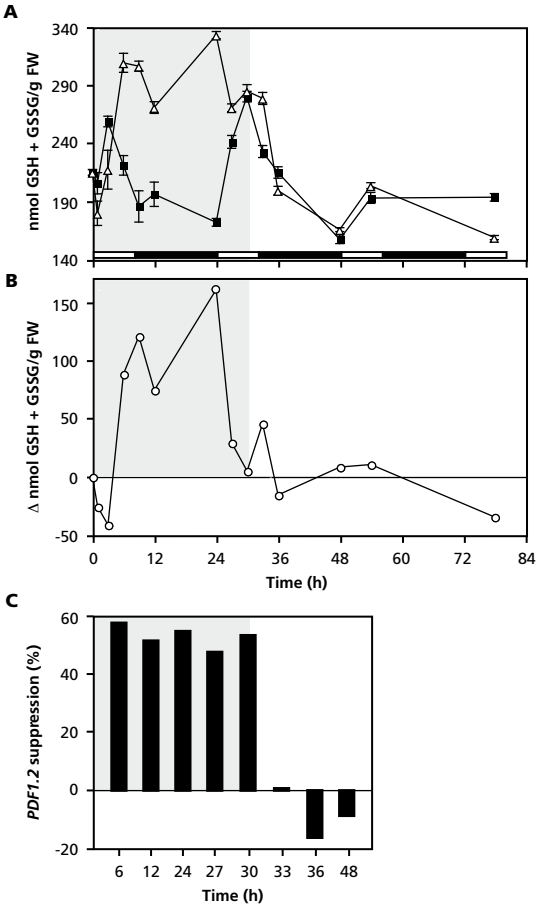



**Figure 6.** Suppression of *PDF1.2* by SA coincides with increased glutathione levels.
A, Total glutathione levels (GSH+GSSG) in wild-type Col-0 plants, harvested 0 to 78 h after foliar drench with 1 mM SA (open triangles) or control solution (closed squares). Error bars represent SE *(n* = 5*)*.
B, Glutathione levels after subtraction of control values from SA values. Inset, Increase in glutathione levels upon treatment with 1 mM (open circles), 10 μM (closed circles), or 0.1 μM (closed triangles) SA after subtraction of control values.
C, Percentage of *PDF1.2* suppression after SA/MeJA treatment, compared to MeJA treatment alone. Signal intensities were quantified using a Phosphor imager. Wild-type Col-0 plants were treated with control or 1 mM SA solutions at t=0 h. Subsequently, 0.1 mM MeJA was applied at t=6, 12, 24, 27, 30, 33, 36, and 48 h. Leaf tissue was harvested 24 h after application of MeJA.
The shaded area represents the 30-h window of opportunity of SA to suppress *PDF1.2* expression. The white horizontal bar indicates the light period, and the black horizontal bar indicates the dark period.

level of glutathione that returned to baseline levels after 30 h (Fig. 6A and 6B). A combined treatment with SA and MeJA did not alter this pattern (data not shown). The SA-induced increase in glutathione levels was also observed when lower levels of SA (10 μM and 0.1 μM) were applied, albeit less pronounced in response to the lowest concentration of 0.1 μM SA (Fig. 6B, inset). Interestingly, the change in glutathione levels coincided with the window of opportunity in which SA was able

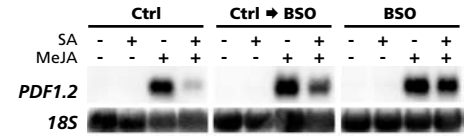



**Figure 7. The glutathione biosynthesis inhibitor BSO affects SA-JA cross-talk.**
**Northern blot analysis of *PDF1.2* expression in 14-day-old Col-0 seedlings grown on MS medium with or without 2.5 mM BSO, 0.5 mM SA, 20 μM MeJA, or a combination of these chemicals. SA and MeJA treatments were performed by transferring 12-day-old seedlings to MS medium with the chemicals indicated. Two days later, seedlings were harvested for northern blot analysis. BSO was included in the medium either at day 12 (Ctrl➔BSO), or during the whole growth period (BSO). Equal loading of RNA samples was checked using a probe for *18S* rRNA.**

to suppress MeJA-induced *PDF1.2* transcription (Fig. 6C). Hence, we postulate that the SA-mediated antagonism on JA signaling pathways is redox modulated.

### Inhibition of glutathione biosynthesis suppresses the antagonistic effect of SA on JA signaling

In order to demonstrate a causal relationship between changes in glutathione levels and the down-regulation of JA-responsive gene expression by SA, we manipulated the glutathione content of the cell and monitored the effect on *PDF1.2* suppression. To deplete glutathione levels, we grew Arabidopsis seedlings on Murashige and Skoog (1962) (MS) medium, supplemented with a non-toxic and highly specific inhibitor of the first enzyme of GSH synthesis, L-buthionine-sulfoximine (BSO) (Griffith and Meister, 1979; May and Leaver, 1993). Inclusion of BSO in the growth medium resulted in a strong reduction in SA-induced glutathione levels (data not shown). To assess the effect of BSO on the ability of SA to suppress JA signaling, BSO was included in the medium either during the whole growth period (2 weeks) or only during the last 48 h prior to harvest. Twelve-day-old seedlings grown on MS or MS supplemented with 2.5 mM BSO were transferred to MS medium supplemented with 2.5 mM BSO and either 0.5 mM SA, 20 μM MeJA, or a combination of both chemicals. Leaf tissue was harvested 48 h after chemical induction and assessed for *PDF1.2* marker gene expression. Fig. 7 shows normal levels of SA-JA signal interaction when the seedlings were grown on MS medium without BSO (Ctrl). However, inclusion of BSO in the growth medium for 2 days clearly reduced the antagonistic effect of SA on MeJA-induced *PDF1.2* expression (Ctrl➔BSO). This effect was even more pronounced when BSO was present in the medium during the whole growth period (BSO). Hence, the glutathione biosynthesis inhibitor BSO affects SA-induced suppression of JA signaling, strengthening our hypothesis that this type of SA-JA signal interaction is redox modulated.

## Discussion

### Kinetics of SA-JA signal interaction demonstrate a conserved and robust mechanism

Cross-talk between defense signaling pathways is thought to play an important role in the regulation of induced defenses in plants. The antagonism between SA and JA signaling emerged as one of the most prominent of all signal interactions studied to date (Dong, 2004; Pieterse and Van Loon, 2004; Bostock, 2005; Nomura et al., 2005; Koornneef and Pieterse, 2008). However, the underlying molecular mechanisms of SA-JA cross-talk are to a large extent unknown. In this paper, we demonstrate that biological or chemical induction of the SA pathway strongly antagonizes the expression of the JA-responsive marker genes *PDF1.2* and *VSP2* as triggered by necrotrophic pathogens or insect herbivores. Moreover, we show that all 18 Arabidopsis accessions tested display SA-mediated attenuation of JA-responsive gene expression, suggesting that this trait is conserved among Arabidopsis ecotypes. Furthermore, we provide insight into how the outcome of the SA-JA signal interaction is influenced by the kinetics of the individual signaling cascades. Activation of the SA pathway resulted in an antagonistic effect on the expression of JA-responsive genes. However, when SA was applied prior to the JA trigger, SA had only a limited time frame to exert its antagonistic effect on the JA pathway. This window of opportunity of SA to downregulate JA-responsive gene expression coincided with a transient SA-induced change in the level of the antioxidant glutathione. Moreover, inhibition of glutathione biosynthesis by BSO strongly affected SA-mediated suppression of MeJA-induced *PDF1.2* expression, suggesting a role for redox modulation in this process.

### Antagonism between SA- and JA-dependent signaling pathways

In this study, we predominantly observed an antagonistic effect of SA on JA-responsive gene expression, while MeJA had virtually no effect on the SA-responsive marker gene *PR-1* (Fig. 1 to 4). Early studies in tomato already revealed that SA and its acetylated form, aspirin, are potent suppressors of the JA-dependent wound response (Doherty et al., 1988; Peña-Cortés et al., 1993; Doares et al., 1995). Thus, activation of the SA pathway, such as upon infection by a biotrophic pathogen, might result in suppression of JA-dependent defenses that are triggered by necrotrophic pathogens and insect herbivores. Indeed, we observed that inoculation with the biotrophic pathogen *H. arabidopsidis* activated the SA pathway, resulting in downregulation of herbivore-induced expression of the JA-responsive gene *VSP2* (Fig. 1C), indicating that during multitrophic interactions, the SA pathway can be prioritized over the JA pathway, potentially resulting in attenuation of resistance against necrotrophs and insect herbivores. Trade-offs between SA-dependent pathogen resistance and JA-dependent defense against insect herbivory have been repeatedly reported (Thaler et

al., 1999; Felton and Korth, 2000; Pieterse et al., 2001; Bostock, 2005). In Arabidopsis, the SA pathway has been shown to inhibit JA-dependent resistance against tissue-chewing herbivores, such as *Spodoptera exigua* (beet armyworm) (Cipollini et al., 2004; Bodenhausen and Reymond, 2007) and *Trichoplusia ni* (cabbage looper) (Cui et al., 2002; Cui et al., 2005), and necrotrophic pathogens, such as *A. brassicicola* (Li et al., 2006; Spoel et al., 2007). Intriguingly, some herbivores have been demonstrated to induce the SA pathway to actively suppress effectual JA-dependent defenses and thereby escape host defense (Zarate et al., 2007). Hence, depending on the plant-attacker combination, the antagonistic effect of SA on JA-dependent defense responses may either be beneficial or deleterious.

While in our study SA-mediated inhibition of JA signaling seems to dominate over the reciprocal effect, several studies have demonstrated that JA-mediated suppression of SA signaling plays an important role in specific plant-pathogen interactions as well. A well-studied example is the suppression of SA-dependent host defenses by the jasmonate-mimicking virulence factor coronatine of the bacterial pathogen *P. syringae* (Zhao et al., 2003; Brooks et al., 2005; Cui et al., 2005; Laurie-Berry et al., 2006). Coronatine produced by *P. syringae* in a susceptible host actively inhibits the SA signaling pathway, thereby promoting susceptibility to this pathogen. Whole genome expression profiling of Arabidopsis plants treated with either SA, MeJA, or a combination of both chemicals revealed that a substantial part of the genes that are sensitive to the SA-JA antagonism are JA-responsive genes that are suppressed by SA. Nevertheless, a significant portion of the genes that are antagonistically affected by the combination treatment with SA and MeJA consists of SA-responsive genes that are suppressed by MeJA (A. Koornneef and C.M.J. Pieterse, unpublished results). Thus, while SA and JA signaling pathways can be mutually antagonistic, the differences observed in the outcome of this signaling interaction between studies are likely to be related to the plant-attacker combination and marker genes tested.

### The onset of SA-mediated suppression of JA signaling requires a transient change in glutathione levels

Our studies on the kinetics of SA and JA signaling in relation to the outcome of the SA-JA signal interaction revealed that low doses of SA are able to suppress JA-responsive *PDF1.2* transcription, suggesting that this down-regulation is highly sensitive (Fig. 3). However, the antagonistic effect was only apparent when the SA pathway was activated after the onset of the JA response, or within a time frame of about 30 h prior to the activation of the JA response, indicating that the ability of SA to suppress JA-responsive gene expression is transient (Fig. 5A and 5B). These experiments were carried out with a single application of SA. Thus, when SA is production is triggered upon pathogen attack, the time frame during which SA is effective may be different (Fig. 1B and 1C). Although our results are to a

large extent consistent with previous findings in tomato, tobacco, and Arabidopsis (Thaler et al., 2002a; Mur et al., 2006), Mur et al. (2006) demonstrated that transient synergistic effects between SA and JA signaling may occur during early stages of the SA-JA signal interaction. However, this synergism was observed only when the chemicals were applied at low doses for short durations, which may account for the differences observed. Also, these experiments were performed with Arabidopsis explants, making it difficult to directly compare the outcome of both studies (Mur et al., 2006). So how does SA manipulate JA-dependent defenses? In this paper, we demonstrated that SA-mediated antagonism coincides with a transient increase in the level of glutathione, and that an inhibitor of glutathione synthesis, BSO, reduced the suppressive effect of SA on MeJA-induced *PDF1.2* expression (Fig. 6 and 7). Glutathione is a major cellular antioxidant and an important determinant of the redox state in eukaryotes (Schafer and Buettner, 2001). Previously, Mou et al. (2003) determined both total glutathione levels and the ratio of reduced (GSH) and oxidized (GSSG) glutathione in Arabidopsis upon application of the SA analog 2,6-dichloroisonicotinic acid (INA), and observed comparable changes in kinetics in both glutathione pool size and redox status. In addition, SA-accumulating mutants with constitutive *PR-1* expression were shown to have an increased glutathione pool size (Mateo et al., 2006). Our data indicate that the SA-induced change in glutathione levels plays an important role in initiating the antagonistic effect on JA-responsive gene transcription. This finding demonstrates that redox modulation is not only important in the activation of SA-dependent genes (Mou et al., 2003), but also in the suppression of JA-responsive gene expression. The involvement of redox modulation is supported by the observation that overexpression of the SA-regulated glutaredoxin GRX480 antagonizes JA-responsive transcription of *PDF1.2* (Ndamukong et al., 2007). In addition, EDS1 and PAD4 have been implicated in transduction of redox signals in response to biotic and abiotic stresses (Wiermer et al., 2005), as well as in the regulation of cross-talk as activators and repressors of SA and JA defenses, respectively (Brodersen et al., 2006).

Previously, it was demonstrated that SA-activated NPR1 is required for the suppression of JA-responsive gene expression by SA (Spoel et al., 2003) and that activation of NPR1 is redox regulated (Mou et al., 2003). In uninduced cells, NPR1 is present as an oligomer formed through intermolecular disulfide bonds. SA mediates a change in the cellular redox potential, resulting in the reduction of the NPR1 oligomer to its active monomeric form. Monomeric NPR1 is then translocated into the nucleus where it functions as a coactivator of SA-responsive genes, such as *PR-1* (Dong, 2004). For the suppression of JA-responsive gene expression, translocation of SA-activated NPR1 into the nucleus is not required, as has been demonstrated in both Arabidopsis and rice (Spoel et al., 2003; Yuan et al., 2007), suggesting an important role for cytosolic NPR1 in SA-JA signal interaction. Thus, although the role of NPR1 in SA-JA cross-talk and SA-induced *PR-1* gene

expression seems to be dissimilar, it is plausible that both defense responses are controlled by active NPR1 monomers that are produced upon changes in the redox state. However, additional SA-dependent signaling components are required for the suppression of JA signaling, because Arabidopsis transgenic plants with constitutively monomerized NPR1 did not affect JA-responsive marker gene expression in the absence of SA (Beckers and Spoel, 2006). Uncovering these players in pathway cross-talk will be the focus of future research.

## Materials and methods

### Cultivation of plants

Seeds of *Arabidopsis thaliana* accessions Col-0 (N1092; Poland), An-1 (N944; Belgium), Bur-0 (CS6643; Ireland), C24 (N906; Portugal), Cvi-0 (N8580; Cape Verde Islands), Di-0 (N1106; France), Eri-1 (CS22548; Sweden), Fei-0 (CS22645; Portugal), Kond (CS6175; Tajikistan), Kyo-1 (W10372; Japan), L*er*-0 (NW20; Poland), Ll-0 (N1338; Spain), Ren-0 (CS22535; Netherlands), RLD-1 (N913; Russia), Sha (CS929; Tajikistan), Uk-4 (N1580; Germany), Wei-0 (N3110; Germany), Ws-2 (CS2360; Belarus), and mutants *npr1-1* and *cpr1-1* (Col-0 background) were kindly provided by M. Koornneef (Wageningen University, Wageningen, the Netherlands) and X. Dong (Duke University, Durham, USA). Seeds were sown in quartz sand. Two weeks later seedlings were transferred to 60-mL pots containing a sand-and-potting soil mixture (5:12 v/v) that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with an 8-h-day (200 μE $m^{-2}$ $s^{-1}$ at 24°C) and 16-h-night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland solution (Hoagland and Arnon, 1938) containing 10 μM Sequestreen (CIBA-Geigy, Basel, Switzerland) once a week.

### Chemical induction

Induction treatments were performed by dipping the leaves of 5-week-old plants in an aqueous solution containing 0.015% (v/v) Silwet L-77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands), supplemented with 0.1, 1, 10, 100, or 1000 μM SA (Mallinckrodt Baker, Deventer, the Netherlands), or 20 or 100 μM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), or a combination of both chemicals. Control plants were treated with 0.015% Silwet L-77 only. MeJA was added to the medium from a 1000-fold stock solution in 96% ethanol. Solutions without MeJA were supplemented with equal amounts of ethanol. Plants were harvested between 1 and 96 h after induction treatment and immediately frozen in liquid nitrogen.

### Pathogen and insect bioassays

*Alternaria brassicicola* strain MUCL 20297 and *Botrytis cinerea* strain B0510 were grown on potato dextrose agar (Difco Laboratories, Detroit, USA) plates for 2 weeks at 22°C. Subsequently, conidia were collected as described previously (Broekaert et al., 1990). Five-week-old Col-0 plants were inoculated by applying 5-µL drops of half-strength potato dextrose broth containing 5 x $10^5$ spores $mL^{-1}$. *Pieris rapae* and *Frankliniella occidentalis* were reared as described previously (De Vos et al., 2005) and transferred to 5-week-old Col-0 plants. Infestation was carried out by transferring 5 first-instar larvae of *P. rapae* or 20 larvae of *F. occidentalis* to each plant using a fine paintbrush. SA (1mM) was applied as a foliar drench 24 h after pathogen inoculation or herbivore infestation and leaf tissue was harvested another 24 h later. Sporangia from *Hyaloperonospora arabidopsidis* strain WACO9 were collected by rinsing sporulating Col-0 leaves in 10 mM $MgSO_4$ as described previously (Van der Ent et al., 2008). Next, 5-week-old Col-0 plants were inoculated by spraying the leaves with the spore suspension containing 5 x $10^4$ sporangia $mL^{-1}$. To ensure infection, plants were placed at 17°C and kept at 100% relative humidity for 24 hours. After this period, plants were kept at 70-80% relative humidity to facilitate growth of the pathogen. MeJA (0.1 mM) was applied as a foliar drench 3 days after *H. arabidopsidis* inoculation. In case of two biological inducers, *P. rapae* larvae were applied 3 days after *H. arabidopsidis* inoculation. Leaf material was harvested 24 h after MeJA or *P. rapae* treatment.

### RNA extraction and northern blot analysis

Total RNA was extracted from 5 to 10 plants as described previously (De Vos et al., 2005). For northern blot analysis, 15 µg RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook et al., 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-$N^+$ membrane (Amersham, 's-Hertogenbosch, The Netherlands) by capillary transfer. The electrophoresis and blotting buffer consisted of 10 and 25 mM sodium phosphate (pH 7.0), respectively. Northern blots were hybridized with gene-specific probes for *PR-1, PDF1.2*, and *VSP2* as described previously (Pieterse et al., 1998). After hybridization with α-[$^{32}$P]-dCTP-labeled probes, blots were exposed for autoradiography and signals quantified using a BioRad Molecular Imager FX (BioRad, Veenendaal, the Netherlands) with Quantity One software (BioRad, Veenendaal, the Netherlands). To check for equal loading, the blots were stripped and hybridized with a probe for *18S* rRNA. The AGI numbers for the genes studied are At2g14610 (*PR-1*), At5g44420 (*PDF1.2*), and At5g24770 (*VSP2*). The probe for *18S* rRNA was derived from an Arabidopsis cDNA clone (Pruitt and Meyerowitz, 1986). All gene expression analyses have been repeated with similar results.

### Glutathione assay

Total levels of glutathione (GSH+GSSG) were measured using the Glutathione Assay Kit (Sigma, Schnelldorf, Germany) according to the manufacturer's protocol. Leaf tissue was frozen in liquid nitrogen and ground to a fine powder. Subsequently, 500 μL of 5% 5-sulfosalicylic acid were added to 0.1 g of pulverized leaf tissue to deproteinize the sample. Glutathione was then determined in a kinetic assay in which the reduction of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) to yellow TNB was spectrophotometrically measured at 415 nm. The amount of total glutathione was calculated using a standard curve of reduced glutathione. Five plants per treatment were harvested at each time point, and each sample was measured 6 times.

### BSO assay

Col-0 seedlings were grown for 12 days on Murashige and Skoog (1962) (MS) medium with or without 2.5 mM L-buthionine-sulfoximine (BSO; Sigma, Schnelldorf, Germany) and with 10 g $L^{-1}$ sucrose and 6 g $L^{-1}$ plant agar, pH 5.7. Seedlings were then transferred to MS plates containing 2.5 mM BSO, 0.5 mM SA, 20 μM MeJA, or a combination of these chemicals. Leaf tissue was harvested 48 h later. BSO was included in the MS medium either continuously or only during the last 48 h, together with the SA and MeJA treatments.

### Acknowledgements

The authors thank Ruth Joosten and Tale Sliedrecht for technical assistance, Marcel Dicke for providing the insect herbivores and Leo Koopman, Frans van Aggelen, André Gidding, and Dick Peeters for insect rearing. This research was supported by grants 813.06.002 and 865.04.002 of the Earth and Life Sciences Foundation (ALW), which is subsidized by The Netherlands Organization of Scientific Research (NWO).

CHAPTER 3

# Ethylene modulates the role of NPR1 in cross-talk between salicylate and jasmonate signaling

**Antonio Leon-Reyes[1], Steven H. Spoel[2], Elvira S. De Lange[1], Hiroshi Abe[3], Masatomo Kobayashi[3], Shinya Tsuda[4], Frank F. Millenaar[5], Rob A.M. Welschen[5], Tita Ritsema[1] and Corné M.J. Pieterse[1]**

[1] Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, the Netherlands
[2] Department of Biology, Duke University, Durham, NC 27708-1000, U.S.A.
[3] Department of Biological Systems, RIKEN BioResource Center, Tsukuba 305-0074, Japan
[4] Department of Plant Pathology, National Agricultural Research Center, Tsukuba 305-8666, Japan
[5] Plant Ecophysiology, Department of Biology, Faculty of Science, Utrecht University, P.O.Box 800.84, 3508 TB Utrecht, the Netherlands

**Plant Physiology** 149: 1797-1809 (2009)

**Abstract**

The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play crucial roles in the signaling network that regulates induced defense responses against biotic stresses. Antagonism between SA and JA operates as a mechanism to fine-tune defenses that are activated in response to multiple attackers. In Arabidopsis, NPR1 was demonstrated to be required for SA-mediated suppression of JA-dependent defenses. Because ET is known to enhance SA/NPR1-dependent defense responses, we investigated the role of ET in the SA-JA signal interaction. Pharmacological experiments with gaseous ET and the ET precursor 1-aminocyclopropane-1-carboxylic acid showed that ET potentiated SA/NPR1-dependent *PR-1* transcription, while it rendered the antagonistic effect of SA on methyl-JA-induced *PDF1.2* and *VSP2* expression NPR1 independent. This overriding effect of ET on NPR1 function in SA-JA cross-talk was absent in the *npr1-1/ein2-1* double mutant, demonstrating that it is mediated via ET signaling. Abiotic and biotic induction of the ET response similarly abolished the NPR1 dependency of the SA-JA signal interaction. Furthermore, JA-dependent resistance against biotic attackers was antagonized by SA in an NPR1-dependent fashion only when the plant-attacker combination did not result in the production of high levels of endogenous ET. Hence, the interaction between ET and NPR1 plays an important modulating role in the fine tuning of the defense signaling network that is activated upon pathogen and insect attack. Our results suggest a model in which ET modulates the NPR1 dependency of SA-JA antagonism, possibly to compensate for enhanced allocation of NPR1 to function in SA-dependent activation of *PR* genes.

## Introduction

Plants have a broad spectrum of mechanisms to cope with adverse conditions such as abiotic stress (*e.g.* flooding and drought) or biotic stresses (*e.g.* pathogen and insect attack). With regard to biotic stress, plants posses both physical and chemical barriers to prevent harmful attackers from causing damage. When these constitutively active layers of defense are overcome, inducible defense systems are recruited to counteract the attacker (Walters et al., 2007). The phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) emerged as key players in regulating the activation of the induced defense responses involved (Dong, 1998; Howe, 2004; Pozo et al., 2004; Grant and Lamb, 2006a; Van Loon et al., 2006a; Von Dahl and Baldwin, 2007; Vlot et al., 2008). Their production varies greatly, depending on the nature of the attacking pathogen or insect. The quantity, composition, and timing of the hormonal blend produced results in the activation of a specific set of defense-related genes that eventually determines the nature of the defense response that is triggered by the

attacker encountered (De Vos et al., 2005; Mur et al., 2006). Other plant hormones, including abscisic acid (Mauch-Mani and Mauch, 2005; de Torres-Zabala et al., 2007; Asselbergh et al., 2008), brassinosteroids (Nakashita et al., 2003), gibberellins (Navarro et al., 2008) and auxins (Navarro et al., 2006; Wang et al., 2007), have also been reported to play a role in the plant's immune response, but their significance is less well-studied.

In *Arabidopsis thaliana*, it was shown that SA-, JA-, and ET-dependent pathways regulate defense responses that are differentially effective against specific types of attackers (Thomma et al., 2001a; Glazebrook, 2005; Thatcher et al., 2005). Pathogens with a biotrophic lifestyle, such as *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis,* are generally more sensitive to SA-dependent responses, whereas necrotrophic pathogens, such as *Botrytis cinerea* and *Alternaria brassicicola,* and herbivorous insects, such as *Pieris rapae* (small cabbage white) and *Frankliniella occidentalis* (Western flower thrips) are commonly deterred by JA- and/or ET-dependent defenses (Thomma et al., 1998; Kessler and Baldwin, 2002; Ton et al., 2002; De Vos et al., 2006; Abe et al., 2008). In nature, plants often deal with simultaneous or subsequent invasion by multiple aggressors, which can influence the primary induced defense response of the host plant (Van der Putten et al., 2001; Bezemer and Van Dam, 2005; Stout et al., 2006; Poelman et al., 2008b). Activation of plant defense mechanisms is associated with ecological fitness costs (Heil and Baldwin, 2002; Heidel et al., 2004; Van Hulten et al., 2006). Hence, plants need regulatory mechanisms to effectively and efficiently adapt to changes in their complex hostile environment. Cross-talk between induced defense signaling pathways provides the plant with such a powerful regulatory potential (Reymond and Farmer, 1998; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Pieterse et al., 2009). Signaling interactions can be either (mutually) antagonistic or synergistic, resulting in negative or positive functional outcomes. Cross-talk helps the plant to minimize fitness costs and create a flexible signaling network that allows the plant to finely tune its defense response to the invaders encountered (Reymond and Farmer, 1998; Pieterse et al., 2001; Kunkel and Brooks, 2002; Bostock, 2005). Yet, it seems that insect herbivores and pathogens have also evolved to manipulate plants for their own benefit by suppressing induced defenses through modulation of the plant's defense signaling network (Pieterse and Dicke, 2007; Robert-Seilaniantz et al., 2007; Walling, 2008).

One of the best studied examples of defense-related signal cross-talk is the interaction between the SA and the JA response pathway (Kunkel and Brooks, 2002; Thaler et al., 2002b; Glazebrook et al., 2003; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Spoel and Dong, 2008). Many studies have demonstrated that endogenously accumulating SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent defenses over JA-dependent ones (Doherty et al., 1988; Peña-Cortés et al., 1993; Gupta et al., 2000; Spoel et al., 2003). As a result of the

negative interaction between SA and JA signaling, activation of the SA response should render a plant more susceptible to attackers that are resisted via JA-dependent defenses and *vice versa*. Indeed, many examples of trade-offs between SA-dependent resistance against biotrophic pathogens and JA-dependent defense against insect herbivory and necrotrophic pathogens have been reported (Pieterse et al., 2001; Bostock, 2005; Stout et al., 2006). In Arabidopsis, Spoel et al. (2007) showed that SA-mediated defenses that are triggered upon infection by a virulent strain of the hemibiotrophic pathogen *P. syringae* rendered infected tissues more susceptible to infection by the necrotrophic pathogen *A. brassicicola* by suppressing the JA signaling pathway. Similarly, infection by the biotrophic pathogen *H. arabidopsidis* strongly suppressed JA-mediated defenses that were activated upon feeding by caterpillars of the small cabbage white *P. rapae* (Koornneef et al., 2008b). Conversely, JA signaling can act antagonistically on SA-dependent defenses. For instance, *P. syringae* produces the phytotoxin coronatine, which functions as a JA mimic and suppresses effectual SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Zhao et al., 2003; Brooks et al., 2005; Cui et al., 2005; Nomura et al., 2005; Uppalapati et al., 2007). Although many reports describe an antagonistic interaction between SA- and JA-dependent signaling, synergistic interactions have been described as well (Schenk et al., 2000; Van Wees et al., 2000; Mur et al., 2006). For example, application of low concentrations of both SA and JA (10 to 100 μM) led to enhanced JA/ET response in the combination treatment compared to JA alone, suggesting that hormone concentration is important for the final output during plant-microbe interactions (Mur et al., 2006).

Pharmacological experiments with Arabidopsis revealed that transcription of JA-responsive marker genes, such as *PDF1.2* and *VSP2*, is highly sensitive to suppression by exogenous application of SA. This SA-mediated suppression of JA-responsive gene expression (hereafter referred to as SA-JA cross-talk) was observed in a large number of *A. thaliana* accessions, highlighting the potential significance of this phenomenon in the regulation of induced plant defenses in nature (Koornneef et al., 2008b). Several lines of evidence point to a role for SA-mediated redox changes in the regulation of SA-JA cross-talk (Ndamukong et al., 2007; Koornneef et al., 2008b). In Arabidopsis, the redox-sensitive protein NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1), an important transducer of SA-induced redox changes (Mou et al., 2003; Dong, 2004; Pieterse and Van Loon, 2004; Tada et al., 2008), was shown to be a key regulator of SA-mediated suppression of JA signaling (Spoel et al., 2003). Induction of the SA-response, either by pathogen infection or by exogenous application of SA, strongly suppressed JA-responsive genes, such as *PDF1.2* and *VSP2*. However, in mutant *npr1-1* plants, this antagonistic effect was completely abolished (Spoel et al., 2003). The *npr1-1* mutant shows enhanced resistance against *Trichoplusia ni* (Cabbage looper) and *Spodoptera littoralis* (Egyptian cotton worm) (Cui et al., 2002; Stotz et al., 2002), indicating

that blocking the NPR1-dependent SA signaling pathway resulted in enhanced JA-dependent defenses against these insect herbivores. Nuclear localization of NPR1, which is essential for SA-mediated defense gene expression (Kinkema et al., 2000), is not required for the suppression of JA-responsive genes, indicating that the antagonistic effect of SA on JA signaling is modulated through a function of NPR1 in the cytosol (Spoel et al., 2003). In rice (*Oryza sativa*), a similar cytosolic function of NPR1 in SA-JA cross-talk was reported (Yuan et al., 2007), over-expression of cytosolic *OsNPR1* suppressed JA-responsive gene transcription and enhanced the level of susceptibility to insect herbivory, whereas NPR1-mediated suppression of the JA response was no longer present in plants expressing OsNPR1 that was constitutively targeted to the nucleus.

Besides SA and JA, ET has also been demonstrated to play an important role in the plant's defense response to pathogen and insect attack (Broekaert et al., 2006; Van Loon et al., 2006a; Adie et al., 2007a; Von Dahl and Baldwin, 2007). In addition to effects on the level of pathogen or insect resistance, ET was shown to function as an important modulator of the plant's response to other hormones, such as JA, SA and ABA (Adie et al., 2007a; Pieterse et al., 2009). For instance, ET enhanced the response of Arabidopsis to SA, resulting in a potentiated expression of the SA-responsive marker gene *PR-1* (Lawton et al., 1994; De Vos et al., 2006). Moreover, in tobacco ET was shown to be essential for the onset of SA-dependent systemic acquired resistance (SAR) against tobacco mosaic virus (Verberne et al., 2003). Also the synergistic interaction between ET and JA has been well established. Many defense-related genes, such as *PDF1.2,* are regulated via a signaling pathway that requires both ET and JA (Penninckx et al., 1998; Broekaert et al., 2006; Adie et al., 2007a). Similarly, co-requirement of ET and JA has been demonstrated for the onset of broad-spectrum induced systemic resistance (ISR) that is triggered after colonization of plant roots by beneficial micro-organisms (Pieterse et al., 1998; Van der Ent et al., 2008; Van Wees et al., 2008), highlighting the important modulating role of ET in plant defense.

In many plant-attacker interactions, ET is part of the signal signature that is produced upon pathogen or insect attack (De Vos et al., 2005). The established role of ET in modulating SA- and JA-dependent defense responses, prompted us to investigate the effect of ET on the interaction between SA and JA signaling. Here, we demonstrate that ET bypasses the NPR1 dependency of the SA-mediated antagonistic effect on JA signaling, thereby shaping the final outcome of the plant defense signaling network that is activated upon pathogen or insect attack.

## Results

### ET modulates the NPR1 dependency of the SA-JA signal interaction

In Arabidopsis, pharmacological experiments revealed that SA can antagonize the expression of JA-responsive genes, such as *PDF1.2* and *VSP2* (Spoel et al., 2003; Koornneef et al., 2008b). To investigate whether ET affects this SA-JA cross-talk, we analyzed the effect of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) on SA- and NPR1-dependent suppression of JA-responsive gene expression. To this end, we made use of mutant *npr1-1* that contains a missense mutation that alters a key ankyrin repeat in the NPR1 protein and disrupts NPR1-dependent regulation of both SA- and JA-dependent genes (Cao et al., 1997; Glazebrook et al., 2003). Twelve-day-old seedlings of wild-type Col-0 and mutant *npr1-1* plants were grown on Murashige Skoog (MS)-agar medium with or without increasing concentrations of ACC and either 0.5 mM SA, 0.02 mM methyl-JA (MeJA), or a combination of both chemicals. Two days later, the expression of the SA-responsive marker gene *PR-1* and the JA-responsive marker gene *PDF1.2* was analyzed by northern blot analysis (Fig. 1). In the absence of ACC, the single treatments of Col-0 with SA or MeJA activated *PR-1* and *PDF1.2,* respectively. In addition, the combination treatments with SA and MeJA resulted in effective SA-mediated suppression of MeJA-induced *PDF1.2* expression. As expected, neither *PR-1* induction nor *PDF1.2* suppression was apparent in the *npr1-1* mutant, supporting previous findings that SA-JA cross-talk is dependent upon wild-type NPR1 function (Spoel et al., 2003). However, addition of ACC into the medium at different concentrations resulted in effective SA-mediated suppression of MeJA-induced *PDF1.2* expression in both Col-0 and *npr1-1* plants, suggesting that ET relieved the NPR1 dependency of the SA-JA signal interaction. Similar results were obtained with the JA-responsive gene *VSP2* (Supplemental Figure S1).

In order to corroborate our observation with media-grown seedlings that ET overrules the NPR1 dependency of SA-JA cross-talk, we investigated the effect of ET on SA-JA cross-talk in 5-week-old, soil-grown plants using both ACC and gaseous ET. The plants were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals and either 0.1 mM ACC or gaseous ET (2 $\mu l \cdot L^{-1}$), and harvested 6 h later for northern blot analysis. As reported previously (Lawton et al., 1994; De Vos et al., 2006), ACC and ET both enhanced the SA-induced expression of *PR-1* in adult wild-type plants (Fig. 2). However, these chemicals failed to restore *PR-1* expression in the *npr1-1* mutant, suggesting that ET stimulates SA signaling through the wild-type function of NPR1. In addition, Fig. 2 shows that in the absence of ACC or ET, MeJA-induced *PDF1.2* gene expression was effectively suppressed by SA in SA/MeJA-treated Col-0 plants, but not in mutant *npr1-1* plants. However, like observed in seedlings, addition of ACC (Fig. 2A) or gaseous ET (Fig. 2B)

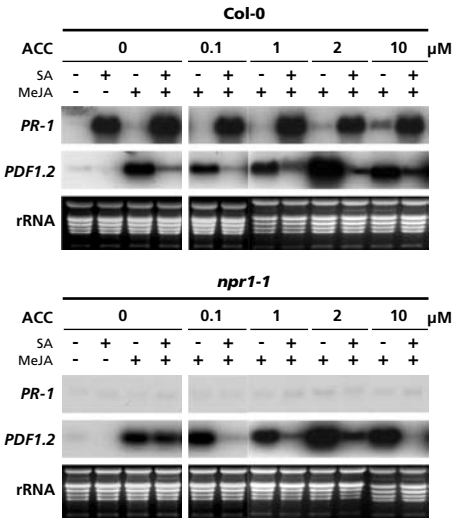



**Figure 1. ACC modulates the NPR1 dependency of SA-JA cross-talk in Arabidopsis seedlings.**
**Northern blot analysis of *PR-1* and *PDF1.2* mRNA levels in Col-0 and *npr1-1* seedlings that were treated with SA, MeJA, or a combination of both chemicals in the absence or presence of the ET precursor ACC. Pharmacological assays were performed with seedlings that were grown for 12 days on MS medium after which they were transferred to fresh MS medium supplemented with increasing concentrations of ACC and either 0.5 mM SA, 0.02 mM MeJA, or a combination of both chemicals. Seedlings were harvested after 2 days for RNA extraction. Ribosomal RNA is presented as a loading control.**

into the SA/MeJA treatment resulted in a partial restoration of SA-mediated suppression of MeJA-induced *PDF1.2* gene expression in the *npr1-1* background. Together, these results indicate that ET affects the dependency of SA-mediated suppression of JA-responsive gene expression on wild-type NPR1 function. The observation that neither ACC nor ET treatment bypassed the NPR1 dependency of SA-induced *PR-1* expression (Figs. 1 and 2), indicates that NPR1 has a dual role in the suppression of JA-dependent genes on the one hand, and in the activation of SA-dependent gene expression on the other.

### Modulation of the NPR1 dependency of SA-JA cross-talk by ET is EIN2 dependent

To test if the modulation of the NPR1 dependency of SA-JA cross-talk by ET is governed by the ET signaling pathway, we performed cross-talk experiments with an *npr1-1/ein2-1* double mutant (Clarke et al., 2000). Because the *ein2-1* mutation completely blocks the ET signaling pathway (Alonso et al., 1999) and *PDF1.2* expression requires an intact response to both JA and ET (Penninckx et al., 1998), we performed these experiments with the JA-responsive marker gene *VSP2*, which is similarly sensitive to the antagonistic effect of SA (Spoel et al., 2003; Koornneef et al., 2008b). Five-week-old Col-0, *ein2-1*, *npr1-1* and *npr1-1/ein2-1* plants were treated with SA, MeJA, or a combination of both chemicals in

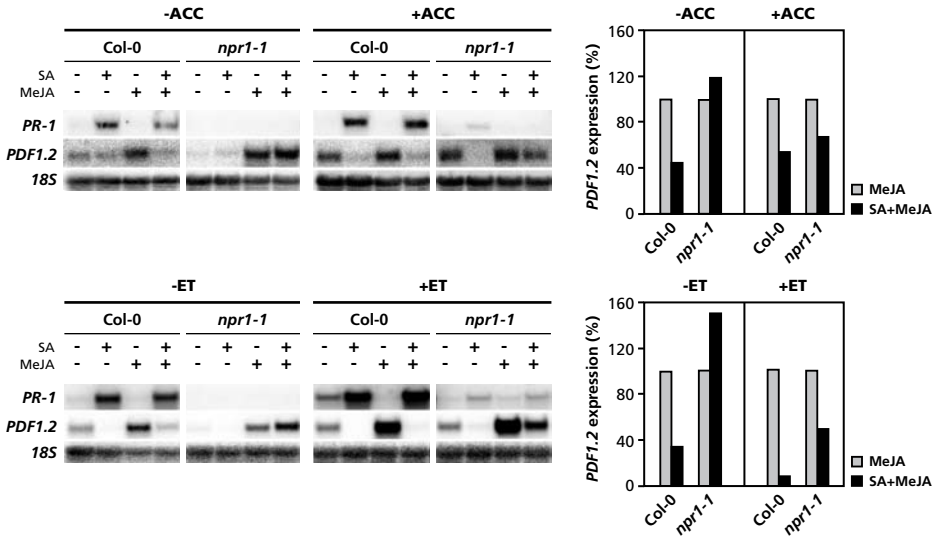



**Figure 2. ACC and gaseous ET enable SA-JA cross-talk in the absence of NPR1 in 5-week-old Arabidopsis plants.** Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in 5-week-old Col-0 and *npr1-1* plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals in the absence (-) or presence (+) of 0.1 mM ACC (A) or 2 µl·L-1 (v/v) of gaseous ET (B). Leaf tissue was harvested 6 h after chemical treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities of the depicted northern blots were quantified using a Phosphor imager (right panels). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.

the absence or presence of ACC. In the absence of ACC, MeJA-induced expression of *VSP2* was effectively suppressed by SA in Col-0 and *ein2-1* but not in the *npr1-1* and *npr1-1/ein2-1* background (Fig. 3), confirming the critical role of wild-type NPR1 in SA-JA cross-talk under low ET conditions. In ACC-treated plants, the NPR1 dependency of SA-JA cross-talk was again relieved as demonstrated by the SA-mediated suppression of MeJA-induced *VSP2* expression in the *npr1-1* background. Compared to *npr1-1* plants, however, SA-JA cross-talk remained blocked upon ACC treatment of the *npr1-1/ein2-1* double mutant. These data indicate that the modulation of the NPR1 dependency of SA-JA cross-talk by ET is dependent upon EIN2 and is thus regulated by the ET signaling pathway.

### Abiotic induction of endogenous ET relieves the NPR1 dependency of SA-JA cross-talk

To test the biological relevance of the effect of ET on the role of NPR1 in SA-JA cross-talk, we performed SA-JA cross-talk experiments under abiotic conditions in which Arabidopsis produces enhanced levels of ET. To this end, 5-week-old plants were placed in trays with open or closed lids. As shown in Fig. 4A, Col-0 and *npr1-1* plants grown in trays with closed lids showed a typical hyponastic response, which is a phenomenon demonstrated to be mediated by ET (Millenaar et al., 2005). Mutant *ein2-1* did not display this hyponastic response, confirming the ET dependency of

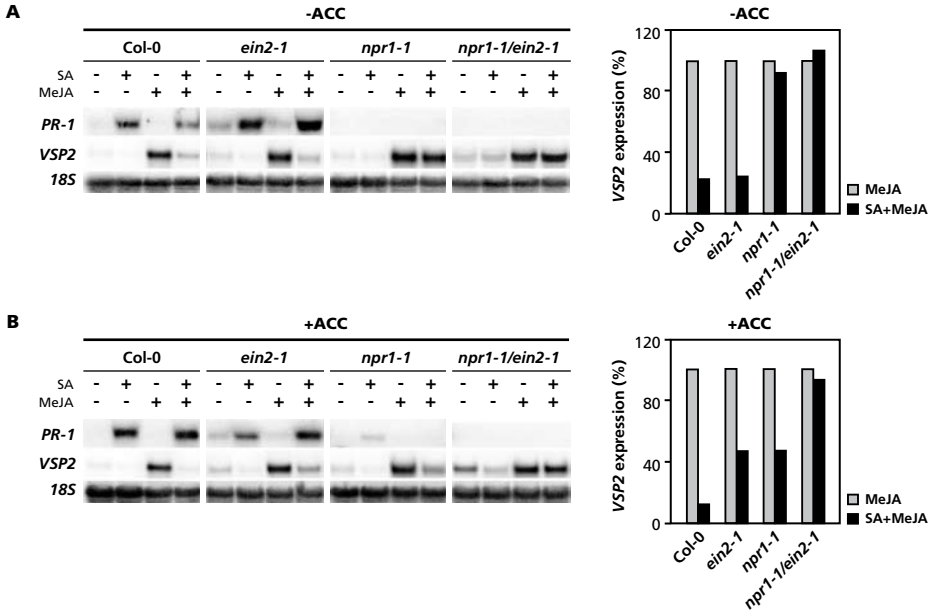



**Figure 3. NPR1-independent SA-JA cross-talk facilitated by ET is EIN2 dependent.**
**Northern blot analysis of *PR-1* and *VSP2* transcript levels in 5-week-old Col-0, *npr1-1*, *ein2-1* and *npr1-1/ein2-1* plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals in the absence (-) or presence (+) of 0.1 mM ACC. Leaf tissue was harvested 6 h after treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities of the depicted northern blots were quantified using a Phosphor imager (Right panels). *VSP2* transcript levels in the single MeJA treatments were set to 100%.**

this phenomenon. Besides the ET-dependent hyponastic response, plants grown in trays with closed lids produced more ET (Fig. 4B) and accumulated enhanced transcript levels of the ET-responsive genes *ERS2* and *EBF2* (Fig. 4C) (Millenaar et al., 2005; Van der Ent et al., 2008), indicating that growth of the plants in closed trays results in enhanced ET signaling. To investigate the effect of endogenously produced ET on the NPR1 dependency of SA-JA cross-talk, plants grown in trays with open or closed lids were treated with SA, MeJA, or a combination of both chemicals and harvested 24 h later for northern blot analysis of *PR-1* and *PDF1.2* gene expression. Fig. 4D shows that the antagonistic effect of SA on MeJA-induced expression of *PDF1.2* is blocked in *npr1-1* mutant plants when grown in trays with open lids (basal ET signaling). However, when the cross-talk experiment was performed with plants grown in trays with closed lids (enhanced ET signaling), the level of SA-mediated suppression of *PDF1.2* in *npr1-1* plants was similar to that observed in Col-0 plants. Hence, abiotic induction of the ET response relieves the dependency of SA-JA cross-talk on wild-type NPR1 function.

### Attacker-induced ET enables NPR1-independent SA-JA cross-talk

Next, we wanted to investigate whether ET produced during a plant-attacker interaction affects the NPR1 dependency of SA-JA cross-talk. To this end, we made

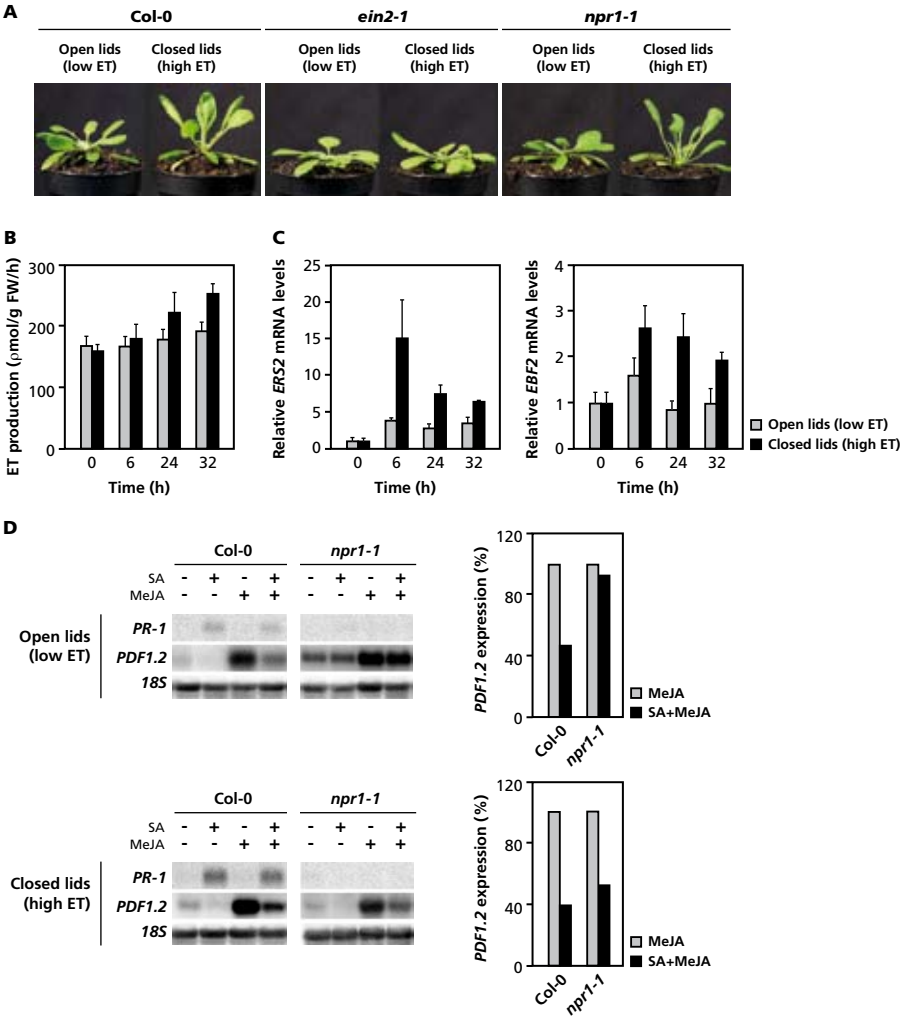



**Figure 4.** **Abiotic induction of ET relieves the NPR1 dependency of SA-JA cross-talk.**
**To enhance the ET response in Arabidopsis plants in a biological manner, 5-week-old plants were placed in trays with the lids open (low ET) or closed (high ET). (A) Arabidopsis Col-0 and *npr1-1* plants grown for 24 h under high ET conditions displayed a hyponastic response, whereas the ET-signaling mutant *ein2-1* did not (B). ET production by Col-0 plants incubated for 6, 24, and 32 h in trays with open or closed lids. (C) Q-RT-PCR analysis of the ET-responsive genes *ERS2* and *EBF2* in Col-0 plants incubated for 6, 24, and 32 h in trays with open or closed lids. D) Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in Col-0 and *npr1-1* plants that were treated with SA, MeJA, or a combination of both chemicals and incubated for 24 h in trays with open lids (low ET signaling) or closed lids (high ET signaling). Chemical treatments were performed by dipping the leaves into a solution of 0.015% (v/v) Silwet L77 containing 1 mM SA, 0.1 mM MeJA, or a combination of these chemicals. Leaf tissue was harvested 24 h after chemical treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities of the depicted northern blots were quantified using a Phosphor imager (right panels). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

use of two JA-inducing attackers: the necrotrophic fungal pathogen *A. brassicicola* and the herbivorous insect *F. occidentalis*. *A. brassicicola* stimulates the biosynthesis of both JA and ET, while *F. occidentalis* induces only JA production (De Vos et al.,

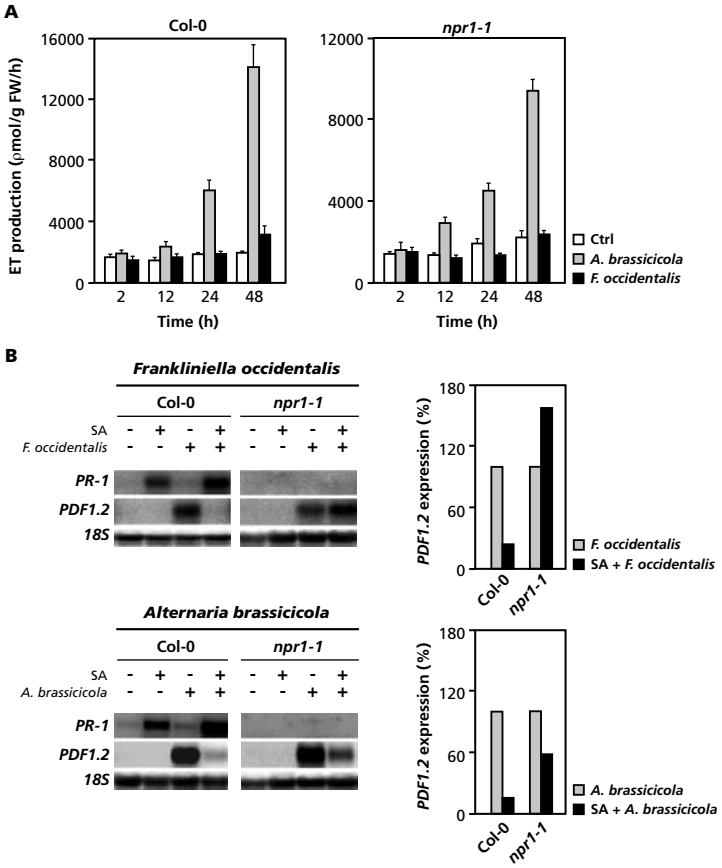



**Figure 5. Attacker-induced ET enables NPR1-independent SA-JA cross-talk.**
**(A) ET production in Col-0 and *npr1-1* after infection with the necrotrophic fungus *Alternaria brassicicola* or infestation with larvae of the Western flower thrips *Frankliniella occidentalis*. (B) Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in Col-0 and *npr1-1* plants that were infested with *F. occidentalis* or inoculated with *A. brassicicola* and treated or not with 1 mM SA. Leaf tissue was harvested for RNA analysis 24 h after application of SA. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities of the depicted northern blots were quantified using a Phosphor imager (right panels). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

2005). Inoculation of Col-0 and *npr1-1* plants with *A. brassicicola* indeed resulted in a strong increase in the production of ET, whereas infestation with thrips had no effect (Fig. 5A).

To investigate the NPR1 dependency of SA-mediated suppression of JA-responsive gene expression during both Arabidopsis-attacker combinations, Col-0 and *npr1-1* plants were infested with *F. occidentalis* or infected with *A. brassicicola* 24 h prior to SA treatment. Twenty-four h later, leaf material was harvested for northern blot analysis of *PR-1* and *PDF1.2* transcript levels. Fig. 5B shows that *F. occidentalis* and *A. brassicicola* both induced the expression of *PDF1.2* and that this expression was strongly suppressed by SA in Col-0 plants. In the *npr1-1* mutant, this

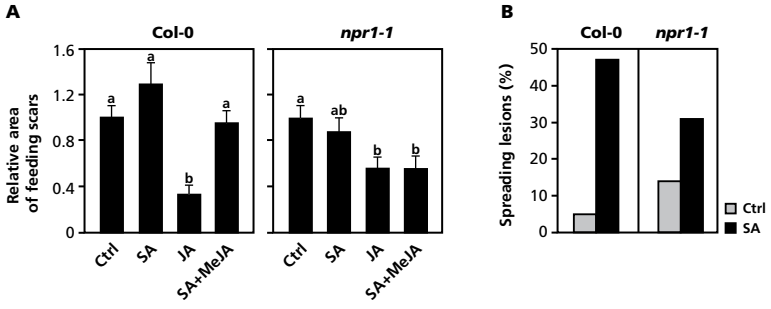



**Figure 6. Antagonistic effect of SA on JA-dependent resistance against *F. occidentalis* and *A. brassicicola* in Col-0 and *npr1-1*.**
**(A) *F. occidentalis* resistance assay with 3-week-old Col-0 and *npr1-1* plants that were pretreated for 24 h with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Presented are the means ±SD ($n$ = 10) of the relative area of feeding scars (control treatment is set to 1) on Col-0 and *npr1-1* leaf discs after 2 days of thrips feeding. Different letters indicated statistically significant differences between treatments (Tukey-Kramer's HSD test, $P < 0.05$). (B) *A. brassicicola* resistance assay with 5-week-old Col-0 and *npr1-1* plants that were treated or not with 1 mM SA. Data represent the percentage of leaves ($n$ = 80) that developed spreading lesions after inoculation with *A. brassicicola*.**

SA-mediated suppression of *PDF1.2* transcription was not observed when the JA response was activated by the non-ET-inducer *F. occidentalis*, indicating that in this plant-attacker combination SA-JA cross-talk is NPR1 dependent. However, when the JA response was activated by the JA- and ET-inducer *A. brassicicola*, *PDF1.2* transcription was suppressed in the *npr1-1* mutant background. These results indicate that attacker-induced ET largely overrules the NPR1 dependency of SA-JA cross-talk, and hence potentially affects the outcome of the defense response that is induced upon attack by multiple invaders.

### NPR1 is not required for SA-mediated suppression of JA-dependent resistance against ET-inducing attackers

In Arabidopsis, resistance against *F. occidentalis* and *A. brassicicola* has been demonstrated to be mediated by the JA response pathway (Thomma et al., 1998; Abe et al., 2008). To investigate the role of NPR1 in the antagonistic effect of SA on the JA-dependent resistance against these attackers, we performed resistance assays in Col-0 and *npr1-1* plants. We hypothesized that the antagonistic effect of SA on JA-dependent resistance against ET-noninducing thrips would be NPR1 dependent, while the negative effect of SA on JA-dependent resistance against the ET-inducing fungal pathogen would function independently of NPR1.

In the thrips resistance assays, Col-0 and *npr1-1* plants were pretreated with 1 mM SA, 0.1 mM MeJA or a combination of both. Twenty-four h later, leaf discs from this material were taken and infested with *F. occidentalis*. Two days later, the level of thrips resistance was determined by measuring the level of feeding scar damage that was inflicted by thrips feeding (Abe et al., 2008). As shown in Fig. 6A, SA treatment had no significant effect on the basal level of thrips resistance in Col-

0 plants. However, MeJA-treated Col-0 plants showed a significantly reduced area of feeding scars, indicating that MeJA treatment enhanced the level of resistance to thrips feeding. Col-0 plants treated with both SA and MeJA showed a basal level of thrips resistance that was not significantly different from that in control plants, suggesting that SA suppressed the level of MeJA-induced resistance against thrips feeding. In mutant *npr1-1* plants, MeJA and SA/MeJA treatments both lead to a significant increase in the level of thrips resistance, indicating that the SA-mediated suppression of MeJA-induced resistance to *F. occidentalis* is controlled by NPR1.

Wild-type Col-0 plants are highly resistant to *A. brassicicola* infection, but this resistance is lost in JA-insensitive *coi1-1* mutant plants (Thomma et al., 1998), indicating that JA is an important regulator of basal resistance against this pathogen. Previously, Spoel et al. (2007) demonstrated that SA suppresses this JA-dependent resistance against *A. brassicicola,* resulting in enhanced susceptibility of Col-0 plants to *A. brassicicola* infection. Indeed, exogenous application of SA to Col-0 plants broke the JA-dependent resistance to *A. brassicicola* (Fig. 6B). However, treatment of *npr1-1* plants with SA only moderately reduced the level of JA-dependent resistance against this pathogen. These results suggest that the SA-mediated suppression of JA-dependent resistance against *A. brassicicola* is functioning, at least partly, independently of NPR1. Since *A. brassicicola*-infected tissues produced high levels of ET, while thrips-infested tissues did not (Fig. 5A), it is likely that the regulatory role of NPR1 in antagonism between SA and JA is determined by the presence or absence of ET in the signal signature of the plant-attacker combination.

## Discussion

### ET modulates the role of NPR1 in cross-talk between SA and JA signaling

Cross-communication between defense signaling pathways is thought to play an important role in the regulation and fine tuning of the defense responses that are activated upon pathogen and insect attack. The antagonism between SA and JA signaling emerged as one of the most prominent of all signal interactions studied to date (Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Pieterse et al., 2009). Pharmacological experiments revealed that the suppression of JA-responsive genes such as *PDF1.2*, *VSP2*, and *LOX2* by SA is regulated by NPR1 (Spoel et al., 2003). Following a whole-genome transcript profiling approach to identify Arabidopsis genes that are sensitive to SA-JA cross-talk we recently identified 258 MeJA-responsive genes of which the expression was significantly affected by SA (A. Koornneef and C.M.J. Pieterse, unpublished results). Sixty percent of the JA-responsive genes that were suppressed by SA displayed this suppression in an NPR1-dependent manner, demonstrating that NPR1 is involved in the SA-mediated

down-regulation of a large number of MeJA-responsive. Because ET is an important modulator of plant defense and a major constituent of the blend of defense signals that is produced during many plant-attacker interactions (Broekaert et al., 2006; Van Loon et al., 2006a; Adie et al., 2007a; Von Dahl and Baldwin, 2007), we investigated the effect of ET on the SA-JA signal interaction. Here, we demonstrate that ET strongly affects the requirement of wild-type NPR1 in the antagonistic effect of SA on JA-dependent defenses. Exogenous application of the ET precursor ACC or gaseous ET (Fig. 1-3), as well as endogenously produced ET during induction of the hyponastic response (Fig. 4) or pathogen attack (Fig. 5), bypassed the NPR1 dependency of SA-JA cross-talk. Experiments in the mutant *ein2-1* background showed that this ET effect is EIN2-dependent, and thus mediated through the ET signaling pathway (Fig. 3). These findings indicate that the final outcome of the SA-JA signal interaction during the complex interaction of plants with their attackers can be shaped by ET. Indeed, the antagonistic effect of SA on MeJA-induced resistance against feeding by ET-noninducing thrips was controlled by NPR1. By contrast, SA-mediated suppression of JA-dependent resistance against the JA- and ET-inducing necrotroph *A. brassicicola* functioned independently of NPR1 (Fig. 6), highlighting the modulating role of ET in the SA-JA signal interaction. In Figure 7, we present a schematic model of the interplay between SA, JA, ET and NPR1 in the Arabidopsis-attacker interactions studied.

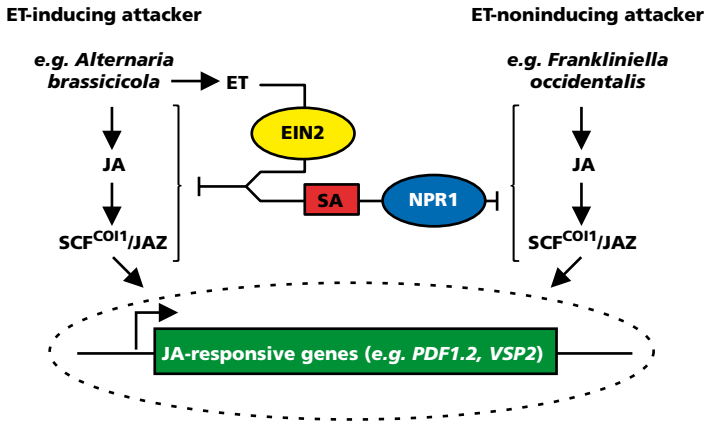



**Figure 7. Working model illustrating the role of ET in modulating the NPR1 dependency of SA-JA cross-talk.**
**Working model illustrating the role of ET in modulating the NPR1 dependency of SA-JA cross-talk. Attack of Arabidopsis by the necrotrophic fungus *Alternaria brassicicola* and the herbivorous insect *Frankliniella occidentalis* results in the biosynthesis of JAs and the activation of the JA signaling pathway in which the E3 ubiquitin ligase SCF$^{COI1}$ and Jasmonate ZIM-domain (JAZ) proteins that repress transcription of JA-responsive genes are central components (Chini et al. 2007; Thines et al. 2007). Activation of the JA signaling cascade leads to the activation of JA-responsive genes such as *PDF1.2* and *VSP2*. SA suppresses JA-responsive gene expression in an NPR1-dependent manner. However, when ET signaling is stimulated, such as upon infection by the ET-inducer *A. brassicicola*, the NPR1-dependency of SA-JA cross-talk is bypassed resulting in wild-type levels of suppression of JA signaling in the *npr1-1* mutant background.**

## Dual role of NPR1

NPR1 is a regulatory protein that was originally identified in Arabidopsis through several genetic screens for SAR-compromised mutants (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). Mutant *npr1-1* plants are not only compromised in SAR, but also in basal resistance against many types of pathogens that are sensitive to SA-dependent defenses (Dong, 2004). In addition, mutant *npr1-1* plants appeared to be blocked in the activation of ISR by beneficial rhizobacteria, an induced defense response that requires regulators of ET and JA signaling (Pieterse et al., 1998; Van Wees et al., 2008). Moreover, NPR1 has been implicated in JA- and ET-dependent resistance against the soil-borne fungus *Verticillium longisporum* (Johansson et al., 2006). The fact that NPR1 also functions as an important regulator of SA-JA cross-talk (Spoel et al., 2003; Yuan et al., 2007) demonstrates that NPR1 plays a central role in the induced defense signaling network that is controlled by SA, JA and ET (Dong, 2004; Pieterse and Van Loon, 2004). Our finding that the requirement of NPR1 in SA-JA cross-talk is bypassed under conditions in which ET production is induced provides a direct link between ET and NPR1 function.

In the present study we demonstrate that ET bypasses the need of NPR1 in SA-JA cross-talk, while it enhances NPR1-dependent, SA-responsive *PR-1* expression. This clearly indicates that NPR1 plays a dual role in regulating SA-mediated suppression of JA-responsive gene expression on the one hand, and SA-mediated activation of SA-responsive *PR* gene expression on the other hand. This raises the question: how does ET signaling differentially affect the NPR1 dependency of these two SA-dependent cellular responses? The differential effect of ET on NPR1 function may be caused by the fact that the role of NPR1 in SA-JA antagonism is mediated by a cytosolic function of NPR1 (Spoel et al., 2003; Yuan et al., 2007), whereas the role of NPR1 as co-activator of SA-responsive *PR* gene expression is exerted in the nucleus (Kinkema et al., 2000; Dong, 2004). Previously, Glazebrook et al. (2003) demonstrated that two different alleles of the *npr1* mutant *(npr1-1* and *npr1-3*) behaved differently in terms of transcriptome changes upon infection by *P. syringae*. The *npr1-1* mutant, which has a mutation in a key ankyrin-repeat domain, was affected in the expression of SA- as well as JA- and ET-dependent genes. However, the *npr1-3* mutant, which produces a truncated cytoplasmatically localized NPR1 protein that misses the carboxyterminal domain with the nuclear localization signal (Dong, 2004), was only affected in SA-dependent gene expression, suggesting that the cytoplasmatic function of NPR1 plays a role in the control of JA- and ET-dependent responses. In agreement with this, the antagonistic effect of SA on JA-responsive gene expression was much less affected in *npr1-3* then in the *npr1-1* (Supplemental Figure S2). These results suggest a model in which the cytosolic function of NPR1 plays a role in SA-JA cross-talk and can

be bypassed by ET, and in which the nuclear function of NPR1 plays a role in the activation of SA-responsive genes and can be stimulated by ET.

Previously, the glutaredoxin GRX480 and the transcription factor WRKY70 were identified as important players in SA/NPR1-dependent suppression of JA-responsive gene expression (Li et al., 2004; Ndamukong et al., 2007). In wild-type plants, transcription of *GRX480* and *WRKY70* was activated by SA in an NPR1-dependent manner, indicating that the roles of GRX480 and WRKY70 in the suppression of JA-responsive genes acts downstream of the NPR1-dependent induction of *GRX480* and *WRKY70* by SA. However, considering the fact that SA/NPR1-dependent gene expression is hampered in mutant *npr1-3*, while SA/NPR1-dependent suppression of JA-responsive gene expression is still intact in this mutant, suggests that the antagonistic effect of SA on JA signaling can function independently of GRX480 or WRKY70. This is corroborated by previous findings that *grx480* and *wrky70* knock-out mutants showed wild-type levels of SA-mediated suppression of MeJA-induced *PDF1.2* gene expression (Ndamukong et al., 2007; A. Leon-Reyes and C.M.J. Pieterse, unpublished results).

### Interaction between ET and NPR1

NPR1 is an important transducer of the SA signal. In uninduced cells, NPR1 is present as an oligomer formed through intermolecular disulfide bonds (Mou et al., 2003). SA mediates a change in the cellular redox potential, resulting in the reduction of the NPR1 oligomer to its active monomeric form. Monomeric NPR1 is then translocated into the nucleus where it functions as a co-activator of SA-responsive genes, such as *PR-1*, by enhancing the binding of TGA transcription factors to SA-responsive promoter elements (Després et al., 2003; Mou et al., 2003; Rochon et al., 2006; Tada et al., 2008). Recently, we demonstrated that SA-mediated redox modulation also plays an important role in the SA-mediated attenuation of the JA signaling pathway (Koornneef et al., 2008b). Hence, it is plausible that the cytosolic function of NPR1 in SA-JA cross-talk is controlled by active NPR1 monomers that are produced upon SA-mediated changes in the redox state.

With our current knowledge on NPR1 function, we can only speculate on how ET affects the NPR1 dependency of the SA-JA signal interaction. On the one hand, ET potentiates the NPR1-dependent expression of the SA-responsive marker gene *PR-1* in Arabidopsis (Fig. 2 and 3; Lawton et al., 1994; De Vos et al., 2006). On the other hand, our study clearly shows that ET bypasses the need for NPR1 in SA-JA cross-talk. These results suggest a model in which ET modulates the allocation of NPR1's positive and negative functions. Since SA-activated NPR1 functions in the nucleus to activate *PR* genes, and in the cytosol to suppress JA-responsive genes, it is tempting to speculate that ET signaling allocates more NPR1 to the nucleus to support SA signaling, thereby making less NPR1 available in the cytosol for SA-JA cross-talk. At the same time possible negative effects of this trade

off on SA-JA cross-talk are compensated because in combination with ET, SA can suppress JA-responsive gene expression in an NPR1-independent manner.

So how could ET modulate the NPR1-dependency of SA-JA cross-talk? In the absence of ET, SA-activated NPR1 monomers may bind a positive regulator of JA-responsive gene expression in the cytosol, which is then prevented from entering the nucleus, resulting in suppression of JA-responsive gene expression. Alternatively, NPR1 may activate a negative regulator of the JA pathway. A simplistic explanation for the role of ET in these scenarios may be that ET signaling results in a similar effect on the putative positive c.q. negative regulator, rendering the function of NPR1 redundant in SA-JA cross-talk. However, other scenarios are plausible as well. For instance, various genetic screens revealed mutations that restored the SAR-compromised phenotype of the *npr1-1* mutant. Mutations in genes such as *SNI1*, *SSI1,* and *CPR6* were demonstrated to restore SA-mediated *PR* gene expression and SAR in the absence of a functional NPR1 protein (Clarke et al., 1998; Li et al., 1999; Shah et al., 1999; Durrant et al., 2007). This clearly indicates that the NPR1 dependency of important SA-mediated cellular responses can be bypassed by inactivation of proteins such as SNI1, SSI1, and CPR6. Future research will be focused on elucidating the targets of ET through which this hormone is able to affect NPR1 function during SA-JA cross-talk.

## Materials and methods

### Plant material

Seeds of *Arabidopsis thaliana* accession Col-0, mutants *npr1-1*, *npr1-3* (Cao et al., 1994), *ein2-1* (Alonso et al., 1999) and double mutant *npr1-1/ein2-1* (Clarke et al., 2000) were sown in quartz sand. After two weeks, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min (Pieterse et al., 1998). Plants were cultivated in a growth chamber with an 8-h day (24°C) and 16-h night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10 mM Sequestreen (CIBA-Geigy) once a week. For experiments with *in vitro*-grown plants, seedlings were grown on plates containing Murashige and Skoog (1962) (MS) medium, ph 5.7, supplemented with 20 $g \cdot L^{-1}$ sucrose and 0.8% (w/v) plant agar. In all experiments 5-week-old plants were used, except in the experiment presented in Fig. 1, in which 12-day-old seedlings grown on MS-agar medium were used, as described by Spoel et al. (2003).

### *Alternaria brassicicola* assays

For induction of JA-responsive gene expression and ET production, Col-0 plants were inoculated with *Alternaria brassicicola* strain MUCL 20297 as described previously (De Vos et al., 2005). Briefly, the fungus was grown on PDA (Potato Dextrose Agar) for 2 to 3 weeks at 22°C. Spores were collected as described by Broekaert et al. (1990). Five-week-old plants were inoculated with 5-µL drops of 50% Potato dextrose broth containing $5 \cdot 10^5$ spores per ml. For assessing the effect of SA on the level of resistance against *A. brassicicola,* leaves of 5-week-old plants were pressure-infiltrated with a solution of 10 mM MgSO4 supplemented with or without 1 mM SA (Spoel et al., 2007). After 24 h, the treated leaves were inoculated with *A. brassicicola* by applying a 3-µl drop of 50% Potato dextrose broth containing $1 \cdot 10^6$ spores per ml. At 4 days after inoculation the percentage of leaves with spreading lesions was assessed.

### *Frankliniella occidentalis* assays

For induction of JA-responsive gene expression, thrips infestations were performed on 5-week-old plants by transferring 20 larvae of *Frankliniella occidentalis* to each plant using a fine paintbrush (De Vos et al., 2005). For determination of thrips resistance, the leaf disc assay described by Abe et al. (2008) was used. Briefly, isolated leaf discs from 3-week-old plants that were pretreated for 24 h with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals (see below) were floated on 1.5 ml of distilled water in wells of a white 19 1.5-ml sample tube stand. A single adult female that had been starved for 2 to 3 h was placed on a single leaf disc. Thrips were allowed to feed for 1 or 2 days at 22°C. The area of thrips feeding scars on the surface of each leaf disk was measured by ImageJ software (Abramoff et al., 2004) on digitized images.

### Chemical treatments

Plants were treated with SA, MeJA and/or ACC by dipping the leaves into a solution of 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands) containing 1 mM SA (Mallinckrodt Baker, Deventer, the Netherlands), 0.1 mM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), 0.1 mM ACC (Sigma, Schnelldorf, Germany), or a combination of these chemicals as described previously (Spoel et al., 2003; Koornneef et al., 2008b). Control treatments were dipped into a solution containing 0.015% (v/v) Silwet L77. Chemical induction of plants grown on MS medium was performed by transferring 12-day-old seedlings to fresh MS medium supplemented with 0.5 mM SA, 0.02 mM MeJA, 0.1 to 10 µM ACC, or a combination of these chemicals (Spoel et al., 2003). MeJA was added to the solutions from a 1,000-fold concentrated stock in 96% ethanol. To the solutions without MeJA, a similar volume of 96% ethanol was added.

Application of gaseous ET to the plants was performed as described by Millenaar et al. (2005). In brief, gaseous ET (100 $\mu L \cdot L^{-1}$; Hoek Loos, Amsterdam, the Netherlands) and air (70% relative humidity) were mixed using flow meters (Brooks Instruments, Veenendaal, the Netherlands) to generate an output concentration of 2 $\mu L \cdot L^{-1}$ ethylene, which was flushed continuously through glass cuvettes (13.5 x 16.0 x 29.0 cm) at a flow rate of 75 $L \cdot h^{-1}$ and then vented to the outside of the building. The concentration of ET in the airflow was verified using gas chromatography as described by Millenaar et al. (2005). For the duration of the gaseous ET treatment, 5-week-old plants were placed in the cuvette, which were placed under climate chamber conditions as described above. Control plants were treated in a similar manner but without ET in the air flow.

### Ethylene measurements

To measure ET production in plants challenged with either *A. brassicicola* or *F. occidentalis*, rosettes of inoculated or infested plants were detached from the roots, weighed and placed individually in 35-mL gas-tight serum flasks ($n$=10) that were subsequently incubated under climate chamber conditions. At different time intervals, 1-mL gas samples were withdrawn through the rubber seal. The concentration of ET was measured by gas chromatography as described by De Laat and Van Loon (1982)

To measure the ET production by plants grown in trays with open and closed lids, plants were removed from the trays and whole rosettes of about 300 mg were immediately transferred into a syringe with a volume of 1.5 mL. ET was allowed to accumulate in the syringe for 15 min after which the head space was analyzed for ET levels using gas chromatography as described by Millenaar et al. (2005).

### RNA extraction and northern blot analysis

For RNA extraction, at least 5 plants per treatment were harvested at the time points indicated. RNA isolation was performed, as described previously by Van Wees et al. (2000). For RNA-blot analysis, 15 mg of RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook et al., 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-$N^+$ membranes (Amersham, 's-Hertogenbosch, the Netherlands) by capillary transfer. The buffers used for electrophoresis and blotting were 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA blots were hybridized with probes for *PR-1, PDF1.2* and *VSP2* as described previously by Pieterse et al. (1998). To check for equal loading, rRNA bands were stained with ethidium bromide or the blots were stripped and hybridized with a probe for *18S* ribosomal RNA. The AGI numbers for the genes studied are At2g14610 (*PR-1*), At5g44420 (*PDF1.2*) and At5g24770 (*VSP2*). After hybridization with $\alpha$-$^{32}$P-dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of

*PDF1.2* or *VSP2* mRNA on the northern blots were quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad). The *PDF1.2* and *VSP2* mRNA levels of the MeJA treatment were set to 100% and compared to *PDF1.2* or *VSP2* mRNA levels of the rest of the treatments. All gene expression analyses have been repeated with similar results.

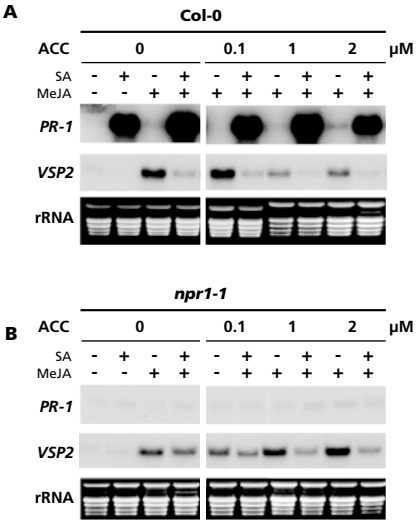
### Quantitative teal-time PCR

Q-RT-PCR analysis was basically performed as described previously (Czechowski et al., 2004; Van der Ent et al., 2008). Gene-specific primers for the ET-responsive genes *EBF2* (Guo and Ecker, 2003) (At5g25350; *EBF2*-FOR 5'- CTTTCACGGTGTCCTGGAAT-3' and *EBF2*-REV 5'-GTGGGCAGCTCCTGATAGAG-3') and *ERS2* (Hua et al., 1998) (At1g04310; *ERS2*-FOR 5'-ACGCTTGCCAAAACATTGTA-3' and *ERS2*-REV 5'-TGAGACGCTTTTCACCAAAC-3') were designed and checked as described (Czechowski et al., 2004; Millenaar et al., 2005).

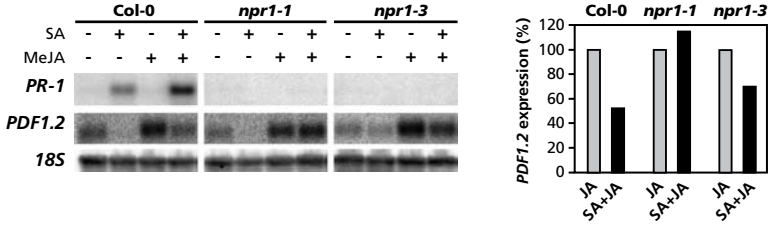
### Acknowledgements

We thank Ruth Joosten, Hans van Pelt, Ingrid van den Berg, Johanna Schild, Kelly Goris, Demitri Demirel and Robert de Zeeuw for their technical assistance, and Marcel Dicke and Dick Peeters of the Wageningen University for facilitating Western flower thrips experiments. This research was supported by VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which is subsidized by the Netherlands Organization of Scientific Research, and by NIH grant 1R01-GM69594 to Dr. Xinnian Dong who supported SHS.

# Supplemental figures




**Supplemental Figure S1.** ACC modulates the NPR1 dependency of SA-mediated suppression of MeJA-induced *VSP2* expression in Arabidopsis seedlings .
Northern blot analysis of *PR-1* and *VSP2* mRNA levels in Col-0 (A) and *npr1-1* (B) seedlings that were treated with SA, MeJA, or a combination of both chemicals in the absence or presence of the ET precursor ACC. Pharmacological assays were performed with seedlings that were grown for 12 days on MS medium after which they were transferred to fresh MS medium supplemented with increasing concentrations of ACC and either 0.5 mM SA, 0.02 mM MeJA, or a combination of both chemicals. Seedlings were harvested after 2 days for RNA extraction. Ribosomal RNA is presented as a loading control.




**Supplemental Figure S2.** Differential effect of SA-mediated suppression of MeJA-responsive *PDF1.2* expression in mutants *npr1-1* and *npr1-3*.
Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in 5-week-old Col-0, *npr1-1,* and *npr1-3* plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 6 h after chemical treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. *PR-1* gene expression was completely blocked in both *npr1-1* and *npr1-3.* SA-mediated suppression of MeJA-induced *PDF1.2* expression, as observed in SA+MeJA-treated Col-0 plants, was abolished in the *npr1-1* background. However, in mutant *npr1-3 PDF1.2* gene expression was substantially suppressed in the SA+MeJA treatment when compared to the MeJA treatment alone, suggesting that cytoplasmatically localized truncated NPR1 protein in *npr1-3* is still partially functional in the regulation of SA-mediated suppression of *PDF1.2* gene expression.

CHAPTER 4

# Potentiation of jasmonate signaling by ethylene counteracts salicylate-mediated suppression of the jasmonate response via the AP2/ERF transcription factor ORA59

**Antonio Leon-Reyes[1], Du Yujuan[1], Annemart Koornneef[1], Silvia Proietti[3], Ana Korbes[2], Johan Memelink[2], Corné M.J. Pieterse[1] and Tita Ritsema[1]**

[1] Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, the Netherlands
[2] Institute of Biology Leiden, Clusius Laboratory, Leiden University, 2333 AL Leiden, the Netherlands
[3] Dipartimento di Agrobiologia e Agrochimica, Universita' della Tuscia, Viterbo, 01100, Italy

**Abstract**

Jasmonates (JAs), ethylene (ET), and salicylic acid (SA) are plant hormones with important regulatory roles in induced defense against harmful pathogens and insects. Their signaling pathways are interconnected providing the plant with a great regulatory potential to tailor its defense response to the invader encountered. Antagonism between SA and JA signaling is thought to operate as a mechanism to fine-tune defenses that are activated in response to multiple attackers. In *Arabidopsis thaliana*, pharmacological assays revealed that transcription of JA-responsive marker genes, such as *PDF1.2* and *VSP2*, is highly sensitive to suppression by SA. In this study, 45 well-characterized Arabidopsis mutant and transgenic lines that are affected in hormone signaling or other defense-related processes were screened for their ability to express SA-mediated suppression of JA-responsive gene expression. From this screen, mutant *cev1* (*constitutive expression of VSP1*), which has constitutive expression of JA and ET responses, appeared to be insensitive to SA-mediated suppression of *PDF1.2* and *VSP2* transcription. Accordingly, strong activation of the JA and ET response by the necrotrophic pathogens *Botrytis cinerea* and *Alternaria brassicicola* prior to SA treatment counteracted the ability of SA to suppress JA-responsive gene expression. Pharmacological assays, mutant analysis, and studies with the ET-signaling inhibitor 1-methylcyclopropene (1-MCP) revealed that ET-mediated potentiation of the JA response plays an important role in neutralizing the antagonistic effect of SA on JA signaling. The AP2/ERF transcription factor ORA59 is an important integrator of the JA and ET signaling pathways. The *ORA59* gene is activated by JA and ET, and necrotrophic pathogens. Overexpression of *ORA59* in transgenic *35S:ORA59* plants and in wild-type Col-0 protoplasts transiently expressing *35S:ORA59* resulted in insensitivity to SA-mediated suppression of *PDF1.2* transcription. Collectively, these results point to a model in which ET potentiates the JA response through the action of ORA59. Above a threshold level of ORA59 activity, SA-mediated suppression of JA-responsive gene expression is blocked. Strong induction of the JA and ET pathways by necrotrophic pathogens renders the plant insensitive for future SA-mediated suppression of JA-dependent defenses, which may prioritize the JA/ET-pathway over the SA-pathway during multi-attacker interactions.

## Introduction

During evolution, plants acquired several layers of defense to protect themselves against a large variety of harmful pathogens and insects. The first layer of defense that attackers encounter is based on pre-formed structural and chemical barriers (Walters et al., 2007). When attackers overcome this pre-invasive layer of defense,

inducible defense responses can be activated to prevent further pathogen ingress. The phytohormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) play pivotal roles in the regulation of these induced defenses (Dong, 1998; Howe, 2004; Pozo et al., 2004; Grant and Lamb, 2006a; Van Loon et al., 2006a; Loake and Grant, 2007; Von Dahl and Baldwin, 2007; Vlot et al., 2008; Pieterse et al., 2009). The accumulation of these hormones triggers the activation of a cascade of defense signaling pathways. However, the final outcome of the defense response is greatly influenced by the production, timing and composition of the hormonal blend produced (De Vos et al., 2005; Mur et al., 2006; Koornneef et al., 2008b; Pieterse et al., 2009). Although there are exceptions, in general it can be stated that SA-dependent defenses are active against pathogens with a biotrophic lifestyle, whereas JA/ET-dependent defenses are active against pathogens with a necrotropic lifes style (Glazebrook, 2005) and insect herbivores (Kessler and Baldwin, 2002; Howe, 2004). Besides SA, JAs, and ET, other phytohormones, such as abscisic acid (ABA), auxins, brassinosteriods, cytokinins, and gibberellins have been shown to affect defense signaling as well, but their role in plant defense is less well characterized (Pieterse et al., 2009).

In nature, plants often deal with simultaneous or subsequent invasion by multiple pathogens and insects, which can influence the primary induced defense response of the host plant (Van der Putten et al., 2001; Bezemer and Van Dam, 2005; Stout et al., 2006; Poelman et al., 2008b). Since activation of plant defense mechanisms is associated with ecological fitness costs (Walters and Heil, 2007), plants need regulatory mechanisms to effectively adapt to changes in their environment. Recent advances in defense signaling research revealed that SA, JAs and ET function in a complex network of interconnecting signaling pathways (Pieterse et al., 2009). Interactions between these pathways provide the plant with a powerful regulatory potential that may allow the plant to tailor its defense response to the invaders encountered (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Bostock, 2005; Pieterse and Dicke, 2007; Pieterse et al., 2009).

One of the best-studied examples of signal cross-talk is the antagonistic interaction between SA and JA signaling. Many studies have demonstrated that endogenously accumulating SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent resistance over JA-dependent defense (Bostock, 1999; Felton and Korth, 2000; Kunkel and Brooks, 2002; Thaler et al., 2002b; Glazebrook et al., 2003; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Spoel and Dong, 2008). For example, induction of the SA pathway in Arabidopsis by exogenous application of SA or infection by the SA-inducing pathogen *Pseudomonas syringae* suppressed JA signaling and rendered infected leaves more susceptible to the necrotrophic fungus *Alternaria brassicicola* (Spoel et al., 2007). Similarly, the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* strongly suppressed JA-mediated defenses that were activated upon feeding by caterpillars

of the small cabbage white *Pieris rapae* (Koornneef et al., 2008b). Pharmacological experiments with Arabidopsis revealed that JA-responsive marker genes, such as *PLANT DEFENSIN 1.2* (*PDF1.2*) and *VEGETATIVE STORAGE* PROTEIN2 (*VSP2*), are highly sensitive to suppression by exogenous application of SA (Spoel et al., 2003; Koornneef et al., 2008b). SA-mediated suppression of JA-responsive gene expression was observed in a large number of Arabidopsis accessions collected from very different geographic origins, highlighting the potential significance of this phenomenon in the regulation of induced plant defenses in nature (Koornneef et al., 2008b).

Although many reports describe an antagonistic interaction between SA- and JA-dependent signaling, synergistic interactions have been described as well (Schenk et al., 2000; Van Wees et al., 2000; Mur et al., 2006). For example in Arabidopsis, treatment with low concentrations of methyl JA (MeJA) and SA resulted in a synergistic effect on the JA- and SA-responsive genes *PDF1.2* and *PR-1* (*PATHOGENESIS-RELATED-1*), respectively. However, at higher concentrations the effects were antagonistic, demonstrating that the outcome of the SA-JA interaction is dependent on the relative concentration of each hormone (Mur et al., 2006). Koornneef et al. (2008b) demonstrated that timing and sequence of initiation of SA and JA signaling are also important for the outcome of the SA-JA signal interaction. Hence, the kinetics of phytohormone biosynthesis and signaling during the interaction of a plant with its attacker(s) could be highly decisive in the final outcome of the defense response to the attacker encountered.

Interplay between defense pathways may provide the plant with a powerful regulatory potential, it is also a possible target for plant attackers to manipulate the plant defense signaling network for their own benefit (Pieterse and Dicke, 2007; Robert-Seilaniantz et al., 2007; Pieterse et al., 2009). A well-studied example of a pathogen that suppresses the host's innate immune response by manipulating the plant's defense signaling network is *P. syringae* (Nomura et al., 2005). *P. syringae* is able to inject different virulence effector proteins into the host cell that suppress host defense responses. One of these virulence factors is the phytotoxin coronatine, which functions as a JA mimic and suppresses SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Kloek et al., 2001; Zhao et al., 2003; Brooks et al., 2005; Cui et al., 2005; Laurie-Berry et al., 2006; Uppalapati et al., 2007).

Several proteins with an important regulatory role in SA-JA cross-talk have been identified in Arabidopsis. Mutation or ectopic expression of the corresponding genes were shown to have contrasting effects on SA and JA signaling and on resistance against biotrophs and necrotrophs (Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Pieterse et al., 2009). The defense regulatory protein NPR1 (NONEXPRESSOR OF *PR* GENES) was identified as a key signaling node in the interaction between the SA and JA pathways, as mutant *npr1* plants

were blocked in SA-mediated suppression of JA-responsive genes (Spoel et al., 2003). Other molecular players in the regulation of the SA-JA signal interaction are the mitogen-activated protein kinase MPK4 (Petersen et al., 2000), the lipase-like proteins EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) and PAD4 (PHYTOALEXIN-DEFICIENT4) (Brodersen et al., 2006), the fatty acid desaturase SSI2 (SUPPRESSOR OF SA INSENSITIVITY2) (Kachroo et al., 2001), the glutaredoxin GRX480 (Ndamukong et al., 2007), and WRKY transcription factor proteins such as WRKY70 (Li et al., 2004). The majority of the identified cross-talk regulators play pivotal roles in SA signal transduction in which NPR1 plays a central role. NPR1 acts downstream of EDS1 and PAD4 in the SA signaling pathway (Brodersen et al., 2006). In addition, NPR1 regulates the SA-mediated expression of *GRX480* and *WRKY70*, which encode proteins that suppress JA-dependent gene expression (Li et al., 2004; Ndamukong et al., 2007). Recently, it was demonstrated that ET bypasses the need of NPR1 in SA-JA cross-talk, while it enhances NPR1-dependent, SA-responsive *PR-1* expression (De Vos et al., 2006; Leon-Reyes et al., 2009). These findings indicate that the final outcome of the SA-JA signal interaction during the complex interaction of plants with their attackers can be shaped by ET.

In many cases, ET has been shown to act as an important modulator of plant responses to SA and JAs (Adie et al., 2007a; Kazan and Manners, 2008). The interaction between ET and JAs is often a synergistic one. A classic example is the regulation of *PDF1.2*, which requires concomitant activation of the JA and the ET response pathway (Penninckx et al., 1998). Two members of the large plant-specific APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) superfamily of transcription factors, ERF1 and ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS 59), emerged as principal integrators of the JA and ET signaling pathways (Lorenzo et al., 2003; Pré et al., 2008). The expression of *ERF1* and *ORA59* is induced by JA and ET and is synergistically activated by both hormones. Overexpression of *ERF1* in the *ethylene insensitive2* mutant *ein2-1*, and the JA-insensitive mutant *coi1-1* and overexpression of *ORA59* in *coi1-1,* constitutively activated the *PDF1.2* gene, indicating that these transcription factors are important nodes of convergence of the JA and ET signaling pathways (Lorenzo et al., 2003; Pré et al., 2008). Ectopic expression of ERF1 was shown to enhance resistance of Arabidopsis to necrotrophic pathogens such as *Botrytis cinerea* and *Plectosphaerella cucumerina*, and *Fusarium oxysporum* (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004; Pré et al., 2008). Moreover, silencing of *ORA59* was shown to enhance susceptibility to *B. cinerea* (Pré et al., 2008), indicating that these AP2/ERF-type transcription factors play an important role in the regulation of JA/ET-dependent defenses. Another point of convergence between JA and ET signaling is CEV1 (CONSTITUTIVE EXPRESSOR OF VSP1) (Ellis and Turner, 2001), which is also known as cellulose synthase CeSA3 (Ellis et al., 2002b). Arabidopsis

*cev1* mutants constitutively express JA- and ET-dependent responses, as evidenced by high *PDF1.2* and *VSP2* transcript levels and enhanced pathogen resistance (Ellis and Turner, 2001; Ellis et al., 2002a).

In this study we searched for novel components involved in the regulation of SA-JA cross-talk. Since plant defenses are controlled by a signaling network in which various defense-related signaling pathways are interconnected, we screened 45 well-characterized Arabidopsis mutants and transgenic lines with an altered phytohormone or defense-related phenotype for their ability to display SA-mediated suppression of JA-responsive expression of *PDF1.2* or *VSP2*. Here we show that mutant *cev1* is impaired SA-JA cross-talk. Moreover, we provide evidence that the synergistic effect of ET on JA signaling is responsible for counteracting the antagonistic effect of SA on JA signaling, and that this is regulated via the AP2/ERF transcription factor ORA59.

## Results

### Screening for novel key players of SA-JA crosstalk

To investigate the interaction between SA and JA signaling we used a previously described SA-JA cross-talk assay (Spoel et al., 2003; Koornneef et al., 2008b; Leon-Reyes et al., 2009). In brief, 5-week-old Arabidopsis plants were treated with 1 mM SA, 0.1 mM methyl JA (MeJA), or a combination of these treatments, after which the expression of the SA-responsive marker gene *PR-1* and the JA-responsive marker genes *PDF1.2* and *VSP2* was analyzed. In wild-type Col-0 plants, the JA-responsive marker genes are typically suppressed by SA, while in mutant *npr1-1* plants SA-JA cross-talk is blocked (Fig. 1A). Since plant defense responses are regulated by a complex network of interconnecting signaling pathways (Kazan and Manners, 2008; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Pieterse et al., 2009), we attempted to identify novel key players in SA-JA cross-talk by screening 45 well-characterized hormone- and defense-related mutants and transgenic lines for their ability to display SA-mediated suppression of JA-responsive gene expression. The Arabidopsis genotypes used in this screen (Fig. 1B) were affected in biosynthesis of or the response to the phytohormones SA, JAs, ET, ABA, auxin, or gibberellin, or in redox regulation or production of antimicrobial camalexin. Five-week-old plants were treated with MeJA or a combination of MeJA and SA. Twenty-four hours later leaf tissue was harvested and the expression of *PDF1.2* was assessed by northern blot analysis and quantified using a Phosphor imager. For those genotypes in which *PDF1.2* was not expressed, we analyzed the expression level of the JA-responsive marker gene *VSP2* (mutants indicated with a Δ in Fig. 1B). In Fig. 1B the results of the SA-JA cross-talk experiments with the 45 Arabidopsis genotypes is depicted. In all genotypes tested, the single MeJA treatment resulted

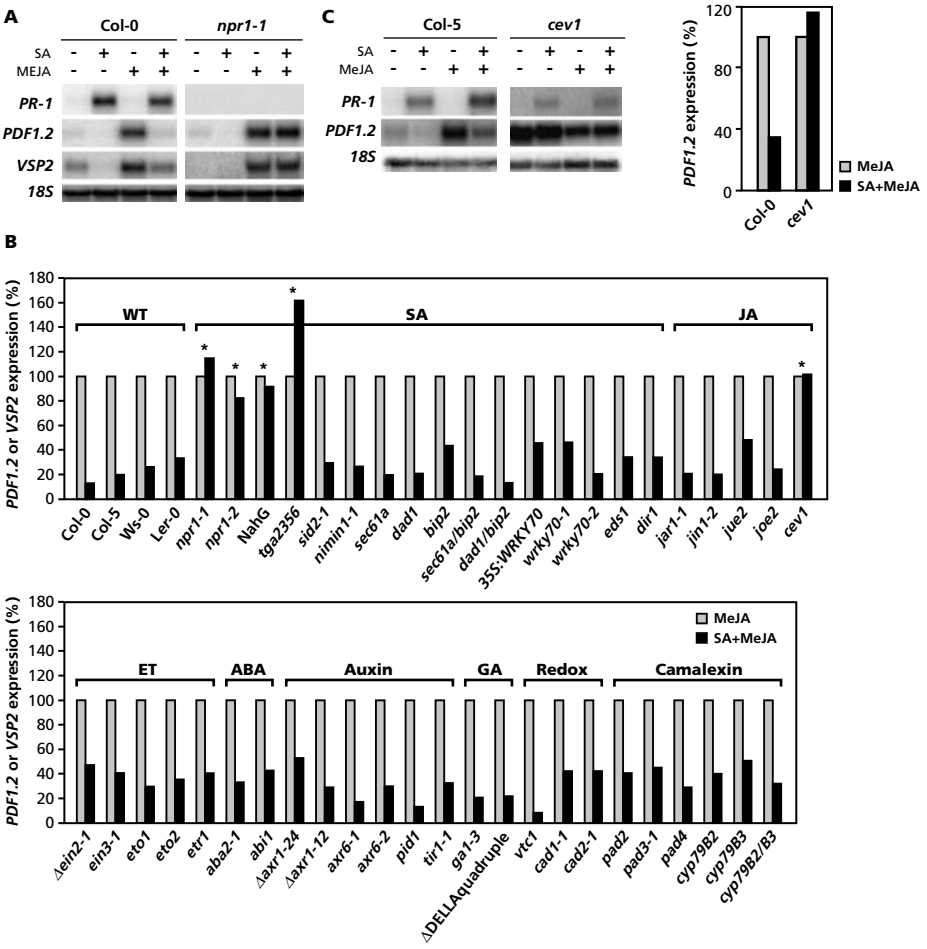



**Figure 1.** **Quantification of SA-JA cross-talk in Arabidopsis genotypes affected in hormone- or defense-related responses.**

**(A) Northern blot analysis of the SA-responsive gene *PR1* and the JA-responsive genes *PDF1.2* and *VSP2* in Col-0 and *npr1-1* after treatment with 1 mM SA, 0.1 mM MeJA or a combination of both chemicals. Leaf tissue was harvested 24 h after chemical treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA.**

**(B) Analysis of *PDF1.2* or *VSP2* (Δ) gene expression in 5-week-old Arabidopsis genotypes after treatment with 0.1 mM MeJA or a combination of 0.1 mM MeJA and 1 mM SA. Leaf tissue was harvested 24 h after chemical treatment. Signal intensities on northern blots were quantified using a Phosphor imager. *PDF1.2* and *VSP2* (Δ) transcript levels in the single MeJA treatments were set at 100%. Asterisks indicate genotypes in which SA-mediated suppression of JA-responsive gene expression in the combination treatment is less than 50% of that in the single MeJA treatment. SA-related genotypes (genetic background in parenthesis): *npr1-1* [Col-0], *npr1-2* [Col-0], NahG [Col-0], *tga2-1/tga3-1/tga5-1/tga6-1* quadruple mutant [Col-0], *sid2-1* [Col-0], *nimin1-1* [Col-0], *sec61a* [Col-0], *dad1* [Col-0], *bip2* [Col-0], *sec61a/bip2* [Col-0], *dad1/bip2* [Col-0], *35S:WRKY70* [Col-0], *wrky70-1* [Col-0], *wrky70-2* [Col-0], *eds1* [Ler-0], *dir1* [Ws-0]; JA-related genotypes: *jar1-1* [Col-0], *jin1-2* [Col-0], *jue2* [Col-0], *joe2* [Col-0] and *cev1* [Col-5]; ET-related genotypes: *ein2-1* [Col-0], *ein3-1* [Col-0], *eto1* [Col-0], *eto2* [Col-0], and *etr1* [Col-0]; ABA-related genotypes *aba2-1* [Col-0] and *abi1* [Ler-0]; auxin-related genotypes: *axr1-24* [Col-0], *axr1-12* [Col-0], *axr6-1* [Col-0], *axr6-2* [Col-0], *pid1* [Ler-0], and *tir1-1* [Col-0]; gibberelin (GA)-related genotypes: *ga1-3* [Ler-0] and DELLA-quadruple [Ler-0]; redox-related genotypes: *vtc1* [Col-0], *cad1-1* [Col-0] and *cad2-1* [Col-0]; camalexin-related genotypes: *pad2* [Col-0], *pad3-1* [Col-0], *pad4* [Col-0], *cyp79B2* [Ws-0], *cyp79B3* [Ws-0] and *cyp79B2B3* [Ws-0].**

**(C) Northern blot analysis of the *PR-1* and *PDF1.2* transcript levels in 5-week-old Col-5 and *cev1* plants that were treated or not with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 24 h after chemical treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. *PDF1.2* transcript levels in the single MeJA treatments were set at 100%.**

in the induction of *PDF1.2* or *VSP2* (transcript levels set at 100%). SA suppressed MeJA-induced *PDF1.2* expression by 70 to 85% in wild-type Col-0, Col-5, L*er*-0 and Ws-0 plants, confirming previous findings (Koornneef et al., 2008b). In the SA-related mutants *npr1-1, npr1-2* and transgenic SA-degrading NahG plants, SA did not suppress MeJA-induced *PDF1.2* expression, corroborating previous findings that SA-activated NPR1 is required for SA-JA cross-talk (Spoel et al., 2003). NPR1 was shown to interact with TGA transcription factors, which play an important role in the regulation of SA-responsive *PR* genes (Després et al., 2000; Dong, 2004; Kesarwani et al., 2007). The quadruple mutant *tga2-1/tga3-1/tga5-1/tga6-1,* which is impaired in four of the seven TGA transcription factors that interact with NPR1, and is affected in its ability to mount systemic acquired resistance (SAR) and *PR*-gene expression (Kesarwani et al., 2007), was also incapable of suppressing JA-responsive gene expression by SA (Fig. 1B). This is in line with experiments performed by Ndamukong et al. (2007), who showed that the triple mutant *tga2-1/tga5-1/tga6-1* is blocked in its ability to display SA-JA cross-talk. All other genotypes with an aberrant phenotype in SA signaling displayed near wild-type levels of SA-JA cross-talk, suggesting that the corresponding proteins have no major effect on the antagonistic effect of SA on JA signaling.

Of all other genotypes tested, only mutant *cev1* was blocked in SA-JA cross-talk (Fig. 1B). Mutant *cev1* was originally identified as a mutant with constitutively enhanced JA and ET signaling (Ellis and Turner, 2001). As a result, *cev1* displayed high *PDF1.2* transcript levels in all treatments (Fig. 1C). In both wild-type Col-5 and mutant *cev1* plants, the SA treatment triggered the expression of *PR-1*. In Col-5 plants, MeJA-induced *PDF1.2* expression was suppressed by SA when SA and MeJA were simultaneously applied. However, in the *cev1* mutant SA had no negative effect on the *PDF1.2* transcript levels (Fig. 1C) and *VSP2* (data not shown). These results point to a model in which stimulation of JA and ET signaling prior to activation of the SA response renders Arabidopsis plants insensitive to SA-mediated suppression of JA-responsive gene expression. This hypothesis is further investigated in this study.

### ET plays a dominant role in counteracting SA-mediated suppression of *PDF1.2*

To investigate whether JA or ET signaling is responsible for neutralizing the antagonistic effect of SA on JA signaling, we made used of mutant *cas1* (*constitutive allene oxide synthase1*) (Kubigsteltig and Weiler, 2003), which exhibits a constitutive JA response, and mutant *ctr1* (*constitutive triple response1*) (Kieber et al., 1993), which displays a constitutive ET response. Five-week-old plants were subjected to the pharmacological SA-JA cross-talk assay. As expected, mutant *cas1* plants (in C24 background) constitutively expressed *PDF1.2* in mock-treated plants (Fig. 2A). Treatment with SA or SA and MeJA resulted in suppression of *PDF1.2* expression

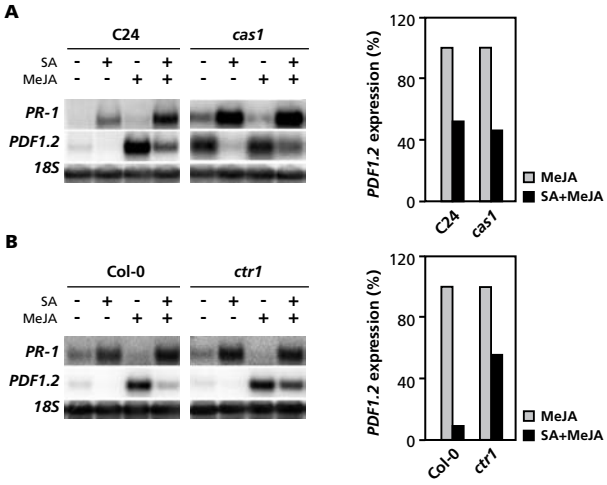



**Figure 2. SA-mediated suppression of *PDF1.2* in *cas1* and *ctr1*.**
**Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in C24 and *cas1* (constitutive JA response) plants (A), and in Col-0 and *ctr1* (constitutive ET response) plants (B) that were treated or not with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 24 h after chemical treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panels). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

to a level that was comparable to that observed in wild-type C24 plants, suggesting that constitutive expression of JA-dependent responses alone has no negative effect on the ability of SA to suppress JA-responsive gene expression. On the other hand, constitutive expression of the ET response in mutant *ctr1* (in Col-0 background) strongly reduced the suppressive effect of SA on MeJA-induced *PDF1.2* expression (Fig. 2B). In Col-0, SA suppressed the level of *PDF1.2* transcription by 85%, whereas in *ctr1* the level of suppression was only 40% (Fig. 2B, right panel). These results suggest that ET plays a dominant role in counteracting the suppressive effect of SA on MeJA-induced expression of *PDF1.2*.

To corroborate the genetic evidence obtained with *cas1* and *ctr1*, we followed a pharmacological approach in which we stimulated the JA response with increasing concentrations of MeJA or with 0.1 mM MeJA and increasing concentrations of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC). First, Col-0 plants were treated with 0.1, 0.5, 1.0, or 2.0 mM MeJA. The plants were allowed to activate the JA response for 24 h after which 1 mM SA was applied to the leaves. Twenty-four h after SA treatment, *PDF1.2* transcript levels were assessed to monitor the antagonistic effect of SA on JA-responsive gene expression. Fig. 3A shows that SA suppressed MeJA-induced *PDF1.2* expression to a similar extent for all MeJA concentrations used, suggesting that the strength of the activated JA response does not affect the capacity of SA to suppress JA signaling.

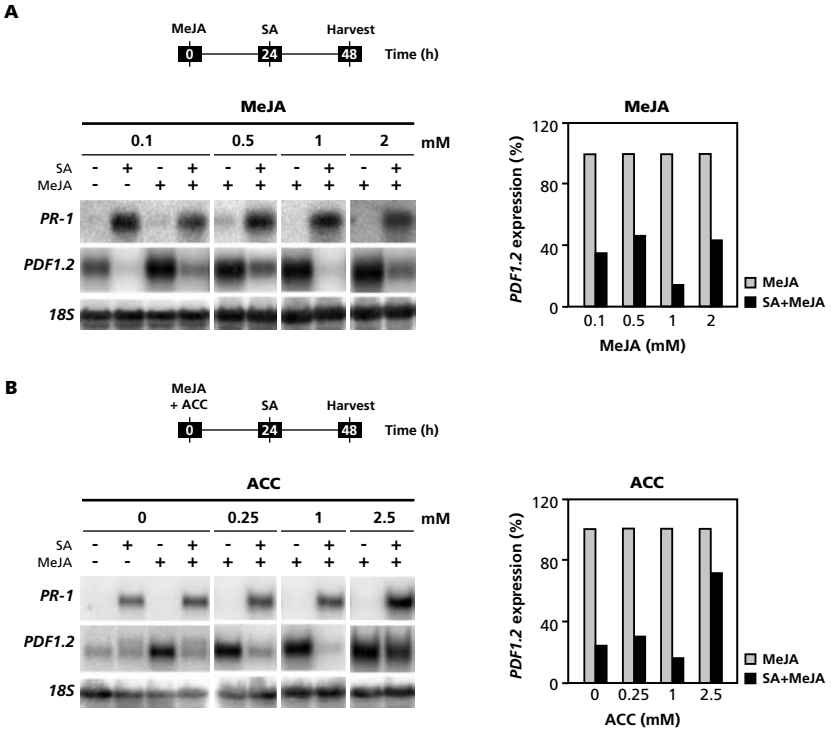



**Figure 3. Effect of increasing concentrations of MeJA and ACC on SA-mediated suppres-sion of MeJA-induced *PDF1.2* expression.**

**Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in Col-0 plants that were treated with (A) increasing concentrations of MeJA (0.1, 0.5, 1.0, or 2.0 mM), or (B) 0.1 mM MeJA and increasing concentrations of ACC (0, 0.25, 1.0 or 2.5 mM). Twenty-four h later plants were treated with 1 mM SA or received a mock treatment. Leaf tissue was harvested 24 h after application of SA for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panels). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

To investigate the effect of ET, 5-week-old plants were treated with 0.1 mM MeJA and either 0.25, 1.0, or 2.5 mM ACC. One day later, the plants were treated with SA and 24 h later harvested for RNA analysis. In the absence of SA, ACC potentiated the expression of MeJA-induced *PDF1.2* (Fig. 3B), supporting previous findings (Penninckx et al., 1998). SA strongly suppressed MeJA-induced *PDF1.2* transcription in the presence of up to 1 mM ACC. However, when 2.5 mM ACC was applied, the antagonistic effect of SA on MeJA-induced *PDF1.2* expression was strongly reduced. These data support the genetic evidence provided in Fig. 2 that under conditions in which JA- and ET-dependent responses are strongly expressed, ET is predominantly responsible for neutralizing the antagonistic effect of SA on JA-responsive gene expression.

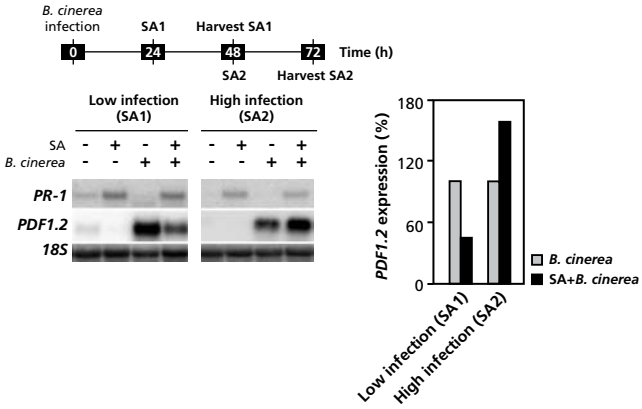



**Figure 4.** ***B. cinerea*** **infection renders Arabidopsis plants insensitive to SA-mediated suppression of** ***PDF1.2*** **expression.**
**Northern blot analysis of** ***PR-1*** **and** ***PDF1.2*** **transcript levels in Col-0 plants that were inoculated with the necrotropic pathogen** ***B. cinerea.*** **After 24 and 48 h, plants had developed mild (low infection) and severe (high infection) disease symptoms, respectively. At 24 (SA1) or 48 h (SA2) after inoculation, plants were treated or not with 1 mM SA and 24 h later harvested for RNA analysis. Equal loading of RNA samples was checked using a probe for** ***18S*** **rRNA. Signal intensities were quantified using a Phosphor imager (right panel).** ***PDF1.2*** **transcript levels in the** ***B. cinerea*****-inoculated plants that were not treated with SA were set to 100%.**

### Pathogen-induced JA/ET signaling renders plants insensitive to SA-mediated suppression of JA-responsive gene expression

Our mutant and pharmacological analysis suggested that potentiation of the JA response by ET prior to induction of the SA response renders Arabidopsis plants insensitive to SA-mediated suppression of JA signaling. In a biological context this would mean that plants that are infected with a JA/ET-inducing pathogen become insensitive for future SA-mediated suppression of JA signaling. To test this hypothesis, we triggered the JA/ET-response in Col-0 plants by inoculating 5-week-old plants with the necrotrophic fungus *B. cinerea,* which has previously been shown to activate JA/ET-dependent responses (Thomma et al., 2000; Thomma et al., 2001b). Twenty-four h after inoculation, only mild disease symptoms were visible (low infection), whereas 48 h after inoculation the plants were severely diseased (high infection; data not shown). Plants with a low or high infection level were treated with 1 mM of SA and 24 h later the ability of SA to suppress *B. cinerea*-induced *PDF1.2* expression was assessed. In Col-0 plants with a low infection level, *B. cinerea*-induced *PDF1.2* expression was suppressed by SA (Fig. 4, left panel). However, in highly infected plants, this suppression was not apparent (Fig. 4, right panel). These results suggest that severe infection with a pathogen that triggers JA- and ET-dependent responses can counteract the ability of SA to suppress JA signaling.

To further substantiate this finding we used the necrotrophic fungus *A. brassicicola* to stimulate JA and ET responses. Previously, we demonstrated that *A. brassicicola* strongly activates JA and ET biosynthesis and signaling in Arabidopsis

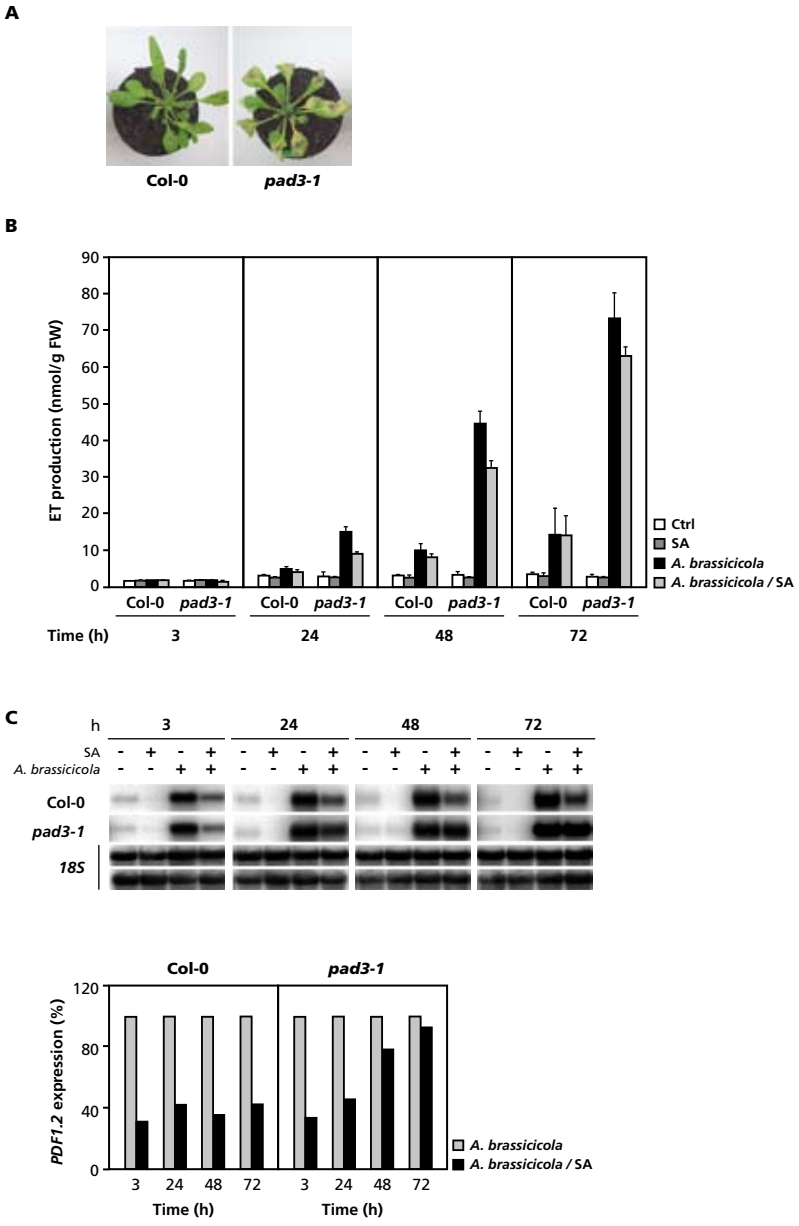



**Figure 5.** High ET levels in susceptible *A. brassicicola*-infected *pad3-1* plants correlate with inability of SA to suppress *A. brassicicola*-induced *PDF1.2* expression.
(A) Disease symptoms in resistant Col-0 and susceptible *pad3-1* plants, 5 days after inoculation with *A. brassicicola.* (B) Kinetics of ET production in Col-0 and *pad3-1* plants after inoculation with *A. brassicicola* and treatment with SA. (C) Northern blot analysis of *PDF1.2* transcript levels in Col-0 and *pad3-1* plants that were inoculated with *A. brassicicola* and treated with 1 mM SA at 3, 24, 48 and 72 h post-inoculation. Leaf tissue was harvested for RNA analysis 24 h after application of SA. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panel). *PDF1.2* transcript levels in *A. brassicicola* inoculated plants that were not treated with SA were set to 100%.

without stimulating the SA pathway (De Vos et al., 2005). We made use of two Arabidopsis genotypes with different levels of resistance against this pathogen: Col-0, which is resistant, and the phytoalexin-deficient mutant *pad3-1*, which is susceptible (Thomma et al., 1999). Upon inoculation with *A. brassicicola,* Col-0 developed necrotic lesions that remained contained to the inoculation site, whereas *pad3-1* plants developed rapidly spreading lesions (Fig. 5A). In the absence of SA, *A. brassicicola* induced similar levels of *PDF1.2* transcription in Col-0 and *pad3-1* at 3, 24, 48 and 72 h after inoculation (Fig. 5C). However, susceptible *pad3-1* plants accumulated significantly higher levels of ET upon *A. brassicicola* infection than resistant Col-0 plants (Fig. 5B). Application of SA at 3, 24, 48 or 72 h after inoculation strongly suppressed *A. brassicicola*-induced *PDF1.2* expression in Col-0. However, in *pad3-1* the antagonistic effect of SA on *A. brassicicola*-induced *PDF1.2* expression was notably reduced at the time points 48 and 72 h. At these time points, the infected *pad3-1* plants produced large amount of ET, again suggesting that potentiation of the JA response by ET, such as upon infection by *A. brassicicola,* renders the plant tissue insensitive to SA-mediated suppression of JA signaling.

### 1-MCP restores sensitivity to SA-mediated suppression of *PDF1.2*

Since our genetic and pharmacological experiments suggested that ET plays a dominant role in counteracting the antagonistic effect of SA on JA signaling (Fig. 2 and 3), we tested whether inhibition of ET signaling in *A. brassicicola*-infected *pad3-1* plants can restore the sensitivity of the tissue to SA-mediated suppression of JA signaling. To this end, we performed SA-JA cross-talk assays with the ET signaling inhibitor 1-methylcyclopropene (1-MCP). Five-week-old *pad3-1* plants were treated with 1 $\mu L.L^{-1}$ of 1-MCP or air for 2 h after which the plants were inoculated with *A. brassicicola*. At 24, 48, and 72 h after inoculation, the plants were treated or not with 1 mM of SA and one day later leaf tissue was harvested for RNA analysis. The 1-MCP treatment was repeated daily for the duration of the experiment. In the absence of 1-MCP, the antagonistic effect of SA on *A. brassicicola* induced-*PDF1.2* expression was visible at 24 h after inoculation (~40%), but at the 48 and 72 h time points, the suppressive effect of SA was clearly reduced (~10%; Fig. 6). In the presence of 1-MCP, *PDF1.2* mRNA levels were lower in *A. brassicicola*-inoculated plants. Since JA and ET signaling are co-required for *PDF1.2* expression (Penninckx et al., 1998), this observation confirms that 1-MCP inhibited the ET pathway. In plants treated with 1-MCP, SA-mediated suppression of *A. brassicicola*-induced *PDF1.2* was clearly more pronounced at all time points (Fig. 6), suggesting that ET plays a main role in counteracting the antagonistic effect of SA on JA signaling.

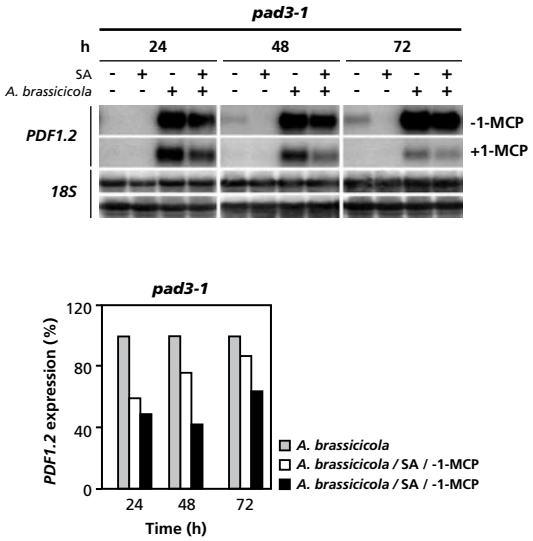



**Figure 6. Inhibition of ET signaling with 1-MCP restores SA-mediated suppression of *A. brassicicola*-induced *PDF1.2* expression.**
**Northern blot analysis of *PDF1.2* transcript levels in *pad3-1* plants that were pre-treated with the ET inhibitor 1-MCP (1 μL·L$^{-1}$) or air and inoculated with *A. brassicicola*. At 24, 48 and 72 h after inoculation, plants were treated or not with 1 mM SA and 24 h later leaf tissue was harvested for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panel). *PDF1.2* transcript levels in *A. brassicicola*-inoculated plants that were not treated with SA were set to 100%.**

## ET-mediated inhibition of SA-JA antagonism coincides with expression of the AP2/ERF transcription factor gene *ORA59*

To identify possible molecular mechanisms underlying the observed interplay between ET, JA and SA signaling, we investigated the role of the AP2/ERF transcription factor ORA59 in this phenomenon. Previously, ORA59 was found to function as an important integrator of the JA and ET signaling pathways (Pré et al., 2008). Overexpression of ORA59 in JA-insensitive *coi1-1* resulted in high levels of *PDF1.2* expression, whereas silencing of *ORA59* blocked *PDF1.2* transcription (Pré et al., 2008), indicating that ORA59 is a dominant regulator of JA/ET-responsive genes that functions downstream of the F-box protein COI1. To investigate whether the inhibitory effect of ET on SA-JA antagonism is regulated at the level of ORA59, we analyzed the expression of *ORA59* in the SA-JA cross-talk assay in the presence or absence of gaseous ET. Col-0 plants were pre-treated with 10 μL.L$^{-1}$ gaseous ET or air for 6 h Thereafter, plants were treated or not with 1 mM SA, 0.1 mM MeJA or a combination of both chemicals and placed back in the ET or air treatment. Twenty-four h later, *ORA59* and *PDF1.2* expression was assessed. In the absence of additional ET, MeJA mildly activated *ORA59,* but this coincided with high expression levels of *PDF1.2* (Fig. 7A). In the ET-treated plants, *ORA59* transcripts accumulated in all four treatments. Albeit at an elevated level, *PDF1.2* followed the expression pattern of *ORA59*, confirming previous findings

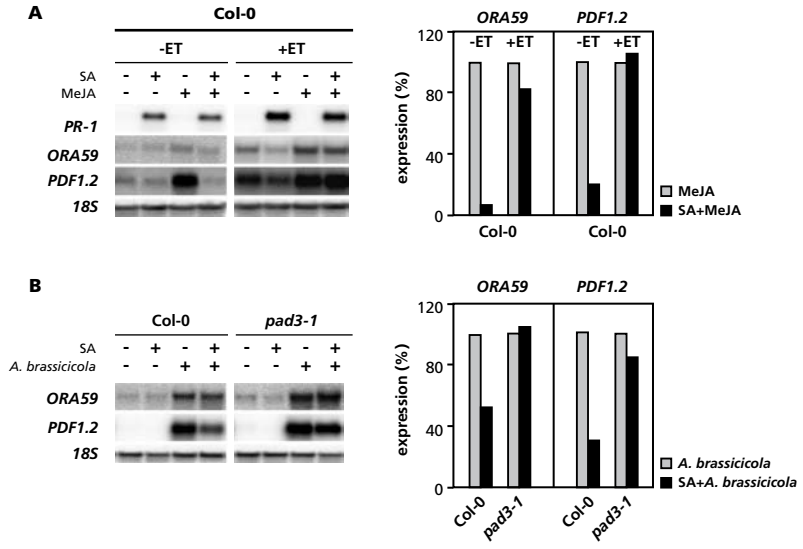



**Figure 7. SA-mediated suppression of *ORA59* and *PDF1.2* is inhibited by ET and the ET-inducing pathogen *A. brassicicola*.**

**Leaf tissue was harvested 24 h after SA and/or MeJA treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (Right panels). *ORA59* and *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

**(A) Northern blot analysis of *PR-1, ORA59* and *PDF1.2* transcript levels in Col-0 plants that were treated 10 $\mu L \cdot L^{-1}$ of gaseous ET or air. Six h after the start of the ET treatment, plants were treated or not with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. (B) Northern blot analysis of *ORA59* and *PDF1.2* transcript levels in Col-0 and *pad3-1* plants that were inoculated or not with the fungus *A. brassicicola*. Forty-eight h after inoculation, plants were treated or not with 1 mM SA.**

that *ORA59* is activated by ET and plays a central role in the regulation of *PDF1.2* gene expression (Pré et al., 2008). In the absence of additional ET, SA strongly suppressed MeJA-induced *ORA59* and *PDF1.2*. However, in ET-treated plants the antagonistic effect of SA on MeJA-responsive *ORA59* and *PDF1.2* expression was blocked. These results suggest that the inhibitory effect of ET on SA-JA antagonism is regulated via the transcription factor ORA59.

To corroborate these findings, we induced the JA response with *A. brassicicola* instead of MeJA (Fig. 7B). *A. brassicicola* induced the expression of *ORA59* in both resistant Col-0 and susceptible *pad3-1* plants. Similar to what we observed for *PDF1.2* in Fig. 5, SA suppressed *A. brassicicola*-induced *ORA59* transcription in Col-0 (low ET production), but not in *pad3-1* plants (high ET production). Again *PDF1.2* showed the same expression pattern as *ORA59*. Together, these results suggest that ORA59 plays a role in mediating the inhibitory effect of ET on SA-JA cross-talk.

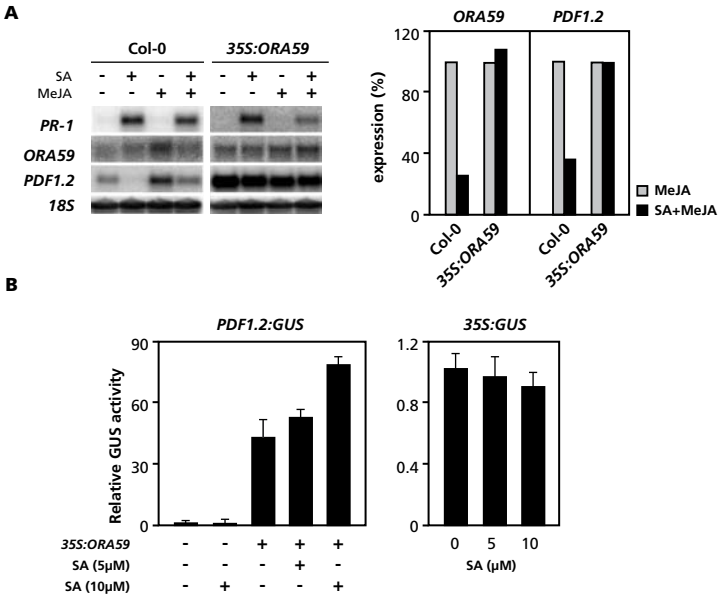



**Figure 8. Overexpression of ORA59 interferes with SA-mediated suppression of *PDF1.2*.**
**(A) Northern blot analysis of *PR-1, ORA59* and *PDF1.2* transcript levels in 5-week-old Col-0 and *35S:ORA59* plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 24 h after treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panel). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%. (B) Transient expression assays with protoplasts isolated from mesophyll cells of Col-0 plants. Protoplasts were transformed with plasmids containing *35S:ORA59*, *PDF1.2:GUS*, or *35S:GUS*, and treated or not with 5 or 10 μM SA immediately after the transformation. After 24 h of incubation in the dark, the GUS activity was measured in the protoplasts of each treatment. A reference plasmid carrying the *Renilla LUC* gene fused to the *35S* promoter was co-transformed to correct for transformation and protein extraction efficiencies. Bars represent the average of GUS/LUC activity ratios from 5 transformations ± SE expressed relative to the vector control.**

### Overexpression of *ORA59* blocks SA-JA cross-talk

To provide further evidence for the role of ORA59 in ET-mediated inhibition of SA-JA cross-talk, we subjected the transgenic *ORA59*-overexpressing line *35S:ORA59* (Pré et al., 2008) to the SA-JA cross-talk assay. Transgenic *35S:ORA59* plants constitutively expressed *ORA59* and showed high constitutive levels of *PDF1.2* mRNA (Fig. 8A), confirming previous findings (Pré et al., 2008). In Col-0 plants MeJA activated both *ORA59* and *PDF1.2* transcription, which was strongly suppressed when SA was included in the treatment. However, in *35S:ORA59* plants the suppression of *PDF1.2* transcription by SA was completely blocked, indicating that overexpression of *ORA59* attenuates the antagonistic effect of SA on JA-responsive gene expression.

To substantiate these findings, we performed a transient expression assay using leaf mesophyll protoplasts. Protoplasts were isolated from 4-week-old Col-0 plants after which they were transfected with plasmid DNA using the polyethylene

glycol (PEG)-calcium transfection protocol described by Yoo et al. (2007). In this transient expression assay we monitored *PDF1.2* expression by determining the activity of the β-glucuronidase (*GUS*) gene that was fused to the *PDF1.2* promoter. Transformation of protoplasts with the *PDF1.2:GUS* reporter plasmid resulted in high levels of GUS activity when the *35S:ORA59* plasmid was co-transformed (Fig. 8B), indicating that ectopic expression of *ORA59* activated the *PDF1.2* promoter in the protoplasts. Application of 5 or 10 μM of SA activated *PR-1* gene expression (data not shown), demonstrating that the protoplasts were capable of activated the SA pathway. However, SA treatment did not result in a reduction of the GUS activity in protoplasts that were co-transformed with the *35S:ORA59* and *PDF1.2:GUS* plasmids. These results again indicate that overexpression of *ORA59* blocks the capacity of SA to suppress *PDF1.2* expression. SA had no effect on the GUS activity in protoplasts transformed with the *35S:GUS* plasmid (Fig. 8B), indicating that the results obtained with SA and the *35S:ORA59* plasmid are not affected by effects of SA on the *35S* promoter.

## Discussion

### Potentiation of JA-responsive gene expression by ET counteracts the antagonistic effect of SA on the JA response

Previously, we demonstrated that exogenous application of SA suppresses JA-responsive genes, such as *PDF1.2* and *VSP2* (Van Wees et al., 1999; Spoel et al., 2003; Koornneef et al., 2008b; Leon-Reyes et al., 2009). Also induction of the SA response upon infection by the biotrophic oomycete *H. arabidopsidis* antagonized JA-responsive gene expression (Koornneef et al., 2008b), suggesting that during multi-attacker interactions, the SA response is prioritized over the JA response in Arabidopsis. However, in the above-mentioned studies the SA response was either activated at the same time, or prior to the activation of the JA/ET response. In the present study we provide evidence that stimulation of JA- and ET-dependent responses prior to the activation of the SA response can render the plant insensitive to SA-mediated suppression of JA signaling. First we showed that the constitutive expressor of JA and ET responses *cev1* mutant, was insensitive to SA-mediated suppression of *PDF1.2* transcription (Fig. 1). Similarly, induction of the JA and ET response upon inoculation with the necrotrophic pathogens *B. cinerea* and *A. brassicicola* resulted in insensitivity of the leaf tissue to suppression of the JA response by SA (Fig. 4 and 5). Analysis with the overexpressor of the JA response *cas1* and the overexpressor of the ET response *ctr1*, revealed that ET plays a dominant role in counteracting the antagonistic effect of SA on JA-responsive gene expression (Fig. 2). This was corroborated by the observation that potentiation of the JA response by application of high concentrations of ACC similarly neutralized the

antagonistic effect of SA (Fig. 3). In addition, the ET signaling inhibitor 1-MCP abolished the effect of ET on SA-JA cross-talk during the Arabidopsis-*A. brassicicola* interaction. It can thus be concluded that potentiation of the JA response by ET renders Arabidopsis plants insensitive to future SA-mediated suppression of the JA response.

### ORA59 is an important regulator in counteracting SA-mediated antagonism

The interplay between JA and ET is one of the best-studied examples of synergistic defense signal interactions (Penninckx et al., 1998; Broekaert et al., 2006; Adie et al., 2007a). Simultaneous activation of the JA and ET response results in a boosted expression pattern of JA/ET-responsive genes, such as *PDF1.2* (Penninckx et al., 1998). The AP2-domain transcription factors, ERF1 and ORA59 have been described as important integrators of the JA and ET pathways (Lorenzo et al., 2003; Lorenzo and Solano, 2005; Pré et al., 2008). In the *PDF1.2*-overexpressing mutant *cev1, ORA59* was constitutively expressed (data not shown). Also MeJA- and *A. brassicicola*-induced *PDF1.2,* correlated with enhanced *ORA59* gene transcription (Fig. 7 and 8), supporting previous findings that ORA59 is an important transcriptional regulator of JA/ET-responsive gene expression (Pré et al., 2008). In this paper, we show that besides its role in the integration of the JA and ET pathway, ORA59 also plays an important role in counteracting SA-mediated suppression of *PDF1.2* expression. Overexpression of *ORA59* in stable *35S:ORA59* transformants resulted in constitutive *PDF1.2* expression, which could not be suppressed by SA (Fig. 7). Similarly, in mesophyll protoplasts overexpression of *ORA59* resulted in strong activation of the *PDF1.2* promoter, which was also insensitive to suppression by SA (Fig. 8). Overexpression of *ERF1* had a much smaller effect on counteracting the antagonistic function of SA (data not shown), suggesting that ORA59 plays a dominant role in this phenomenon.

This raises the question, how does ORA59 counteract the antagonistic effect of SA on JA-responsive gene expression? Previously, it was demonstrated that in uninduced cells ORA59 is localized in the cytosol, and that after activation by JA, ORA59 is translocated to the nucleus where it binds to the GCC-box motif in the promoters of JA-responsive genes (Zarei, 2007). A plausible explanation for the neutralizing effect of ORA59 on SA antagonism may be that the suppressive effect of SA on JA-responsive gene expression functions through the GCC-box as well. In this scenario, SA induces an inhibitor that binds to the GCC box, thereby blocking this site for positive activators of JA-responsive gene expression, such as ORA59. When ORA59 is strongly activated prior to activation of the SA response, then ORA59 outcompetes the putative SA-induced inhibitor resulting in insensitivity to SA-mediated suppression of JA-responsive gene expression (Fig. 9).

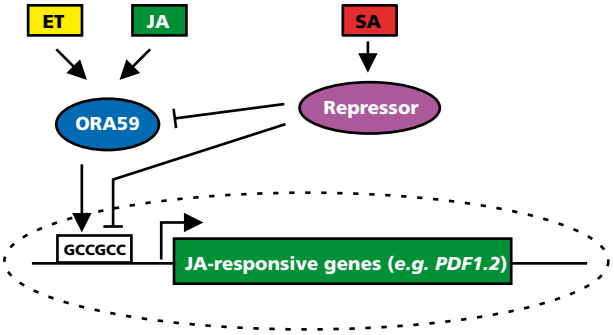



**Fig. 9. Model illustrating possible mechanism by which ORA59 counteracts the antagonistic effect of SA on JA-responsive gene expression.**

### Defense-related life history influences the immune response?

Our results clearly indicate that the final outcome of the interplay between SA, JA and ET signaling is dependent on the sequence in which these plant hormones are produced. If the SA pathway is activated prior, or at the same time as the JA response, then the SA response will suppress the JA pathway. When the JA pathway is activated first in the absence of ET, then SA can still suppress the JA pathway. However, when the JA and ET pathways are activated simultaneously, then the potentiated JA response becomes insensitive to suppression by SA. These findings provide novel insights into our notion that concentration, timing, and sequence of initiation of the SA, JA, and ET signaling pathways are important for the outcome of the SA-JA signal interaction (Mur et al., 2006; Koornneef et al., 2008b). The blend of defense signals produced during a plant-attacker interaction varies greatly and is highly dependent on the type of invader encountered (De Vos et al., 2005). Hence, the kinetics of phytohormone biosynthesis and signaling during the interaction of a plant with its attacker(s) could be highly decisive in the final outcome of the defense response to the attacker encountered.

In nature, plants often have to deal with multiple attackers. Ecological studies have shown that the induced defense response of a plant to a specific invader can be influenced by the history of the plant in terms of the type of attackers that the plant has encountered in the past (Poelman et al., 2008b). In view of our findings, it is tempting to speculate that when Arabidopsis is first attacked by a SA-inducing pathogen, the SA pathway will suppress JA responses. This will result in enhanced resistance against pathogens that are sensitive to SA-dependent defenses (e.g. biotrophs), but it will also lead to enhanced susceptibility to necrotophic pathogens and insects that are sensitive to JA-dependent defenses. Vice versa, when a plant is first attacked by a pathogen that strongly activates both the JA and the ET response, then these plants develop enhanced resistance to pathogens and insects that are sensitive to JA/ET-dependent defenses. When a secondary, SA-inducing pathogen

comes into play, the JA/ET-dependent defenses will remain active as they can not be suppressed by the SA response that is activated by this pathogen. It must be noted that the data we provide in this paper do not necessarily hold true for other plant species. In other plant species similar molecular mechanisms may operate but the order of signal prioritization may be different among plant species. This may be affected by the ecological context in which a plant species has evolved. A relatively high disease pressure of SA-inducing pathogens may have caused a different signal prioritization then a relatively high pressure of JA/ET-inducing pathogens or insect herbivores. The research described in this paper provides novel insight the interplay between SA-, JA- and ET-signaling and highlights the complexity of the induced defense signaling network that controls the immune response of plants to harmful organisms. Moreover, it provides a basis for future research on the regulation of plant defense responses in a multi-attacker context.

## Materials and methods

### Plant material

Seeds of *Arabidopsis thaliana* accession of Col-0, Col-5, L*er*-0, Ws-0, C24 and the mutant and transgenic genotypes *npr1-1* [Col-0] (Cao et al., 1994), *npr1-2* [Col-0] (Cao et al., 1994), NahG [Col-0] (Delaney et al., 1994), *tga2-1/tga3-1/tga5-1/tga6-1* [Col-0] (Kesarwani et al., 2007), *sid2-1* [Col-0] (Nawrath and Métraux, 1999), *nimin1-1* [Col-0] (Weigel et al., 2005), *sec61a* [Col-0] (Wang et al., 2005), *dad1* [Col-0] (Wang et al., 2005), *bip2* [Col-0] (Wang et al., 2005), *sec61a/bip2* [Col-0] (Wang et al., 2005), *dad1/bip2* [Col-0] (Wang et al., 2005), *35S:WRKY70* [Col-0] (Li et al., 2004), *wrky70-1* [Col-0] (Li et al., 2006), *wrky70-2* [Col-0] (Li et al., 2006), *eds1-1* [L*er*-0] (Falk et al., 1999), *dir1* [Ws-0] (Maldonado et al., 2002), *jar1-1* [Col-0] (Staswick et al., 1992), *jin1-2* [Col-0] (Lorenzo et al., 2004), *jue2* [Col-0] (Jensen et al., 2002), *joe2* [Col-0] (Jensen et al., 2002), *cev1* [Col-5] (Ellis and Turner, 2001), *aba2-1* [Col-0] (Koornneef et al., 1982), *abi1* [Ler] (Assmann et al., 2000), *pad2* [Col-0] (Glazebrook and Ausubel, 1994), *pad3-1* [Col-0] (Glazebrook and Ausubel, 1994), *pad4* [Col-0] (Glazebrook and Ausubel, 1994), *cyp79B2* [Ws-0] (Zhao et al., 2002), *cyp79B3* [Ws-0] (Zhao et al., 2002), *cyp79B2B3* [Ws-0] (Zhao et al., 2002), *ein2-1* [Col-0] (Guzmán and Ecker, 1990), *ein3-1* [Col-0] (Guzmán and Ecker, 1990), *eto1* [Col-0] (Wang et al., 2004), *eto2* [Col-0] (Woeste et al., 1999), *etr1-1* [Col-0] (Bleecker et al., 1988), *axr1-24* [Col-0] (Tiryaki and Staswick, 2002), *axr1-12* [Col-0] (Lincoln et al., 1990), *axr6-1* [Col-0] (Hellmann et al., 2003), *axr6-2* [Col-0] (Hellmann et al., 2003), *pid1* [Ler] (Lee and Cho, 2006), *tir1-1* [Col-0] (Ruegger et al., 1998), *ga1-3* [Ler], (Koornneef and Van der Veen, 1980), DELLA-quadruple [Ler] (Cheng et al., 2004), *vtc1* [Col-0] (Conklin et al., 1997), *cad1-1* [Col-0], (Howden et al., 1995), *cad2-1* [Col-0] (Howden et al., 1995), *cas1* [C24]

(Kubigsteltig and Weiler, 2003), *ctr1* [Col-0] (Kieber et al., 1993), and *35S:ORA59* line 19-2 [Col-0] (Pré et al., 2008) were sown in quartz sand. After two weeks, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min (Pieterse et al., 1998). Plants were cultivated in a growth chamber with an 8-h day (24°C) and 16-h night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10 mM Sequestreen (CIBA-Geigy) once a week.

### Pathogen bioassays

*Alternaria brassicicola* strain MUCL 20297 and *Botrytis cinerea* strain B0510 were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) plates for 2 weeks at 22°C. Subsequently, conidia were collected as described (Broekaert et al., 1990; Thomma et al., 1998). Plants were inoculated when 5 weeks old by applying 5-µL droplets of half-strength potato dextrose broth containing 5 x $10^5$ spores per mL, as described previously (Van der Ent et al., 2008).

### Chemical treatments

Plants were treated with SA, MeJA and/or ACC by dipping the leaves into a solution of 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands) containing the indicated concentrations of SA (Mallinckrodt Baker, Deventer, the Netherlands), MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), and/or ACC (Sigma, Schnelldorf, Germany), or a combination of these chemicals as described previously (Spoel et al., 2003; Koornneef et al., 2008b). Control treatments were dipped into a solution containing 0.015% (v/v) Silwet L77. MeJA was added to the solutions from a 1,000-fold concentrated stock in 96% ethanol. To the solutions without MeJA, a similar volume of 96% ethanol was added.

Application of gaseous ET to the plants was performed as described by Millenaar et al. (2005). In brief, gaseous ET (100 µL.$L^{-1}$; Hoek Loos, Amsterdam, the Netherlands) and air (70% relative humidity) were mixed using flow meters (Brooks Instruments, Veenendaal, the Netherlands) to generate an output concentration of 10 µL.$L^{-1}$ of ET, which was flushed continuously through glass cuvettes (13.5 x 16.0 x 29.0 cm) at a flow rate of 75 L·$hr^{-1}$ and then vented to the outside of the building. The concentration of ET in the airflow was verified using gas chromatography as described by Millenaar et al. (2005). Control plants were treated in a similar manner but without ET in the air flow.

### Inhibition of ET signaling with 1-MCP

To inhibit ET signaling, plants were treated with gaseous 1-methylcyclopropene (1-MCP), which is released from EthylBloc (Floralife, Walterboro, USA). EthylBloc

contains 0.14% (w/w) 1-MCP. To release 1-MCP, EthylBloc was first dissolved in water in an airtight container at 40°C for 12 min (Millenaar et al, 2005). 1-MCP gas was then collected from the headspace with a syringe and injected into an airtight 18-L cuvette in which the plants were placed for the SA-JA cross-talk assay. For a final concentration of 1 $\mu L.L^{-1}$ of 1-MCP, 1.6 g of EthylBloc was used per $m^3$. The *pad3-1* mutant plants were pretreated with 1 $\mu L.L^{-1}$ of 1-MCP for 2 h, after which the cuvettes were opened and plants were inoculated with the fungus *A. brassicicola* as described above. Subsequently, the plants were placed back in airtight cuvettes for 24 h. Thereafter, plants were treated with 1 mM SA at 24, 48 and 72 h post-inoculation. Another 24 h after SA treatment, leaf tissue was harvested for gene expression analysis. During the whole experiment, plants were contantly treated with 1 $\mu L.L^{-1}$ of 1-MCP to assure inhibition of ET signaling.

### Ethylene measurements

To measure ET production in plants challenged with *A. brassicicola,* rosettes of inoculated plants were detached from the roots, weighed and placed individually in 35-mL gas-tight serum flasks ($n$=10) that were subsequently incubated under climate chamber conditions. At different time intervals, 1-mL gas samples were withdrawn through the rubber seal. The concentration of ET was measured by gas chromatography as described by De Laat and Van Loon (1982).

### RNA extraction and northern blot analysis

For RNA extraction, at least 5 plants per treatment were harvested at the time points indicated. RNA isolation and northern blot analysis was performed, as described previously by Van Wees et al. (1999). Northern blots were hybridized with gene-specific probes for *PR-1, PDF1.2* and *VSP2* as described (Van Wees et al., 1999). A probe for *ORA59* was made by PCR amplification of cDNA of MeJA-treated plants and the gene-specific primers *ORA59*-FOR 5'-TTCCCCGGAGAACTCTTCTT-3' and *ORA59*-REV 5'-TCCGGAGAGATTCTTCAACG-3'. To check for equal loading, blots were stripped and hybridized with a probe for *18S* ribosomal RNA. The AGI numbers for the genes studied are At2g14610 (*PR-1*), At5g44420 (*PDF1.2*) At5g24770 (*VSP2*) and At1g06160 (O*RA59*). After hybridization with $\alpha$-$^{32}$P-dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of *PDF1.2*, *VSP2* and *ORA59* probes were quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad, Veenendaal, the Netherlands). All gene expression analyses have been repeated with similar results.

### Transient gene expression assay using mesophyll protoplasts

For the transient gene expression assay, protoplasts were isolated from 4-week-old Col-0 plants and transformed with plasmid DNA using the polyethylene glycol (PEG)-calcium transfection protocol described by Yoo et al. (2007). PEG-calcium

transfection of plasmid DNA was performed with protoplasts at a density of 1 x $10^6$ protoplast per ml. Protoplasts were co-transformed with a reporter plasmid carrying the *PDF1.2* promoter fused to the coding sequence of the β-glucuronidase (*GUS*) reporter gene (*PDF1.2:GUS*), and an effector plasmid carrying the CaMV *35S* promoter fused to the coding sequence of *ORA59* (*35S:ORA59*) (De Sutter et al., 2005; Pré et al., 2008). As an internal control, a reference plasmid carrying the *Renilla LUCIFERASE* (*LUC*) gene under the control of the *35S* promoter was used (Pré et al., 2008). As controls, *PDF1.2:GUS* was co-transformed with the corresponding empty effector vector pRT101. Protoplasts were transformed with the three constructs in a ratio of 2:1:2 (*GUS*:*LUC*:*ORA59*) with a maximum of 5 μg of plasmid per transformation. Protoplasts were harvested at 24 h after transformation and frozen in liquid nitrogen. GUS and LUC activities were measured as described by Pré et al. (2008). GUS activity was related to LUC activity to correct for transformation and protein extraction efficiency. Average GUS-LUC ratios from 5 experiments were expressed relative to the respective vector controls.

**Acknowledgements**

We thank Dieuwertje van der Does, Ruth Joosten, Hans van Pelt, Ientse van der Sluis and Rob Welschen for technical assistance. This work was supported by VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which is subsidized by the Netherlands Organization of Scientific Research.

CHAPTER 5

# Salicylate-mediated suppression of jasmonate signaling does not require downregulation of the jasmonate biosynthesis pathway

**Antonio Leon-Reyes, Elvira S. De Lange, Annemart Koornneef, Corné M.J. Pieterse and Tita Ritsema**

Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, the Netherlands

**Abstract**

Jasmonates (JAs) and salicylic acid (SA) are plant hormones that play a pivotal role in the regulation of induced defenses against microbial pathogens and herbivorous insects. Their signaling pathways cross-communicate to provide the plant with a finely-tuned defense response against the attacker encountered. In *Arabidopsis thaliana*, pharmacological experiments showed that SA strongly antagonizes methyl jasmonate (MeJA)-induced expression of the JA-responsive marker gene *PDF1.2*. Genes encoding key enzymes in the JA biosynthesis pathway, such as *LOX2, AOS*, *AOC3,* and *OPR3*, were also sensitive to SA-mediated suppression, suggesting that the JA biosynthesis pathway may be a target for SA-mediated antagonism. If the antagonistic effect of SA on JA signaling functions through inhibition of JA production, then JA biosynthesis mutants should be affected in SA-JA cross-talk. To test this hypothesis, we made use of the JA biosynthesis mutant *aos/dde2*, which is affected in the *ALLENE OXIDE SYNTHASE* gene. Inoculation of mutant *aos/dde2* plants with the JA-inducing necrotrophic fungus *Alternaria brassicicola* did not result in the activation of the JA-responsive marker gene *PDF1.2*. Bypassing JA biosynthesis by exogenous application of MeJA rescued this JA-responsive phenotype. Application of SA suppressed MeJA-induced *PDF1.2* expression to the same level in the *aos/dde2* mutant as in wild-type Col-0 plants, indicating that SA targets the JA response at a position downstream of the JA biosynthesis pathway.

## Introduction

Plants are at constant risk of being attacked by a wide variety of harmful organisms, such as insect herbivores, fungi, bacteria, and viruses. To defend themselves, plants possess a powerful innate immune system by which they coordinate recognition of the attacker and activate appropriate plant defenses. Since the activation of defenses involves fitness costs, it seems important that plants are capable of fine tuning their defense response, depending on the attacker encountered (Van Hulten et al., 2006; Walters and Heil, 2007). Changes in hormone concentrations, such as induced upon pathogen or insect attack, mediate a whole range of plant adaptive responses that are highly specific for the attacker encountered. The importance of salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) as primary signals in the regulation of the plant's immune response is well established (Howe, 2004; Pozo et al., 2004; Mauch-Mani and Mauch, 2005; Van Loon et al., 2006a; Loake and Grant, 2007; Von Dahl and Baldwin, 2007; Asselbergh et al., 2008). Upon pathogen attack, the quantity, composition, and timing of the phytohormonal blend produced by the plant varies among plant species and depends greatly on the lifestyle and infection strategy of the invading attacker. This so-called 'signal signature' results in

the activation of a specific set of defense-related genes that eventually determines the nature and effectiveness of the immune response that is triggered by the attacker (De Vos et al., 2005). In recent years, molecular, genetic and genomic tools have been used to uncover the complexity of the hormone-regulated induced defense signaling network. Besides balancing the relative abundance of different hormones, intensive interplay between hormone signaling pathways emerged as an important regulatory mechanism by which the plant is able to tailor its immune response to the type of invader encountered.

JA and its derivatives (collectively called jasmonates (JAs)) are lipid-derived compounds that have important signaling functions in the plant's defense response to nectrotrophic pathogens and herbivorous insects (Kessler and Baldwin, 2002; Howe, 2004; Kessler et al., 2004; Pozo et al., 2004; Van Poecke and Dicke, 2004). Upon pathogen or insect attack, JAs are rapidly produced. JAs are members of the oxylipin family and their biosynthesis is very well studied (Reviewed in Creelman and Mulpuri, 2002; Delker et al., 2006; Wasternack, 2007). JAs originate from α-linolenic acid that is released from chloroplast membranes through the action of phospholipases, such as the DAD-like phospholipase $A_1$. Initial biosynthesis steps take place in the chloroplast but later the synthesis translocates to the peroxisome and the cytoplasm. The first conversion in the JA biosynthesis pathway is performed by lipoxygenases (LOXs), which convert α-linolenic acid into 13- hydroperoxyoctadecatrienoic acid (13-HPOT). From the seven lipoxygenase genes identified in *Arabidopsis thaliana* (Arabidopsis), LOX2 seems to be very important, since co-suppression of this gene in the anti-sense transgenic line S-12 led to reduced JA production (Bell and Mullet, 1993). Allene oxide synthase (AOS) is responsible for converting 13-HPOT to allene oxide. *AOS* is present as a single gene copy and a mutation in this gene leads to a complete elimination of JA production (Song et al., 1993; Park et al., 2002; Von Malek et al., 2002). The conversion of allene oxide to 12-oxo-phytodienoic acid (OPDA) is performed by allene oxide cyclases (AOCs). OPDA has been shown to be capable of triggering JA-responsive gene expression and resistance in the absence of JA, indicating that this JA precursor can play an important role in the regulation of JA-dependent defenses (Stintzi et al., 2001). The enzyme responsible of converting OPDA to 3-oxo-2(2'-pentenyl)-cyclopentane-1-octanoic acid (OPC:8) is 12-oxo-phytodienoate reductase (OPR). An important member of the small gene family of six genes encoding OPRs is *OPR3* (Stintzi and Browse, 2000). Mutation of the *OPR3* gene results in the elimination of endogenously JA production. The conversion of OPC:8 to JA is performed by three rounds of β-oxidation. Several β-oxidation enzymes have been characterized, including the enoyl-CoA hydratase AIM1 (Richmond and Bleecker, 1999), and OPC:8: CoA Ligase1 (OPCL1) (Koo et al., 2006). Upon synthesis, JA can be readily metabolized to the volatile methyl-JA through the activity of JA carboxyl methyltransferase (JMT) (Seo et al., 2001). In addition JA can be conjugated to amino acids such as isoleucine via the activity of

the JA conjugate synthase JAR1 (Staswick and Tiryaki, 2004). Conjugation of JA to isoleucine results in a biologically highly active form of JA that has been shown to play an important role in mediating JA responses in the JA signaling pathway (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008; Fonseca et al., 2009).

JA biosynthesis results in positive or negative alteration of a large set of JA-responsive genes. However, JA-related transcriptomes show limited overlap, suggesting that the context in which the JA signal is perceived is crucial in shaping the response (Devoto and Turner, 2003; De Vos et al., 2005; Pauwels et al., 2009). SA emerged as an important antagonist of the JA response (Kunkel and Brooks, 2002; Rojo et al., 2003; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Pieterse et al., 2009). Early studies in tomato revealed that SA and its acetylated form aspirin, are potent suppressors of the JA-dependent wound response (Doherty et al., 1988; Peña-Cortés et al., 1993; Doares et al., 1995). Also in Arabidopsis, SA was shown to suppress JA signaling, resulting in the down regulation of JA-responsive gene expression (Pieterse and Van Loon, 1999; Van Wees et al., 1999; Gupta et al., 2000; Spoel et al., 2003). Trade-offs between SA-dependent pathogen resistance and JA-dependent defense against insect herbivory have been repeatedly reported (Thaler et al., 1999; Felton and Korth, 2000; Pieterse et al., 2001; Bostock, 2005). In Arabidopsis, the SA pathway has been shown to inhibit JA-dependent resistance against tissue-chewing herbivores, such as *Spodoptera exigua* (beet armyworm) (Cipollini et al., 2004; Bodenhausen and Reymond, 2007; Van Oosten et al., 2008) and *Trichoplusia ni* (cabbage looper) (Cui et al., 2002; Cui et al., 2005), and necrotrophic pathogens, such as *Alternaria brassicicola* (Li et al., 2006; Spoel et al., 2007).

The antagonistic effect of SA on JA signaling in plants shows a remarkable resemblance to the effect of the anti-inflammatory drug aspirin on the formation of prostaglandins in animal cells. Prostaglandins are hormonal pain messengers that are structurally related to JAs and play a role in inflammation at sites of infection or tissue injury (Straus and Glass, 2001). JAs and prostaglandins are both synthesized via the oxylipin biosynthesis pathway in which the enzymatic reactions leading to JA and prostaglandin formation are similar (Pan et al., 1998). In animal cells, aspirin antagonizes prostaglandin action by targeting the biosynthesis genes *COX1* and *COX2* (Straus and Glass, 2001). In tomato and rubber tree, SA and aspirin have been shown to suppress JA biosynthesis (Peña-Cortés et al., 1993; Norton et al., 2007). Hence, JA biosynthesis may be a potential target for the SA-mediated suppressive effect on the JA response. In Arabidopsis, induction of the JA response results in the activation of several JA biosynthesis genes (Sasaki et al., 2001), indicating that JA biosynthesis is under control of a positive feedback regulatory system. In this study we investigated whether suppression of the JA biosynthesis pathway is involved in the antagonistic effect of SA on JA-dependent defense-related gene expression.

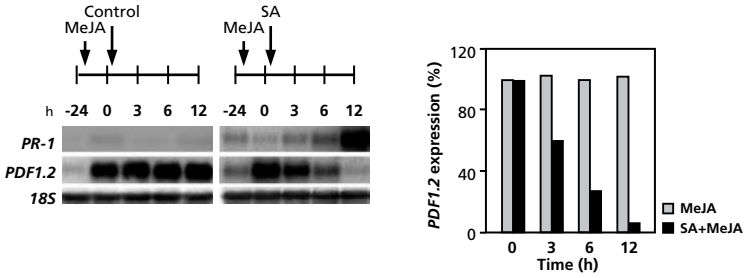



**Figure 1. SA-mediated suppression of MeJA-induced *PDF1.2* expression.**
**Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in 5-week-old Arabidopsis Col-0 plants that were treated with 0.1 mM MeJA and 24 h later with either water (control) or 1 mM SA. Leaves were harvested at the indicated time points. In Col-0 plants exogenous application of 0.1 mM MeJA resulted in a strong activation of *PDF1.2* (left panel). Exogenous application of 1 mM SA, 24 h after MeJA treatment resulted in a strong and fast suppression of *PDF1.2* (right panel). Expression of the SA-responsive marker gene *PR-1* gene was monitored as a control for the effectiveness of the SA treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panel). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

## Results

### SA-mediated suppression of JA biosynthesis genes

In Arabidopsis, pharmacological experiments revealed that SA can antagonize the expression of JA-responsive genes, such as *PDF1.2* (Spoel et al., 2003; Koornneef et al., 2008b; Leon-Reyes et al., 2009). Figure 1 shows that MeJA-induced expression of *PDF1.2* is suppressed within a few hours after application of SA, resulting in almost undetectable *PDF1.2* transcript levels at 12 h after SA application. Several genes encoding key enzymes of the JA biosynthesis pathway (*e.g. LOX2, AOS, AOC3,* and *OPR3*) are regulated by JAs (Bell and Mullet, 1993; Stintzi and Browse, 2000; Sasaki et al., 2001; Park et al., 2002; Von Malek et al., 2002). To investigate whether the JA-responsive expression of *LOX2, AOS, AOC3,* and *OPR3* is antagonized by SA, 5-week-old wild-type Arabidopsis Col-0 plants were treated with 0.1 mM MeJA, 1 mM SA or a combination of both chemicals. Subsequently, leaf tissue was harvested for gene expression analysis at different time points after treatment. All JA biosynthetic genes tested were up-regulated after treatment of the leaves with MeJA (Figure 2). In the combination treatment with SA, MeJA-induced expression of *AOS* was strongly antagonized as early as 1 hour (Fig. 2A), resulting in a two-fold reduction in *AOS* mRNA levels. Although to a lesser extent, *LOX2, AOC3,* and *OPR3* were also antagonized by SA (Fig. 2B), indicating that these JA biosynthesis genes can be antagonized by SA.

### *PDF1.2* gene expression in JA biosynthesis mutants

If the antagonistic effect of SA on JA signaling is targeted at the JA biosynthesis pathway, then Arabidopsis genotypes affected in JA production should be impaired

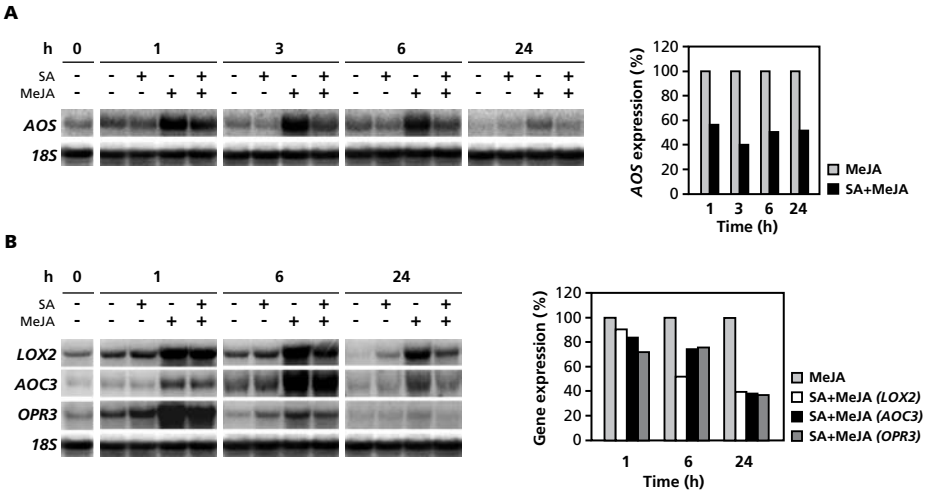



**Figure 2. Antagonistic effect of SA on the JA biosynthesis genes *AOS, LOX2, AOC3,* and *OPR3*.**
**Northern blot analysis of (A) *AOS*, and (B), *LOX2, AOC3,* and *OPR3* transcript levels in 5-week-old Arabidopsis Col-0 plants that were treated with 0.1 mM MeJA or a combination of 0.1 mM MeJA and 1 mM SA. Leaf tissue was harvested at the indicated time points. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panels). Transcript levels in the single MeJA treatments were set to 100%.**

in cross-talk between SA and JA signaling. To investigate this, we first screened previously characterized Arabidopsis mutants and transgenic lines that are impaired in JA biosynthesis for their ability to activate *PDF1.2* upon MeJA treatment or inoculation with the necrotrophic fungus *A. brassicicola*. Arabidopsis lines *dad1*, S-12, *aos/dde2, aoc3*, *AOC*:RNAi lines 5 and 16, *opr3, aim1, opcl1,* and *jmt*, which are affected at different steps in the JA biosynthesis pathway (Fig. 3A), showed wild-type levels of *PDF1.2* expression upon exogenous application of MeJA (Fig. 3B). Because MeJA treatment bypasses the JA biosynthesis pathway, these results indicate that downstream of JA biosynthesis the JA signaling pathway is fully functional in these genotypes. Previously, we demonstrated that *A. brassicicola* induces high levels of JA in Arabidopsis (De Vos et al., 2005). Upon inoculation with *A. brassicicola, PDF1.2* transcripts were readily detectable in wild-type Col-0, Col-5, and Ws-0 plants (Fig. 3B). Although to a lesser extent, the majority of the genotypes affected in JA biosynthesis also activated *PDF1.2* upon inoculation with *A. brassicicola,* indicating that they are either leaky, or still able to produce biologically active oxylipins. The allene oxide synthase mutant *aos/dde2* was the only genotype that was fully blocked in its capacity to express *PDF1.2* upon pathogen infection. At the same time, it showed normal levels of *PDF1.2* expression after MeJA treatment (Fig 3B). In a control experiment, the inability of *aos/dde2* to activate *PDF1.2* upon inoculation with *A. brassicicola* was confirmed (Fig. 3C). Also the induction of the JA-responsive marker gene *VSP2* upon feeding by larvae of the insect herbivore

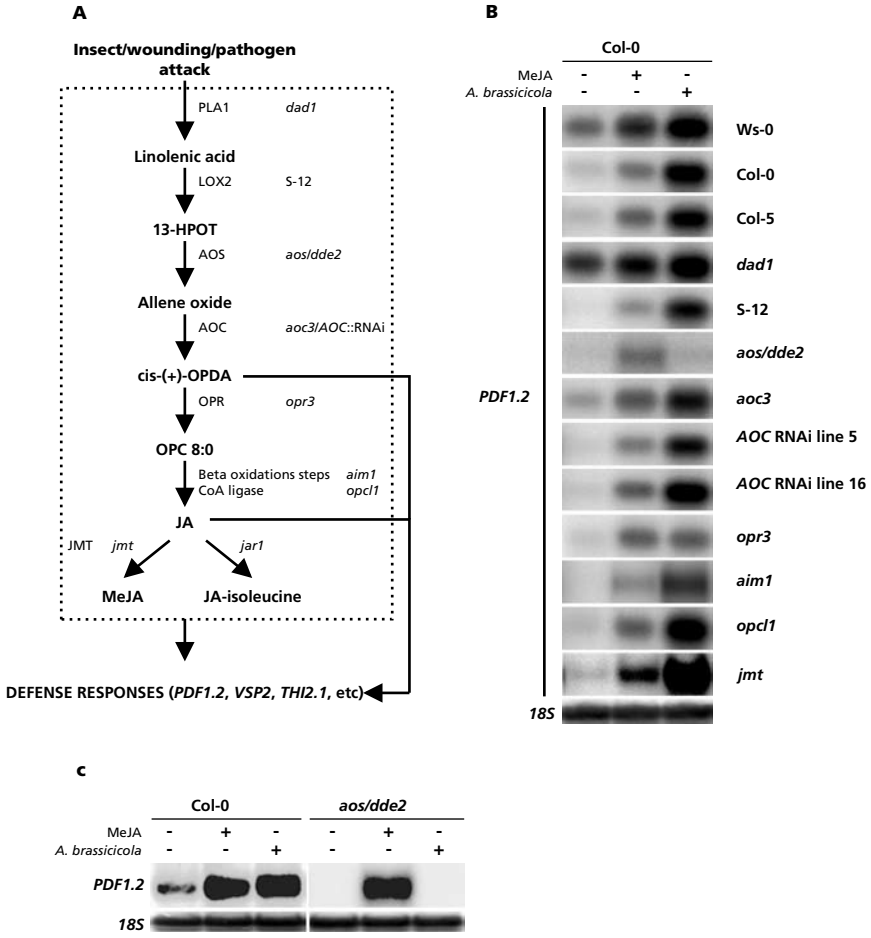



**Figure 3. Characterization of JA-responsive *PDF1.2* expression in JA biosynthesis mutants.**
**(A) JA biosynthesis pathway and corresponding Arabidopsis genotypes that are affected in JA biosynthesis.**
**(B) and (C) Northern blot analysis of *PDF1.2* transcript levels in 5-week-old Arabidopsis plants that were treated with 0.1 mM MeJA or inoculated with the necrotrophic fungus *A. brassicicola.* Genotypes in the Ws-0 background (*dad1, aoc3, opr3, aim1*); genotypes in the Col-0 background (*aos/dde2*, AOC RNAi lines 5 and 16, *opcl1 and jmt*); genotype in Col-5 background (S-12). Leaf tissue was harvested 24 h after treatment with MeJA or 24 h after inoculation with *A. brassicicola.* In (B) since loading was equal, only *18S* from one treatment is shown.**

*Pieris rapae* (small cabbage white) was blocked in this mutant (data not shown). Hence, mutant *aos/dde2* was used to assess whether the antagonistic effect of SA on JA signaling is affected in plants that are unable to produce JAs.

### SA antagonizes JA signaling downstream of JA biosynthesis

If down regulation of the JA biosynthesis pathway is a central target of SA in the suppression of JA signaling, then SA should not have a negative effect on MeJA-induced expression of *PDF1.2* in an *aos/dde2* mutant. To test this hypothesis we

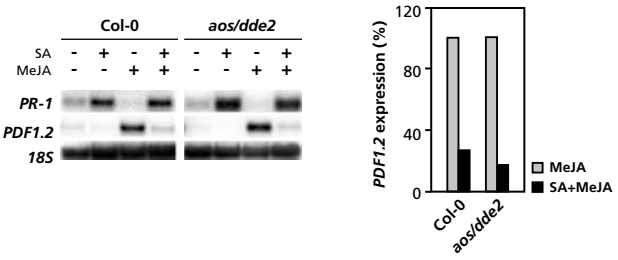



**Figure 4. SA-mediated suppression of JA signaling acts down stream of JA biosynthesis**
**Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in 5-week-old Col-0 and *aos/dde2* mutant plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 24 h after treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panel). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

activated the JA response by dipping the plants in a solution containing 0.1 mM MeJA. As expected, MeJA induced *PDF1.2* expression, wheresas 1 mM SA induced *PR-1* in both Col-0 and *aos/dde2* (Fig. 4). Exogenous application of 1 mM SA induced the SA-responsive marker gene *PR-1*. When applied in combination with MeJA, SA strongly suppressed the expression of *PDF1.2* in both Col-0 and *aos/dde2*. Hence, SA-mediated suppression of MeJA-induced *PDF1.2* expression is fully functional in the JA biosynthesis mutant *aos/dde2*. Also in the other JA biosynthesis genotypes SA was able to suppress MeJA-induced *PDF1.2* transcription (Fig. 5). These results indicate that SA antagonizes JA signaling via a target downstream of the JA biosynthesis pathway.

## Discussion

Several genes encoding key enzymes of the JA biosynthesis pathway are activated by JAs, indicating that JA biosynthesis is regulated by a positive feed forward loop. To gain insight in the molecular mechanisms involved in the antagonistic interaction between SA and JA, we tested the hypothesis that SA-mediated suppression of JA signaling is caused by the down regulation of the expression of JA biosynthesis genes. Indeed, the JA-responsive JA biosynthesis genes *LOX2, AOS, OAC3* and *OPR3* were down regulated by exogenous application of SA. Hence, JA biosynthesis is a potential target of the antagonistic effect of SA on JA signaling (Fig. 2). However, induction of the JA response in the JA biosynthesis mutant *aos/dde2* could be suppressed by SA to wild-type levels (Fig. 4), indicating that down regulation of JA biosynthesis is not required for SA-mediated suppression of JA signaling. Observations by Spoel et al. (2007) and Adie et al. (2007b) that JA levels in Arabidopsis are not negatively affected by SA accumulation support our conclusion that the antagonistic effect of SA on JA signaling acts down stream of JA biosynthesis.

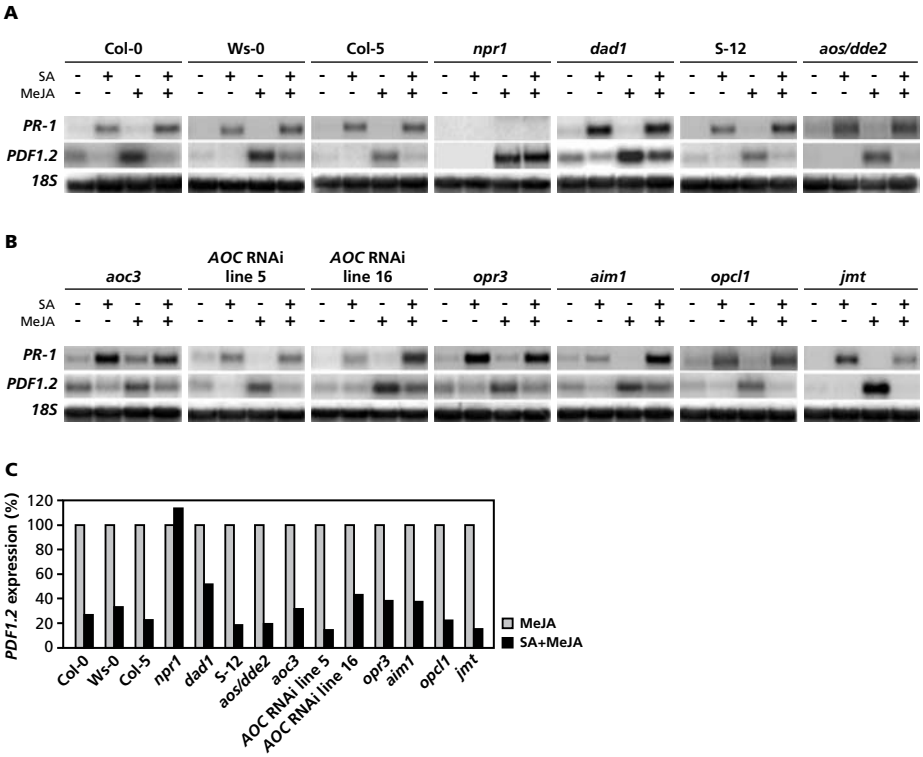



**Figure 5. Antagonistic effect of SA on *PDF1.2* expression in JA biosynthesis mutants.**
**Northern blot analysis of *PR-1* and *PDF1.2* transcript levels in 5-week-old Arabidopsis plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. For details on the JA biosynthesis mutants used, see legend to Fig. 2. Mutant *npr1-1* was used as a negative control for SA-JA cross-talk. Leaf tissue was harvested 24 h after treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager (right panel). *PDF1.2* transcript levels in the single MeJA treatments were set to 100%.**

In animal cells, the SA derivative aspirin inhibits the oxylipin pathway by acetylating the key enzyme cyclooxygenase, ultimately leading to a decrease in prostaglandin formation (Van der Ouderaa et al., 1980). In a similar process in plants, aspirin has been shown to inhibit the activity of the plant counterpart of cyclooxygenase, AOS, which catalyzes the same step in the oxylipin biosynthesis pathway, thereby affecting the formation of JA and the subsequent activation of stress-related gene expression (Pan et al., 1998). Whereas aspirin is able to inhibit prostaglandin and JA biosynthetic enzymes by acetylating them, SA, which lacks the acetyl group, is ineffective in this respect. Indeed, in Arabidopsis and flax plants, no inhibitory effect of SA on AOS activity was observed (Harms et al., 1998; Laudert and Weiler, 1998). Thus, given the fact that the acetylated form of SA does not occur naturally in plants (Pierpoint, 1997), it is unlikely that inhibition of the AOS activity plays a major role in the SA-JA signal interaction in plants.

In conclusion, we demonstrated that the antagonistic effect of SA on JA signaling acts down stream of JA biosynthesis. However, since several JA biosynthetic genes are positively regulated by JA and sensitive to SA-mediated suppression, it can not be excluded that SA-mediated suppression of JA biosynthesis genes may contribute to alleviation of the JA response during plant-pathogen or plant-insect interactions.

## Materials and methods

### Plant material

Seeds of *Arabidopsis thaliana* genotypes were sown in quartz sand. Two-week-old seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min. Plants were cultivated in a growth chamber with an 8-h day (24°C) and 16-h night (20°C) cycle at 70% relative humidity for another three weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10 mM Sequestreen (CIBA-Geigy) once a week. For all the experiments 5-week-old soil-grown plants were used. The following Arabidopsis genotypes were used: Wild-type accessions Col-0, Col-5, and Ws-0, mutants *dad1* [Ws-0] (Ishiguro et al., 2001), *aos/dde2-2* [Col-0] (Von Malek et al., 2002), *AOC* RNAi line 5 and 16 [Col-0] and mutant *aoc3* [Ws-0] (kindly provided by Prof. Claus Wasternack and Dr. Caroline Delker), *opr3* [Ws-0] (Stintzi and Browse, 2000), *aim1* [Ws-0] (Richmond and Bleecker, 1999), S-12 [Col-5] (Bell and Mullet, 1993). The following T-DNA knockout lines [Col-0] were obtained from the SALK Institute Genomic Analysis Institute: SALK_140659 for *opcl1* (At1g20510) (Koo et al., 2006) and Exotic line SM_3_35279 for *jmt* (At1g19640). Gene-specific primers for the *JMT* gene were used for selecting homozygous insertion lines. Disruption of the *JMT* gene was checked by PCR using a specific primers for the insert (*JMT* Spm32exotic FOR 5'- TAC GAA TAA GAG CGT CCA TTT TAG AGT GA -'3) and a primer (*JMT* REV 5'- TGT TTT TGG TAA TTT AAA CTA GTT TCT TG -'3) positioned on opposite site of the predicted T-DNA insertion. Gene-specific primers for *JMT* (*JMT* FOR; 5'- GCA CCA ACT CCT AAG TGG CAA G -3'; *JMT* REV; 5'-AAA GAA GCA AGG TAT GGC AGT AAA ACA TT-3')\ were used as controls for the endogenous gene. For seed production, sterility of the mutants *aos/dde2* and *opr3* was restored by exogenous application of MeJA to the flowers as described (Stintzi and Browse, 2000; Park et al., 2002; Von Malek et al., 2002).

### *Alternaria brassicicola* inoculation

*Alternaria brassicicola* strain MUCL 20297 was grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) plates for 2 weeks at 22°C. Subsequently, conidia

were collected as described (Broekaert et al., 1990). Plants were inoculated when 5 weeks old by applying 5-μL droplets of half-strength potato dextrose broth containing 5 x $10^5$ spores per mL, as described previously (Leon-Reyes et al., 2009). After inoculation plants were kept at 100% relative humidity for optimal fungal germination.

### Chemical treatments

Plants were treated with SA and/or MeJA by dipping the leaves into a solution of 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands) containing 0.1 mM MeJA, 1 mM SA or a combination of both chemicals as described previously (Spoel et al., 2003; Koornneef et al., 2008b; Leon-Reyes et al., 2009). Control treatments were dipped into a solution containing 0.015% (v/v) Silwet L77. MeJA was added to the solutions from a 1,000-fold concentrated stock in 96% ethanol. To the solutions without MeJA, a similar volume of 96% ethanol was added.

### RNA extraction and northern blot analysis

For RNA extraction, at least 5 plants per treatment were harvested at the time points indicated. RNA isolation and northern blot analysis was performed, as described previously by Van Wees et al. (Van Wees et al., 1999). Northern blots were hybridized with gene-specific probes for *PR-1* (At2g14610), *PDF1.2* (At5g44420), *VSP2* (At5g24770), and *LOX2* (At3g45140) and *18S* rRNA as described (Van Wees et al., 1999; Pozo et al., 2008). Probes for the genes *AOS* (At5g42650), *OPR3* (At2g06050), and *AOC3* (At3g25770) were made by PCR amplification on cDNA that was synthesized from mRNA that was isolated from MeJA-treated Arabidopsis plants. Primers used for the amplification of gene-specific probes were: *AOS*-FOR 5'-CCCTTTTCCGATTTCTCTCC-3' and *AOS*-REV 5'-ACGGTAGCCTCCGGTTAGTT-3', *OPR3*-FOR 5'-AAAACAGGTGGCGAGTTTTG-3' and *OPR3*-REV 5'-GCCTTCCAGACTCTGTTTGC-3', *AOC3*-FOR 5'-GCCAAGAAGAACCTCACTGC-3' and *AOC3*-REV 5'-GGCACCTTCAAAGATTCCAG-3'. To check for equal loading, blots were stripped and hybridized with a probe for *18S* ribosomal RNA. After hybridization with α-$^{32}$P-dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of probes were quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad, Veenendaal, the Netherlands). All gene expression analyses have been repeated with similar results.

**Acknowledgements**

We thank Ruth Joosten, Hans van Pelt, and Ientse van der Sluis for technical assistance. We thank the following colleagues for kindly sending Arabidopsis seeds: Dr. Ishiguro and Prof. Okada (*dad1*), Prof. Keller and Dr. Ringli (*aos/dde2-2*), Prof. Claus Wasternack and Dr. Caroline Delker (*AOC* RNAi line 5 and 16, and mutant *aoc3*), Prof. John Browse (*opr3*) and Dr. Brad Brinder (*aim1*). This work was supported by VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which is subsidized by the Netherlands Organization of Scientific Research.

CHAPTER 6

# Suppression of jasmonate signaling by salicylic acid acts downstream of the $SCF^{COI1}$-JAZ complex and targets GCC-box promoter elements

**Antonio Leon-Reyes[1], Annemart Koornneef[1], Dieuwertje van der Does[1], Laurens Pauwels[2], Alain Goossens[2], Nicole Rodenburg[1], Hana Návarová[4], Saskia C.M. van Wees[1], Johan Memelink[3], Tita Ritsema[1], and Corné M.J. Pieterse[1]**

[1] Plant-Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, P.O. Box 800.56, 3508 TB Utrecht, the Netherlands
[2] Department of Plant Systems Biology, Flanders Institute for Biotechnology, Ghent University, 9052 Ghent, Belgium
[3] Institute of Biology Leiden, Clusius Laboratory, Leiden University, 2333 AL Leiden, the Netherlands
[4] Institute of Chemical Technology, Prague, Department of Biochemistry and Microbiology, Prague, 166 28 Czech Republic

**Abstract**

The plant hormones salicylic acid (SA) and jasmonic acid (JA) play a central role in the regulation of induced plant defenses. Cross-communication between their signaling pathways is thought to play an important role in the fine-tuning of the defense response that is triggered upon pathogen and insect attack. In *Arabidopsis thaliana,* chemical or biological induction of the SA pathway results in a strong suppression of the JA response. Here, we investigated the molecular mechanism underlying the antagonistic effect of SA on JA signaling. SA-mediated suppression of JA-responsive marker genes was not affected in mutants with defects in the E3 ubiquitin-ligase $SCF^{COI1}$ complex that targets JASMONATE ZIM-domain (JAZ) transcriptional repressor proteins for proteasome-mediated degradation. In addition, neither the stability nor the JA-induced degradation of JAZ1, JAZ2 and JAZ9 was affected by SA, suggesting that the antagonistic effect of SA on JA signaling is not targeted at the $SCF^{COI1}$-JAZ protein machinery. Ectopic overexpression of the AP2/ERF transcription factor ERF1 in the JA-insensitive mutant *coi1-1* background resulted in constitutive expression of the JA-responsive marker gene *PDF1.2. 35S:ERF1*-mediated expression of *PDF1.2* in *coi1-1* was suppressed by SA, indicating that the antagonistic effect of SA on JA signaling acts downstream of the $SCF^{COI1}$. *In silico* promoter analysis of the SA-JA cross-talk transcriptome revealed that the GCC-box motif (A)GCCGCC was significantly enriched in the 1-kb promoter regions of genes that are activated by methyl JA (MeJA) and suppressed by SA. MeJA and inoculation with the JA-inducing fungus *Alternaria brassicicola* activated *GUS* expression in transgenic *4xGCC:GUS* lines carrying four copies of the GCC-box fused to the β-glucuronidase-encoding *GUS* reporter gene. MeJA-induced activation of *4xGCC:GUS* was antagonized by SA, suggesting that the GCC-box motif is sufficient for SA-mediated suppression of JA-responsive gene expression. Collectively, these data indicate that SA inhibits JA signaling downstream of the $SCF^{COI1}$-JAZ complex and that the antagonistic effect targets the GCC-box motifs in JA-responsive promoters.

## Introduction

Plants possess multiple defense mechanisms to protect themselves against different attackers. Trichomes, thorns, wax layers and pre-formed antimicrobial metabolites are examples of constitutive layers of plant defense. When attackers overcome this pre-invasive layer of defense, inducible defense responses can be activated to prevent further pathogen ingress (Walters et al., 2007). The phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play a major role in the regulation of induced plant defenses (Dong, 1998; Howe, 2004; Pozo et al., 2004; Grant and

Lamb, 2006a;Van Loon et al., 2006a; Loake and Grant, 2007;Von Dahl and Baldwin, 2007;Vlot et al., 2008; Pieterse et al., 2009). The hormonal blend that is produced upon pathogen or insect attack, the so-called signal signature, varies greatly in both composition and timing and is highly attacker specific (De Vos et al., 2005). Endogenous accumulation of SA, JA, and/or ET leads to increased resistance against diverse types of attackers (Thomma et al., 2001a; Glazebrook, 2005; Thatcher et al., 2005). In general, it can be stated that pathogens with a biotrophic lifestyle, such as *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis,* are more sensitive to SA-induced defenses, whereas necrotrophic pathogens, such as *Botrytis cinerea*, *Alternaria brassicicola*, and herbivorous insects, such as *Pieris rapae* (small cabage white) and *Frankiniella occidentalis* (Western flower thrips), are resisted through JA/ET-mediated defenses (Thomma et al., 1998; Kessler and Baldwin, 2002; Ton et al., 2002; Glazebrook, 2005; De Vos et al., 2006; Abe et al., 2008).

SA is a phenolic compound that can be synthesized in the chloroplasts from isochorismate, or from phenylalanine via cinnamic and benzoic acid through the phenylpropanoid pathway (Métraux, 2002). The signaling pathway that is triggered upon endogenous accumulation of SA is well studied (Durrant and Dong, 2004; Loake and Grant, 2007). The redox-sensitive protein NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1) plays a pivotal regulatory role in the SA signaling pathway (Dong, 2004; Pieterse and Van Loon, 2004). The onset of the SA response is characterized by a SA-mediated change in the cellular redox potential, which results in the reduction of oligomeric NPR1 to its active monomeric form (Mou et al., 2003). Monomeric NPR1 is then translocated into the nucleus where it functions as a co-activator of SA-responsive *PATHOGENESIS-RELATED* (*PR*) genes by enhancing the binding of TGA transcription factors to SA-responsive elements in their promoters (Després et al., 2000; Dong, 2004). Several *PR*-genes encode proteins with antimicrobial activity, which is thought to contribute to the SA-mediated resistance that is conferred by SA (Van Loon et al., 2006b).

JA and its structurally related metabolites (collectively called jasmonates (JAs)) are lipid-derived compounds that are synthesized via the oxylipin biosynthesis pathway from α-linolenic acid that is released from chloroplast membranes (Feussner and Wasternack, 2002;Wasternack, 2007). Upon production, JA is rapidly conjugated to isoleucine (Ile) via the activity of the JA conjugate synthase JAR1 (Staswick and Tiryaki, 2004) resulting in the biologically highly active form JA-Ile (Fonseca et al., 2009). The F-box protein COI1 (CORONATINE INSENSITIVE 1) functions as a key regulator of JA signaling (Feys et al., 1994; Xie et al., 1998). Mutant *coi1-1* plants are completely unresponsive to JAs and show alterations in the level of resistance to different pathogens and insect herbivores (Feys et al., 1994; Pozo et al., 2004; Cui et al., 2005; De Vos et al., 2006; Bodenhausen and Reymond, 2007; Van Oosten et al., 2008). COI1 functions in the E3 ubiquitin-ligase Skip-Cullin-F-box complex $SCF^{COI1}$ (Devoto et al., 2002; Xu et al., 2002; Feng et al., 2003).

Binding of JA-Ile to COI1 leads to ubiquitination and subsequent degradation of JASMONATE ZIM-domain (JAZ) transcriptional repressor proteins via the ubiquitin-proteasome pathway (Chini et al., 2007; Thines et al., 2007; Chico et al., 2008; Katsir et al., 2008; Staswick, 2008). In resting cells, JAZ proteins act as transcriptional repressors of JA signaling by binding to positive transcriptional regulators, such as MYC2 (Chini et al., 2007). In Arabidopsis, the JAZ family of repressor proteins consists of 12 members with a similar structure (Chini et al., 2007; Thines et al., 2007). Pull-down and yeast-two-hybrid assays demonstrated that most Arabidopsis JAZ proteins bind to MYC2, suggesting that JAZ proteins have overlapping functions (Chini et al., 2009). JAZ proteins share two conserved domains: the Jas motif, which is required for interactions with COI1 and MYC2, and the ZIM domain, which is important for mediating homo- and heterodimeric interactions between JAZ proteins (Chini et al., 2009; Chung and Howe, 2009). The Jas domain is specific for JAZ proteins and is required for protein breakdown in response to JAs. Overexpression of JAZ proteins that lack the Jas domain causes a strong JA-insensitive phenotype that is similar to that observed in mutant *coi1-1* plants (Chung and Howe, 2009). Derepression of JA signaling that is initiated by the binding of bioactive JAs to COI1 results in the activation of a large number of JA-responsive genes. However, JA-related transcriptomes show limited overlap, suggesting that the context in which the JA signaling pathway is activated is crucial for the final outcome of the JA response (Devoto and Turner, 2003; De Vos et al., 2005; Pauwels et al., 2009).

In their natural habitat, plants often deal with simultaneous or subsequent invasion by multiple attackers, which can influence the primary induced defense response of the host plant (Van der Putten et al., 2001; Bezemer and Van Dam, 2005; Stout et al., 2006; Poelman et al., 2008b). Induced defense mechanisms are associated with ecological fitness costs (Walters and Heil, 2007). Hence, plants need regulatory mechanisms to effectively adapt to changes in their environment. Cross-talk between defense-related signaling pathways provides the plant with a great regulatory potential that may help the plant to "decide" which defense strategy to take, depending on the type of attacker encountered (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Pieterse and Dicke, 2007; Pieterse et al., 2009). Cross-talk between SA and JA signaling has been demonstrated to occur in many plant species (Bostock, 1999; Felton and Korth, 2000; Kunkel and Brooks, 2002; Thaler et al., 2002b; Glazebrook et al., 2003; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Pieterse et al., 2009). Endogenously accumulating SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent resistance over JA-dependent defense. For example, induction of the SA pathway in Arabidopsis by exogenous application of SA or infection by *P. syringae,* suppressed JA signaling and rendered infected leaves more susceptible to the necrotrophic fungus *A. brassicicola* (Spoel et al., 2007). Similarly, the biotrophic

oomycete pathogen *H. arabidopsidis* strongly suppressed JA-mediated defenses that were activated upon feeding by caterpillars of *P. rapae* (Koornneef et al., 2008b). Pharmacological experiments with Arabidopsis revealed that JA-responsive marker genes, such as *PLANT DEFENSIN 1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), are highly sensitive to suppression by exogenous application of SA (Van Wees et al., 1999; Spoel et al., 2003; Koornneef et al., 2008b; Leon-Reyes et al., 2009). This antagonism between SA and JA signaling was observed in a large number of Arabidopsis accessions collected from very different geographic origins, highlighting the potential significance of this phenomenon in the regulation of induced plant defenses in nature (Koornneef et al., 2008b).

Many reports describe an antagonistic interaction between the SA and JA pathways, but synergistic interactions have been reported as well (Schenk et al., 2000; Van Wees et al., 2000; Mur et al., 2006). In Arabidopsis, treatment with low concentrations of methyl JA (MeJA) and SA resulted in a synergistic effect on the JA- and SA-responsive genes *PDF1.2* and *PR-1,* respectively. However, at higher concentrations the effects were antagonistic (Mur et al., 2006). Moreover, timing and sequence of initiation of SA and JA signaling are also important for the outcome of the SA-JA signal interaction (Koornneef et al., 2008b). Hence, the kinetics of phytohormone biosynthesis and signaling during the interaction of a plant with its attacker(s) could be highly decisive in the final outcome of the defense response to the attacker encountered. Pathway cross-talk may enable the plant to tailor its defense response, it is also a possible target for plant attackers to manipulate the plant defense signaling network for their own benefit (Pieterse and Dicke, 2007; Robert-Seilaniantz et al., 2007; Pieterse et al., 2009). For instance, *P. syringae* is able to inject different virulence effector proteins into the host cell that suppress the host immune response. One of these virulence factors is the phytotoxin coronatine, which functions as a JA mimic and suppresses effective SA-dependent defenses, thereby reducing the level of resistance to this pathogen (Kloek et al., 2001; Zhao et al., 2003; Brooks et al., 2005; Cui et al., 2005; Nomura et al., 2005; Laurie-Berry et al., 2006; Uppalapati et al., 2007).

In Arabidopsis, NPR1 was identified as a key signaling node in the regulation of SA-JA cross-talk, because in mutant *npr1-1* plants the antagonistic effect of SA on *PDF1.2* and *VSP2* transcription was completely abolished (Spoel et al., 2003; Leon-Reyes et al., 2009). Several other molecular players in SA-JA crosstalk have been identified in recent years, including the mitogen-activated protein kinase MPK4 (Petersen et al., 2000), the lipase-like proteins EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) and PAD4 (PHYTOALEXIN-DEFICIENT4) (Brodersen et al., 2006), the fatty acid desaturase SSI2 (SUPPRESSOR OF SA INSENSITIVITY2) (Kachroo et al., 2001), the glutaredoxin GRX480 (Ndamukong et al., 2007), and WRKY transcription factor proteins such as WRKY70 (Li et al., 2004). Mutation or ectopic expression of the corresponding genes were shown to

have contrasting effects on SA and JA signaling and on resistance against biotrophs and necrotrophs, indicating that these proteins are important regulators of SA-JA crosstalk.

Although, several molecular players in the regulation of SA-JA cross-talk have been identified, the molecular mechanism by which SA exerts is antagonistic effect on JA signaling is still unknown. In this study, we investigated the site of action at which SA exerts its antagonistic effect on the JA signaling pathway. To this end, we tested whether SA targets the JA signaling pathway at the level of the $SCF^{COI1}$-complex, the JAZ repressor proteins, or at the level of JA-responsive gene transcription. We provide evidence that SA suppresses the JA response downstream of the $SCF^{COI1}$-JAZ machinery and that the GCC-box motif in JA-responsive promoters is sufficient for SA-mediated suppression. Collectively, we show that the antagonistic effect of SA on JA signaling is controlled at the level of gene transcription.

## Results

### SA-mediated suppression of JA signaling functions independently of $SCF^{COI1}$

Ubiquitination and subsequent proteasome-mediated degradation of transcriptional repressor proteins emerged as a common regulatory mechanism of several hormone-regulated signaling pathways, including those of JA, ET, auxin, and gibberellin (Devoto et al., 2003). Several Arabidopsis mutants with defects in one of the proteins in the E3 ubiquitin-ligase $SCF^{COI1}$-complex show a reduced response to JA, demonstrating that this protein complex plays a delicate role in the regulation of the JA signaling pathway. Hence, the $SCF^{COI1}$ protein complex may be a potential target for SA to suppress the JA pathway. To investigate this, we tested a number of mutants that are impaired in functionally important proteins of the $SCF^{COI1}$-complex for their ability to display SA-mediated suppression of MeJA-induced *PDF1.2* or *VSP2* gene expression. To this end, the following genotypes were tested: Col-0 (wild type), L*er*-0 (wild type), *npr1-1* (negative control), *ask1* (mutated in gene encoding SCF subunit SKP1), *axr6-1* and *axr6-2* (mutated in gene encoding SCF subunit CUL1), *coi1-12* and *coi1-16* (mutated in gene encoding F-box protein COI1; leaky mutants with residual JA response), and *axr1-12* and *axr1-24* (mutated in genes that modify the activity of the SCF complex). Five-week-old plants were treated with 0.1 mM MeJA or with a combination of 0.1 mM MeJA and 1 mM SA. Twenty-four h later leaf tissue was harvested and the expression of the JA-responsive marker gene *PDF1.2* was assessed by northern blot analysis and quantified using a Phosphor imager. For those genotypes in which *PDF1.2* was not sufficiently expressed, we analyzed the expression level of the JA-responsive marker

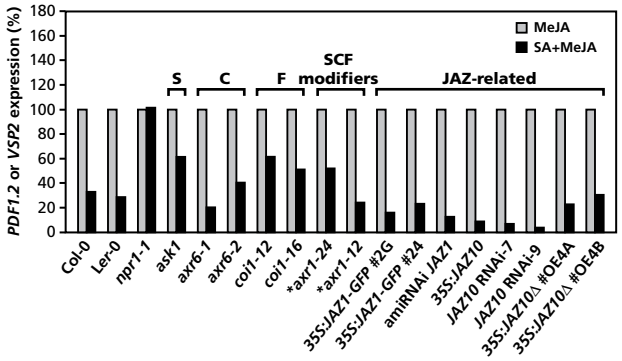



**Figure 1.** **Quantification of SA-JA cross-talk in Arabidopsis genotypes affected in SCF$^{COI1}$-JAZ.**
**Analysis of *PDF1.2* or *VSP2* (*) gene expression in 5-week-old Arabidopsis genotypes after treatment with 0.1 mM MeJA or a combination of 0.1 mM MeJA and 1 mM SA. Leaf tissue was harvested 24 h after chemical treatment. Signal intensities on northern blots were quantified using a Phosphor imager. *PDF1.2* and *VSP2* transcript levels in the single MeJA treatments were set at 100%. The following genotypes were tested (genetic background is given in square brackets): *npr1-1* [Col-0], *ask1* [L*er*-0], *axr6-1* [Col-0], *arx6-2* [Col-0], *coi1-12* [Col-0], *coi1-16* [Col-5], *arx1-12* [Col-0], *arx1-24* [Col-0], *35S:JAZ1-GFP* #2G [Col-0], *35S:JAZ1-GFP* #24 [Col-0], amiRNAi *JAZ1* [Col-0], *35S:JAZ10* [Col-0], *JAZ10* RNAi-7 [Col-0], *JAZ10* RNAi-9 [Col-0], *35S:JAZ10Δ* #OE4A and [Col-0] and *35S:JAZ10Δ* #OE4B [Col-0].**

gene *VSP2* (mutants indicated with an asterisk in Fig. 1). In Fig. 1 the results of the SA-JA cross-talk experiments with the SCF$^{COI1}$ mutants are depicted. In all genotypes tested, the single MeJA treatment resulted in the induction of *PDF1.2* or *VSP2* (transcript levels set at 100%). Exogenous application of SA suppressed *PDF1.2* transcription in wild-type Col-0 and L*er*-0 plants, but not in mutant *npr1-1*, confirming previous findings (Spoel et al., 2003; Koornneef et al., 2008b). Although many of the SCF$^{COI1}$-related mutants showed reduced *PDF1.2* or *VSP2* mRNA levels after MeJA treatment, the MeJA-induced level of gene expression could still be suppressed by SA to near wild-type levels. These results suggest that none of the SCF$^{COI1}$ components tested play a crucial role in SA-JA cross-talk.

### SA does not inhibit degradation of JAZ proteins in Arabidopsis suspension cells

Upon induction of the JA signaling pathway, JAZ transcriptional repressor proteins are ubiquitinated by the SCF$^{COI1}$-complex and subsequently degraded via the proteasome, resulting in the derepression of the JA response (Chico et al., 2008; Katsir et al., 2008; Staswick, 2008). In the auxin signaling pathway, SA was shown to repress auxin signaling by stabilizing auxin repressor proteins (Wang et al., 2007). We hypothesized that SA may act in a similar manner in the suppression of the JA pathway. Therefore, we tested the effect of SA on JA-induced degradation of JAZ proteins. First, we checked whether modification of one of the JAZ proteins (JAZ10) leads to changes in MeJA-induced expression of *PDF1.2* and *VSP2*. To this end, 5-week-old plants of wild-type Col-0, the *JAZ10* overexpressing line *35S:JAZ10* and the *JAZ10* silenced lines *JAZ10* RNAi-7 and *JAZ10* RNAi-9

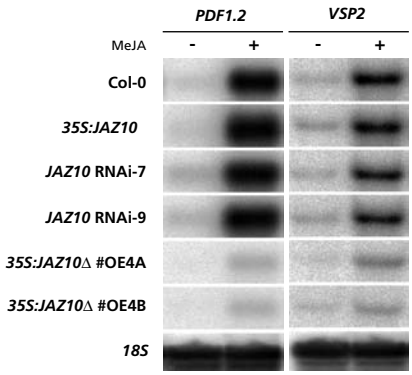



**Figure 2. MeJA-induced expression of *PDF1.2* and *VSP2* in JAZ10-modified transgenic lines.**
**Northern blot analysis of *PDF1.2* and *VSP2* transcript levels in 5-week-old Col-0, *35S:JAZ10*, *JAZ10* RNAi-7, *JAZ10* RNAi-9, *35S:JAZ10Δ* #OE4A and *35S:JAZ10Δ* #OE4B plants. Leaves were harvested 24 h after treatment with 0.1 mM MeJA. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Since loading was equal, only *18S* from one treatment is shown.**

(Yan et al., 2007) were treated with 0.1 mM MeJA. Twentyfour h later, *PDF1.2* and *VSP2* transcript levels were assessed by northern blot analysis. Fig. 2 shows that *PDF1.2* and *VSP2* transcripts accumulated to wild-type levels in *35S:JAZ10* and the *JAZ10* RNAi lines, confirming previous findings that increased or reduced abundance of one member of the JAZ protein family does not affect the JA response (Thines et al., 2007;Yan et al., 2007). Lines *35S:JAZ10*Δ #OE4A and *35S:JAZ10Δ* #OE4B overexpress a truncated form of JAZ10 that lacks the Jas domain that is required for JA-mediated degradation (Yan et al., 2007). In these lines the JA response is constitutively suppressed, resulting in the inability of MeJA to induce *PDF1.2* or *VSP2* expression (Fig. 2). These results indicate that stabilization of a single JAZ repressor protein is sufficient to block the JA response.

To investigate whether SA affects the stability of JAZ proteins, we performed assays with stable Arabidopsis cells cultures overexpressing either *JAZ1*, *JAZ2*, or *JAZ9* fused to the luciferase-encoding reporter gene *LUC*. First, we checked the optimal JA concentration for the initiation of JAZ protein degradation in this assay. Therefore, an Arabidopsis cell suspension culture of *35S:JAZ9-LUC* was treated with 0.001, 0.01 or 0.1 mM JA (Fig. 3A). After 1, 2, 5 and 15 min samples were taken and protein was isolated for LUC activity measurements. After the application of JA, the amount of JAZ9 decreased rapidly suggesting that JAZ proteins were quickly degraded (a half life of ~2.5 min); this in contrast to the control where no degradation was observed (Fig. 3A). Addition of the proteasome inhibitor MG132 to JA-treated cells inhibited the degradation of JAZ9 as previously described (data not shown; Chini et al., 2007; Thines et al., 2007). Since application of 0.001, 0.01, or 0.1 mM JA resulted in the same rate of JAZ9 degradation, we choose the lowest concentration for further experiments. To verify whether SA-JA cross-talk occurs

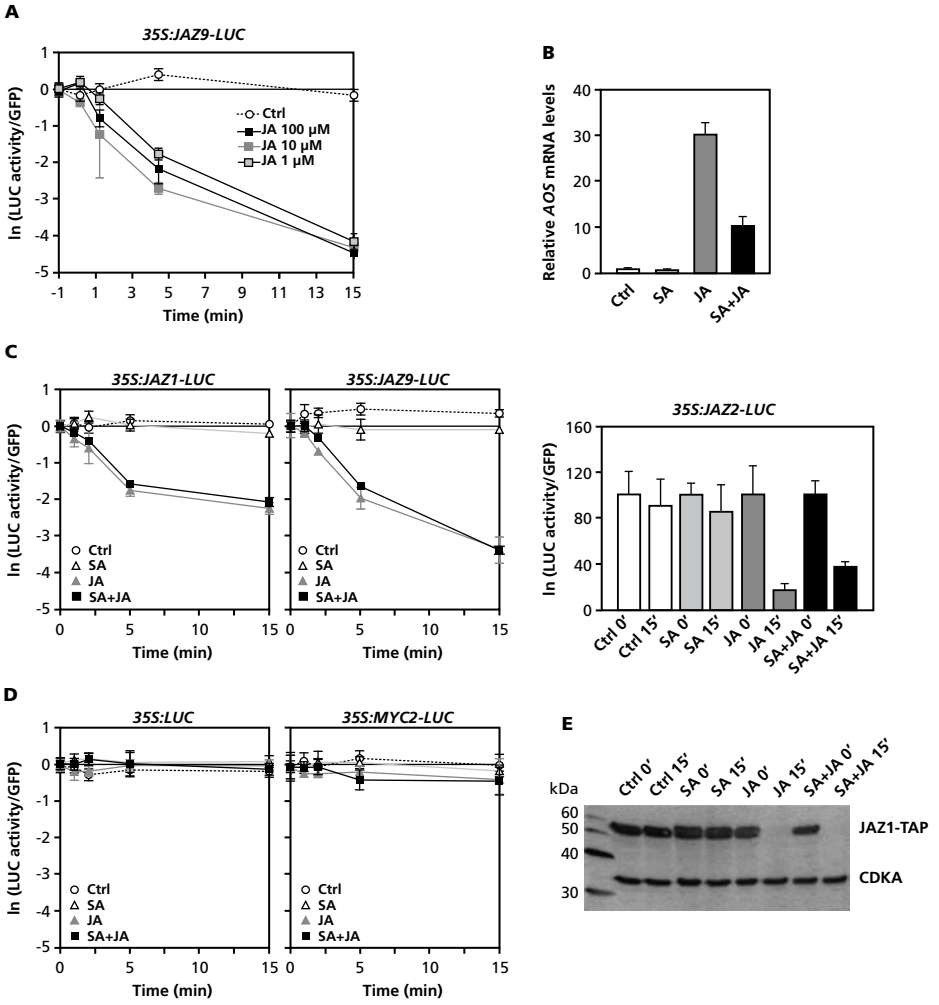



**Figure 3.** **JA-mediated degradation of JAZ proteins in Arabidopsis cell suspension cultures is not affected by SA.**
**(A) Q-RT-PCR analysis of *AOS* mRNA levels in Arabidopsis cells 15 min after treatment with JA, SA or a combination of these chemicals.**
**(B) Luciferase activity in Arabidopsis cells expressing *35S:JAZ9-LUC* and *35S:GFP*, 0, 1, 2, 5, and 15 min after the application of 0.1, 0.01 or 0.001 mM of JA. LUC activities were normalized to GFP internal controls. Three independent protein samples were measured per time point. The natural logarithm (ln) of the normalized LUC activities are given. Error bars represent standard errors (±SE).**
**(C) Luciferase activity in Arabidopsis cells expressing *35S:JAZ1-LUC, 35S:JAZ9-LUC, or 35S-JAZ2-LUC,* or**
**(D) *35S:LUC* or *35S:MYC2-LUC.* SA-treated cells were treated with 0.01 mM SA. JA-treated cells were treated with 0.001 mM JA 3 h after the SA treatment. Samples were taken 0, 1, 2, 5, and 15 min after application of JA, except for the *35S:JAZ2-LUC* cells from which samples were taken at time points 0 and 15 min only. LUC activities were normalized to GFP internal controls. Three independent protein samples were measured per time point. The ln of the normalized LUC activities is given (±SE). Data are from representative experiments that were repeated with similar results.**
**(E) Western blot analysis of the TAP-tagged JAZ1 protein levels in Arabidopsis cells expressing *35S:JAZ1-TAP* 0 and 15 min after treatment of the cells with 0.01 mM SA, 0.001 mM JA or a combination of both chemicals. JAZ1-TAP was detected using an anti-PAP antibody. The constitutively expressed Cdc2 kinase (CDKA) served as an internal control and was detected with an anti-CDKA antibody.**

in the Arabidopsis cell cultures within the time frame of 15 min in which the JAZ degradation assay was performed, we analyzed the expression of the early JA-responsive *ALLENE OXIDE SYNTHASE* (*AOS*) gene in cell cultures that were treated with 0.01 mM SA and 3 h later with 0.001 mM JA. Fig. 3B shows that JA induced *AOS* transcription within 15 min after JA application. In the combination treatment, the *AOS* mRNA level was significantly suppressed, indicating that SA-JA cross-talk was functional in the cell culture assay. Next, we wanted to test the effect of SA on JAZ protein degradation. Therefore, we treated cell culture lines *35S:JAZ1-LUC, 35S:JAZ9-LUC,* and *35S:JAZ2-LUC* with 0.01 mM SA and 3 h later with 0.001 mM MeJA and measured LUC activity at 1, 2, 5 and 15 minutes after JA application (for *35S:JAZ2-LUC* we only measured at 15 min after JA application). Fig. 3C shows that degradation of JAZ1-LUC, JAZ9-LUC and JAZ2-LUC was similar upon application of JA or a combination of JA and SA, suggesting that SA has no effect on the JA-mediated degradation of these JAZ proteins. To confirm that application of SA or JA did not interfere with the LUC reporter system, we tested two controls in our assays: *35S:LUC* and *35S:MYC2-LUC*. Neither JA nor SA affected LUC activity in these control lines, indicating that these chemicals did not affect the LUC reporter system per se (Fig. 3D).

In addition to the LUC reporter assay, we assessed the stability of JAZ1 by western blot analysis. A cell culture expressing *35S:JAZ1-TAP* was treated with 0.01 mM SA, 0.001 mM JA or a combination of both chemicals. Samples were taken just prior to treatment (time point 0) and 15 min after treatment. Fig. 3E shows that JAZ1-TAP was fully degraded 15 min after treatment of the cells with either JA or a combination of JA and SA. These results confirm that SA does not affect the stability of JAZ proteins in Arabidopsis cell suspension cultures.

### SA does not inhibit degradation of JAZ proteins in Arabidopsis plants

To check whether JA-mediated degradation of JAZ proteins is also not affected by SA in intact plants, we made use of the transgenic line *35S:JAZ1-GUS* (Thines et al., 2007). Twelve-day old *35S:GUS* and *35S:JAZ1-GUS* seedlings grown on Murashige and Skoog (MS) agar plates were transferred to fresh medium containing 0.5 mM SA, 0.02 mM MeJA, or a combination of both chemicals and stained for β-glucuronidase (GUS) activity 24 h later. Treatment of *35S:JAZ1-GUS* seedlings with MeJA resulted in reduced GUS staining (Fig. 4A), suggesting that the JAZ1-GUS protein was degraded upon induction of the JA pathway (confirming previous findings; Thines et al., 2007). Inclusion of SA in the medium had no effect on the MeJA-mediated degradation of JAZ1-GUS. Analysis of *GUS, PR-1, PDF1.2,* and *VSP2* transcript levels in these seedlings showed that SA-mediated suppression of *PDF1.2* and *VSP2* gene expression was fully active (Fig. 4B). We therefore

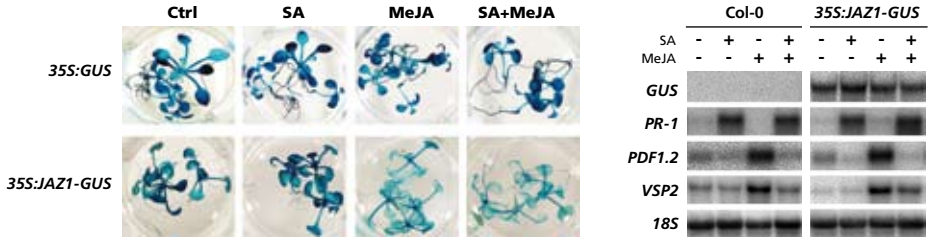



**Figure 4. MeJA-induced degradation of JAZ1 in Arabidopsis seedlings is not affected by SA.**
**Histochemical staining of GUS activity in 2-week-old *35S:JAZ1-GUS* and *35S:GUS* seedlings. Twelve-day-old seedlings grown on MS agar plates were transferred to fresh medium containing 0.5 mM SA, 0.02 mM MeJA, or a combination of both chemicals and stained for GUS activity 24 h later.**
**Northern blot analysis of *GUS*, *PR-1*, *PDF1.2* and *VSP2* transcript levels in 2-week-old Col-0 and *35S:JAZ1-GUS* plants that were treated with 0.5 mM SA, 0.02 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 24 h after chemical treatment for RNA analysis. Equal loading of RNA samples was checked using a probe for *18S* rRNA.**

conclude that the antagonistic effect of SA on JA signaling is not acting through the stabilization of JAZ proteins in Arabidopsis seedlings.

Next, we tested 5-week-old Arabidopsis genotypes with an altered expression of either JAZ1 or JAZ10 for their ability to express SA-JA cross-talk. The following genotypes were tested: *35S:JAZ1-GFP* #2G and *35S:JAZ1-GFP* #24 (*JAZ1* overexpressors), amiRNAi *JAZ1* (*JAZ1* silenced), *35S:JAZ10* (*JAZ10* overexpressor), *JAZ10* RNAi-7 and *JAZ10* RNAi-9 (*JAZ10* silenced), and *35S:JAZ10Δ* #OE4A and *35S:JAZ10Δ* #OE4B (overexpressors of JAZ10 lacking the Jas domain, resulting in constitutive suppression of the JA response). As shown in Fig. 1, the MeJA-induced level of *PDF1.2* expression was strongly suppressed by SA in all JAZ-related genotypes tested. Collectively, these results suggest that the antagonistic effect of SA on JA signaling is not targeted at the $SCF^{COI1}$-JAZ machinery.

### SA represses JA signaling downstream of $SCF^{COI1}$

To investigate whether the antagonistic effect of SA on JA signaling acts downstream $SCF^{COI1}$ in the JA pathway, we made use of transgenic lines that overexpress the AP2/ERF transcription factor ERF1 in the background of wild-type Col-0 and mutant *coi1-1* plants. Overexpression of *ERF1* in the JA-insensitive mutant *coi-1-1* activates *PDF1.2* expression (Lorenzo et al., 2003), indicating that this transcription factor is a direct activator of *PDF1.2* transcription. Exogenous application of 1 mM SA to the leaves of 5-week-old plants resulted in the activation of *PR-1* in all genotypes tested (Fig. 5). Dipping the leaves in 0.1 mM MeJA activated *PDF1.2* in Col-0, but not in JA-insensitive *coi1-1* plants. Overexpression of *ERF1* in the Col-0 background resulted in enhanced *PDF1.2* expression in control-treated plants and boosted the level of *PDF1.2* transcription upon application of MeJA. SA suppressed *PDF1.2* transcription in both Col-0 and *35S:ERF1* plants. Importantly,

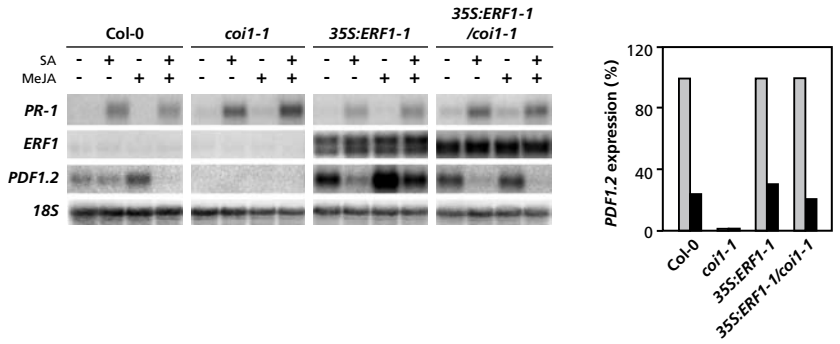



**Figure 5. SA suppresses *35S:ERF1*-mediated *PDF1.2* transcription in the *coi1-1* background.**
**Northern blot analysis of *PR-1, ERF1,* and *PDF1.2* expression in Col-0, *coi1-1, 35S:ERF1* and *35S:ERF1/coi1-1* plants after treatment with 1 mM SA, 0.1 mM MeJA or a combination of both chemicals. Leaf tissue was harvested 24 h after chemical treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager. *PDF1.2* transcript levels in the single MeJA treatments were set to 100% (right panel).**

in the *coi1-1* background *35S:ERF1*-mediated *PDF1.2* transcription was strongly suppressed by SA. These results clearly indicate that SA-mediated suppression of JA signaling acts downstream of the $SCF^{COI1}$ and may act directly at the level of gene transcription.

### The SA-JA cross-talk transcriptome

To gain insight into the regulation and complexity of SA-JA cross-talk at the whole-genome level, we performed a transcript profiling approach to identify JA-responsive genes that are sensitive to SA-mediated suppression, and to subsequently search for cross-talk-related *cis*-acting elements in the promoters of JA-responsive genes that are suppressed by SA. Three independent cross-talk experiments were performed with 5-week-old Col-0 and *npr1-1* plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 28 h after chemical induction. The expression of the marker genes *PR-1* and *PDF1.2* was assessed in each biological replicate by northern blot analysis (Fig. 6A). In all three experiments, SA induced *PR-1* expression and suppressed MeJA-induced expression of *PDF1.2* in Col-0 plants. Both induction of *PR-1* and suppression of *PDF1.2* by SA were blocked in mutant *npr1-1,* confirming that the plant material displayed NPR1-dependent SA-JA cross-talk (Fig. 6A).

The transcript profile of each independent experiment was analyzed using Affymetrix ATH1 whole-genome GeneChips representing approximately 23,750 Arabidopsis genes (Redman et al., 2004). After hybridization, expressed genes were identified using GeneChip Operating Software (GCOS), which uses statistical criteria to generate a "present" or "absent" call for genes represented by each probe set on the array. The fact that each of the three experiments was conducted

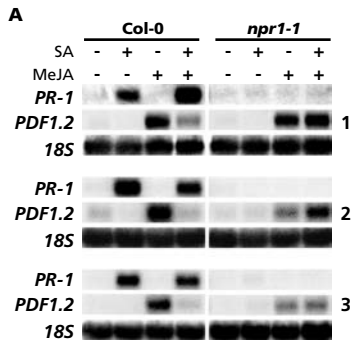
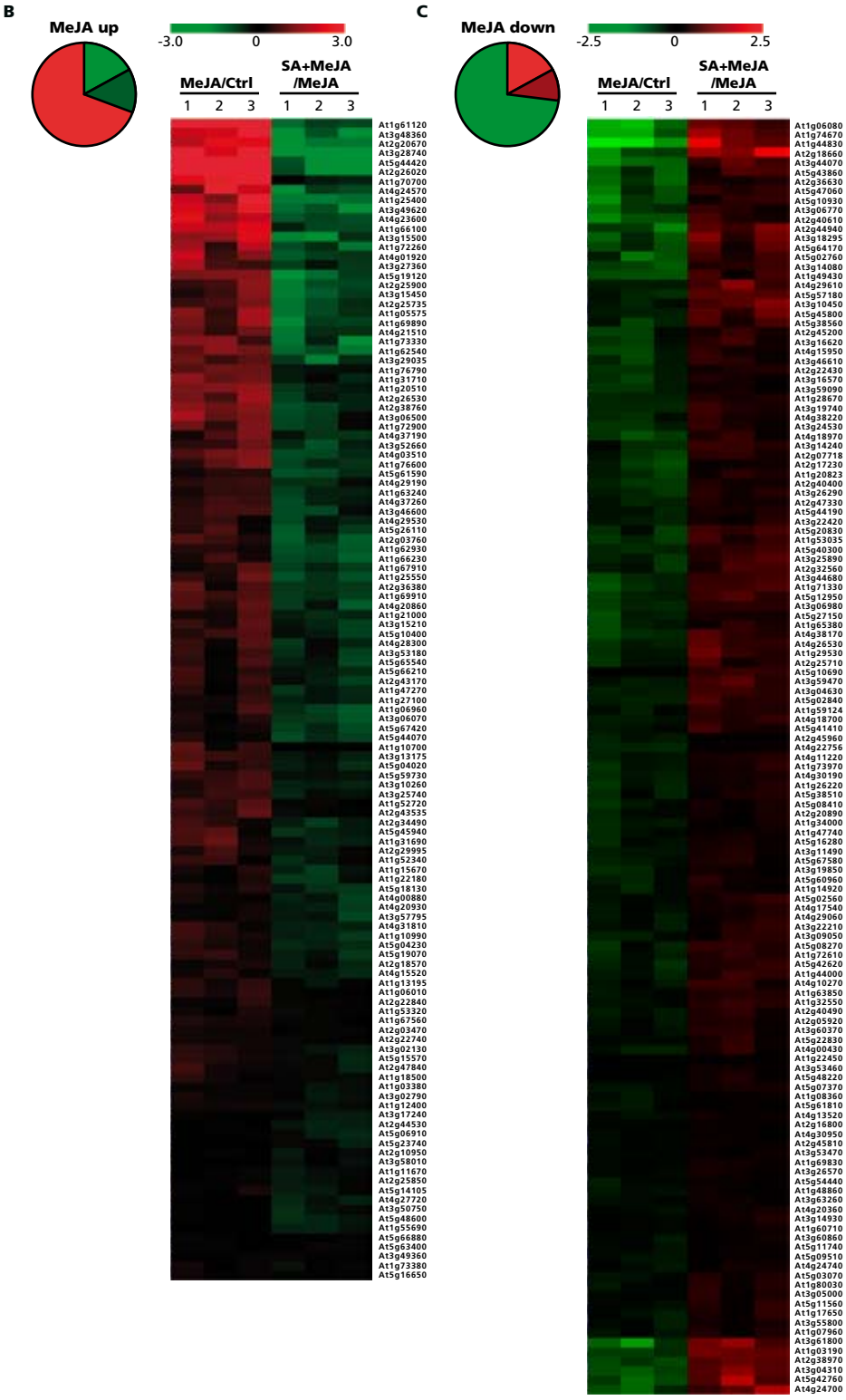



**Figure 6.** **MeJA-responsive genes of which the expression is antagonized by SA.**
**(A) Northern blot analysis of *PR-1* and *PDF1.2* gene expression in Col-0 and *npr1-1* plants after treatment with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. The three biological replicates consistently showed SA-mediated suppression of MeJA-induced *PDF1.2* expression in wild-type Col-0. This suppression was absent in mutant *npr1-1* plants. Leaf tissue was harvested 28 h after chemical treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. RNA from these three biological replicates was used for whole-genome transcript profiling using Affymetrix ATH1 GeneChips.**
**(B) The pie chart represents the proportion of MeJA-upregulated genes that are suppressed by SA (Student's *t* test; $p<0.05$, 123 genes in bright green; $0.05<p<0.1$, 98 genes in dark green) and that are not significantly suppressed by SA (510 genes in red). The log2 fold-changes in the expression of the 123 SA-JA cross-talk genes in the three biological replicates are depicted in the heat map (Saeed et al., 2003).**
**(C) The pie chart represents the number of MeJA-downregulated genes whose repression is relieved by SA (Student's *t* test; $p<0.05$, 135 genes in bright red; $0.05<p<0.1$, 80 genes in dark red) and that are not significantly induced by SA (592 genes in green). The log2 fold-changes of the 135 SA-JA cross-talk genes in the three biological replicates are depicted in the heat map (Saeed et al., 2003).**

at a different time of the year and in different growth chambers contributed significantly to the variation in gene expression observed between the biological replicates. However, by identifying conserved gene expression patterns within the three biological replicates, genes were selected that are likely to contribute to the biological significance of the phenomenon studied. To identify a robust set of MeJA-responsive genes, we selected genes that were statistically significantly up- or downregulated in MeJA-treated plants compared to the mock-treated control (Student's *t* test, $p<0.05$). In addition, the expression level had to be detectable (present call generated by GCOS) in all three MeJA-treated samples (for the upregulated genes) or in all three control treatments (for the downregulated genes). These selection criteria were met by 731 genes that were significantly upregulated upon MeJA treatment (Supplemental Table S1). Among these were genes involved in JA biosynthesis (*LOX2, AOS*, *OPR3*), JA signal transduction (*ERF1*, *ERF4*, *JAZ1*, *JAZ2*, *JAZ5*, *JAZ7*, *JAZ9*), and JA-dependent defenses (*PDF1.2*, *Thi2.1*, *VSP1*, *HEL,* β-*1,3-glucanase*). In addition, a group of 807 genes was significantly downregulated by MeJA (Supplemental Table S1). For SA-responsive genes, a similar selection procedure was followed, resulting in 705 SA-upregulated genes (including *PR-1, ICS1, PAL3*) and 698 SA-downregulated genes (Supplementary Table S1).

To select for MeJA-induced genes that were suppressed by SA, we identified MeJA-upregulated genes that were significantly repressed by the combined treatment with SA and MeJA, compared to MeJA alone. In addition, we selected MeJA-


B
MeJA up
-3.0
0
3.0
MeJA/Ctrl
SA+MeJA /MeJA
1 2 3 1 2 3
C
MeJA down
-2.5
0
2.5
MeJA/Ctrl
SA+MeJA /MeJA
1 2 3 1 2 3

downregulated genes that were significantly upregulated by SA and MeJA, compared to MeJA alone. These selection criteria resulted in the identification of 123 MeJA-inducible genes that are suppressed by SA, and 135 MeJA-downregulated genes that are upregulated by SA (Student's *t* test; $p<0.05$; Figures 6B and 6C; Supplemental Table S2). On average, 17% of all stringently selected MeJA-responsive genes were affected by SA-JA cross-talk, demonstrating that this type of pathway cross-talk is specific for only a subset of the MeJA-responsive genes. Using slightly less stringent selection criteria for the identification of SA-JA cross-talk genes (Student's *t* test; $p<0.1$), another 98 and 80 MeJA-responsive genes were suppressed and upregulated by SA, respectively, demonstrating that depending on the stringency of the selection, up to 30% of MeJA-responsive genes were affected by SA (Figures 6B and 6C; Supplemental Table S2). Among the MeJA-inducible genes that were suppressed by SA were those encoding defense-related proteins PDF1.2a, PDF1.2b, and HEL, and the defense-related transcription factor ERF1, confirming previous findings (Spoel et al., 2003; Ndamukong et al., 2007; Spoel et al., 2007).

NPR1 has been identified as an essential regulator of SA-JA cross-talk in the expression of JA-responsive genes such as *PDF1.2*, *VSP2*, and *LOX2* (Spoel et al., 2003). To investigate the general importance of NPR1 in the regulation of SA-JA cross-talk, we determined the NPR1-dependency of the expression patterns identified from the whole-genome profiles. To compare the SA-JA cross-talk genes in Col-0 and *npr1-1*, we focused on the 123 MeJA-inducible genes that were significantly suppressed by SA in Col-0 (Supplemental Table S2), and thus followed a similar expression pattern as *PDF1.2*. Of the 123 selected SA-JA cross-talk genes, only 45 were also induced by MeJA in the *npr1-1* background (Student's *t* test; $p<0.05$). The remaining 78 genes were discarded from the selection as their expression levels were not significantly enhanced. This may be because MeJA-responsive expression is truly NPR1-dependent, or just did not reach the level of statistical significance due to the relatively large biological variation in the plant material of the three independent experiments. Amongst the remaining robust set of genes with a MeJA-responsive expression profile in the *npr1-1* background was *PDF1.2,* confirming the northern blot data shown in Fig. 6A. Of the 45 selected MeJA-responsive genes that were all significantly suppressed by SA in the Col-0 background, 27 genes (60%) were not significantly suppressed by SA in the *npr1-1* background (Student's *t* test; $p<0.05$; Supplemental Table S3;). Again, *PDF1.2* was amongst this selection. These results suggest that the SA-mediated suppression of MeJA-inducible genes is dependent on NPR1 in approximately 60% of the cases.

### Promoter analysis of MeJA-responsive genes that are suppressed by SA

To search for *cis*-acting motifs with a putative role in SA-JA cross-talk, we performed an *in silico* analysis of the promoter sequences of the selected MeJA-inducible

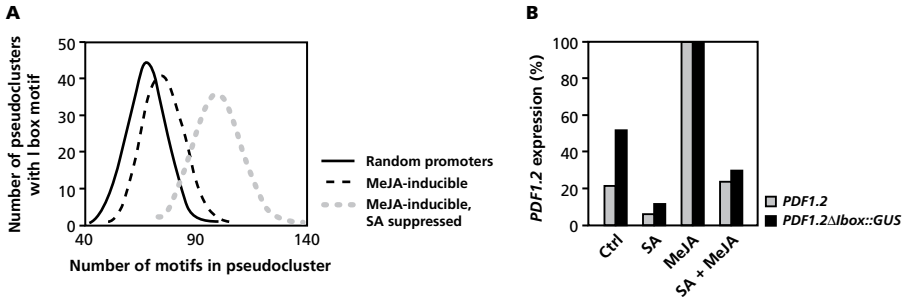



**Figure 7.** **The I-box motif in the *PDF1.2* promoter is not essential for SA-JA cross-talk**
**(A) Frequency distribution of the I-box motif in the promoter sequences of MeJA-inducible and SA-JA cross-talk genes. Occurrence of the I-box motif was determined in the 1-kb sequences upstream of the 5′-UTRs using POBO bootstrapping analysis. The promoters of the MeJA-inducible genes that were not affected by SA (dashed black) and the MeJA-inducible genes that were suppressed by SA (dashed grey) were compared to randomly selected promoter sequences from the Arabidopsis genome (solid black).**
**(B) Northern blot analysis of *PDF1.2* and *PDF1.2ΔIbox:GUS* gene expression. Five-week-old plants were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals. Leaf tissue was harvested 24 h after chemical treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Signal intensities were quantified using a Phosphor imager. *PDF1.2* and *GUS* transcript levels in the single MeJA treatments were set to 100%.**

genes that, like the *PDF1.2* marker gene, were suppressed by SA in the SA/MeJA combination treatment. Functional *cis*-regulatory elements in plant promoters are typically found within the first kilobase (kb) upstream of the ATG translation start site (Rombauts et al., 2003). Therefore, we scanned the 1-kb sequences upstream of the 5'-untranslated regions (UTRs) of the 123 MeJA-inducible genes that were suppressed by SA (Supplemental Table S2), using the visualization tool of the web-based application Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) (O'Connor et al., 2005). The Athena program identified two motifs as being significantly enriched in the promoters of these genes: the I-box motif GATAAG, and the GCC-box motif (A)GCCGCC, suggesting a putative role for these motifs in SA-JA cross-talk (data not shown).

### The I-box motif is not essential for SA-JA cross-talk

The I box motif is a *cis*-acting element that was previously found in the promoters of light-regulated and circadian clock-controlled plant genes (Borello et al., 1993). Because the I-box motif was also overrepresented in MeJA-inducible genes that were not suppressed by SA, we employed the promoter bootstrapping program POBO that allows a three-way comparison between two clusters of co-regulated genes and the genomic background (Kankainen and Holm, 2004). The-I box motif was found to be even more overrepresented in SA-JA cross-talk genes than in the MeJA-inducible genes that were not suppressed by SA (Figure 7A). Therefore, we further investigated the involvement of this promoter element in the regulation of SA-JA cross-talk. The promoter of the *PDF1.2* gene contains a single I-box

motif at position –188 to –183 relative to the ATG start codon. Using site-directed mutagenesis, we knocked out this I-box motif (GATAAG changed into GAATTC), and fused the mutated *PDF1.2ΔIbox* promoter to the *GUS* reporter gene. Four independent transgenic *PDF1.2ΔIbox:GUS* lines were treated with SA, MeJA, or a combination of both chemicals and evaluated for *GUS* and endogenous *PDF1.2* expression. Fig. 7B shows the relative *GUS* and *PDF1.2* transcript levels in Col-0 and a representative transgenic line. Clearly, the MeJA-induced activation of *PDF1.2* and *PDF1.2ΔIbox* was equally sensitive to suppression by SA, indicating that the I-box motif is not an essential regulatory element in the SA-JA antagonism.

### The GCC-box motif is sufficient for SA-mediated suppression of JA-responsive gene transcription

The GCC-box is a binding site for members of the family of AP2/ERF transcription factors (Fujimoto et al., 2000). Mutation of the GCC-box in the *PDF1.2* promoter demonstrated that this motif is essential for induction of the *PDF1.2* gene by MeJA and ET (Brown et al., 2003). Therefore, this motif provides an attractive target for SA-mediated suppression of JA-responsive gene expression, either through interference with binding of positive regulators of JA-responsive gene expression, or through direct competition for binding sites. However, since mutation of the GCC-box abolishes MeJA-inducible *PDF1.2* expression (Brown et al., 2003), the effect of SA cannot be readily determined with a GCC-box mutated line. Therefore, we used transgenic *4xGCC:GUS* lines containing 4 copies of the GCC-box fused to a minimal *35S* promoter and the *GUS* reporter gene (Fig. 8A). With these lines we tested whether the GCC-box is sufficient for SA-mediated suppression of JA-responsive gene expression. To this end, 2-weeks-old seedlings grown on MS medium were transferred to fresh medium containing 0.5 mM SA, 0.02 mM MeJA or a combination of both chemicals. Forty-eight h later, samples were taken for the analysis of GUS activity. *PDF1.2*:*GUS*, *4xGCC:GUS* line #5 and *4xGCC:GUS* line #7 showed induced GUS activity after application of MeJA (Fig. 8B), confirming previous findings that the GCC-box is sufficient for MeJA-responsive gene expression (Brown et al., 2003). Interestingly, SA was able to suppress the induction of the *4xGCC* promoter by MeJA. Quantitative analysis of GUS activity in *PDF1.2:GUS* and the *4XGCC:GUS* lines yielded similar results: the *PDF1.2* and the *4XGCC* promoters were activated by MeJA and suppressed by SA (Fig. 8C). The SA-responsive *PR-1:GUS* and the *35S:GUS* lines were used as controls (Fig. 8C). These results indicate that the GCC-box is sufficient for the suppression of MeJA-induced gene expression by SA.

To further substantiate this finding we investigate whether SA is able to suppress the activation of the *4XGCC* promoter when induced by a pathogen. Therefore, we inoculated *4XGCC:GUS* line #5 with the JA-inducing pathogen *A. brassicicola* and treated the plants or not with 1 mM 24 h later. *A. brassicicola* induced

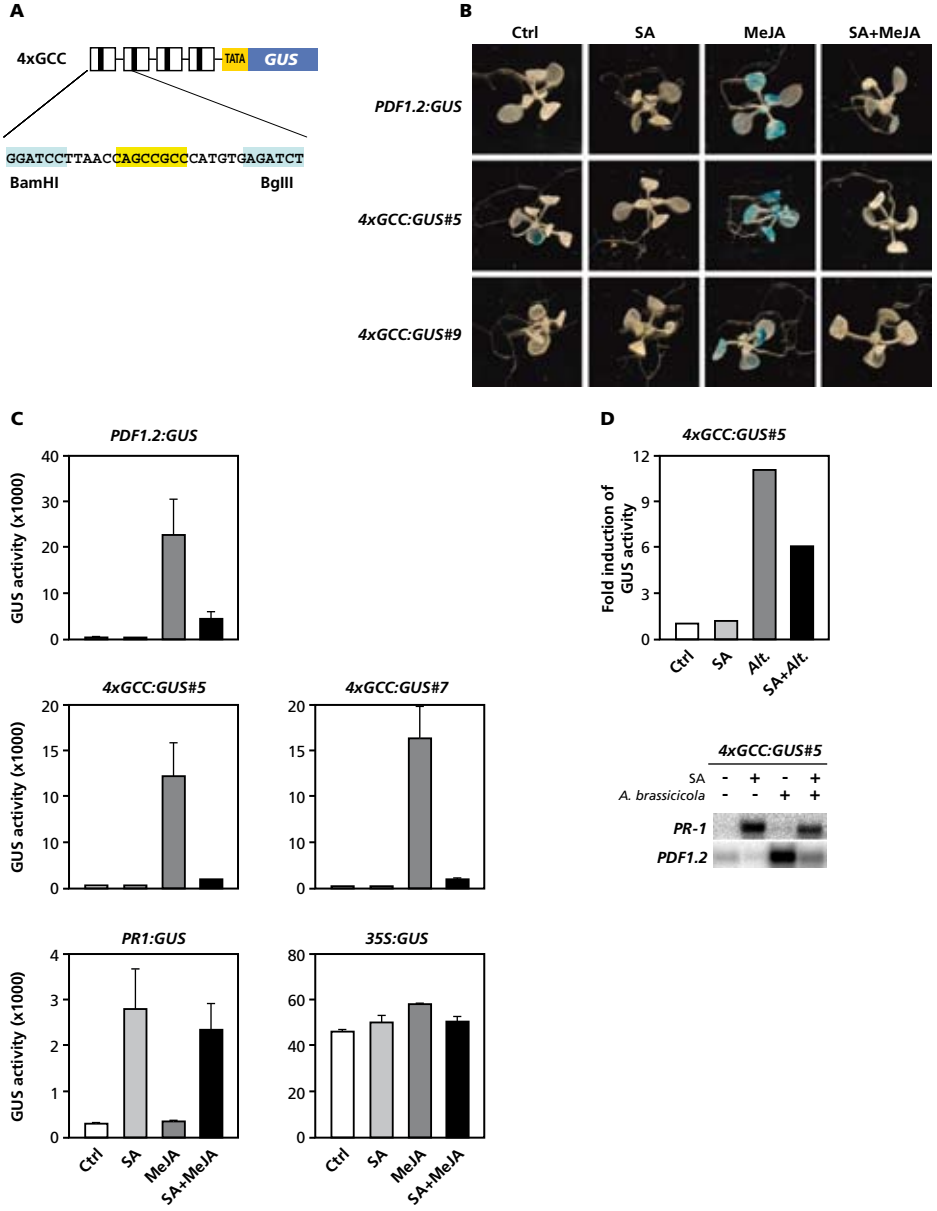



**Figure 8.** The GCC-box is an important motif involved SA-JA antagonism
**(A) Schematic representation of the *4XGCC:GUS* construct.**
**(B) Histochemical staining of GUS activity in 2-week-old seedlings of *PDF1.2:GUS*, *4XGCC:GUS* line #5 and *4XGCC:GUS* line #7. Twelve-day-old seedlings grown on MS agar plates were transferred to fresh medium containing 0.5 mM SA, 0.02 mM MeJA, or a combination of both chemicals and stained for GUS activity 24 h later.**
**(C) Quantitative analysis of GUS activity in 2-week-old seedlings of *PDF1.2:GUS*, *4XGCC:GUS*#5, *4XGCC:GUS*#7, *PR-1:GUS,* and *35S:GUS.* Error bars represent standard errors (±SE).**
**(D) Analysis of GUS activity and *PR-1* and *PDF1.2* transcription in 5-week-old *4XGCC:GUS*#5 plants that were inoculated or not with the fungus *A. brassicicola*. Twenty-four h after inoculation, plants were treated or not with 1 mM SA. Leaf tissue was harvested 24 h after inoculation for the analysis of GUS activity or gene expression. Equal loading of RNA samples was checked using a probe for *18S* rRNA.**

the expression of both *PDF1.2* and *4XGCC:GUS* (Fig. 8D). In addition, SA suppressed pathogen-induced *PDF1.2* and *4XGCC:GUS* expression, corroborating our finding that the GCC-box is sufficient for SA-mediated suppression of JA-responsive gene expression.

## Discussion

Plant defenses are regulated by a complex network of cross-communicating signaling pathways (Pieterse et al., 2009). Elucidation of the mechanisms underlying pathway cross-talk will contribute significantly to our understanding of the complexity of plant defense regulation. The plant hormones SA and JA play a crucial role in the regulation of plant defense after pathogen and insect attacked. Their signaling pathways are mutually antagonistic, but the outcome of the signal interaction greatly depends on the context in which they are induced (Pieterse et al., 2009). In this study, we investigated the molecular mechanism underlying the antagonistic effect of SA on JA signaling. We systematically tested different components of the JA signaling pathway to identify the site of action of SA-mediated antagonism.

### SA inhibits JA signaling downstream of the $SCF^{COI1}$-JAZ machinery

The E3 ubiquitin-ligase $SCF^{COI1}$ complex plays a crucial role in the regulation of the JA response as it targets JAZ transcriptional repressor proteins for degradation upon perception of biologically active JAs (Chico et al., 2008; Katsir et al., 2008; Staswick, 2008). Hence, $SCF^{COI1}$ is a potential target of SA for the suppression of JA signaling. However, mutants with defects in proteins from the $SCF^{COI1}$-complex showed near wild-type levels of SA-JA cross-talk (Fig. 1), suggesting that it is unlikely that SA inhibits the JA signaling pathway via $SCF^{COI1}$. Previously, it was shown that SA inhibits the auxin signaling pathway through the stabilization of members of the Aux/IAA family of transcriptional repressors (Wang et al., 2007). In analogy to the JAs, auxins induce gene expression through direct physical interaction with TIR1-like F-box proteins in the $SCF^{TIR1}$-complex, which in turn target the Aux/IAA family of transcriptional repressors for degradation via the proteasome (Gray et al., 2001; Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). Hence, we postulated that the antagonistic effect of SA on JA signaling might similarly function via the stabilization of JAZ transcriptional respressor proteins. However, we found no evidence for a stabilizing effect of SA on the JAZ proteins (Figs. 3 and 4), suggesting that SA-mediated suppression of the JA response is not targeted at the $SCF^{COI1}$-JAZ machinery.

The AP2-domain/ERF transcription factors ERF1 and ORA59 are important positive regulators of JA-responsive genes such as *PDF1.2* (Lorenzo et al., 2003; Pré et al., 2008). To test whether SA suppresses the JA pathway downstream

of $SCF^{COI1}$-JAZ machinery, we activated *PDF1.2* expression in the JA-insensitive *coi1-1* mutant background through ectopic expression of *35S:ERF1*. SA readily suppressed ERF1-mediated *PDF1.2* transcription (Fig. 4), indicating that SA exerts its suppressive effect on the JA pathway at a position downstream of COI1.

### Inhibition of the JA response by SA is regulated at the transcriptional level

In order to gain insight into transcriptional changes induced by SA and MeJA, we monitored the expression of all 23,750 Arabidopsis genes using Affymetrix ATH1 GeneChips. The effect of SA and (Me)JA on gene expression has been analyzed in several microarray studies in Arabidopsis and sorghum (Schenk et al., 2000; Salzman et al., 2005). In addition, global expression phenotyping of signaling-defective mutants of the SA and JA pathways has been exploited to investigate the network of regulatory interactions among different defense signaling pathways (Glazebrook et al., 2003). These expression profiling studies revealed one-way and mutual antagonism as well as synergistic effects between SA- and JA-dependent signaling pathways. Here, we identified 731 genes that were significantly induced by MeJA, 123 of which were significantly downregulated by SA. *In silico* analysis of the 1-kb promoter region of the 123 MeJA-inducible genes that were suppressed by SA revealed that the I-box motif GATAAG and the GCC-box motif (A)GCCGCC were significantly overrepresented, suggesting that these elements may be involved in the regulation of the SA-JA antagonism.

### The GCC-box is sufficient for SA-mediated downregulation of JA-responsive gene expression

Previously, *PDF1.2* promoter deletion constructs fused to the *GUS* reporter gene were tested for their ability to show SA-JA cross-talk (Spoel et al., 2003). All constructs tested were susceptible to SA-mediated suppression, except for the construct lacking the first 304 bp upstream of the ATG start codon, which include the I-box and the GCC-box (Spoel et al., 2003). This supports our hypothesis that SA-mediated suppression of *PDF1.2* expression may be targeted at either the I-box or the GCC-box. Site-directed mutagenesis of the I-box motif in the *PDF1.2* promoter did not alter the response to either SA, MeJA, or both chemicals, demonstrating that the-I box motif is not essential for SA-JA cross-talk. Previously, Brown et al. (2003) demonstrated that the GCC-box is essential for MeJA-responsiveness of the *PDF1.2* promoter. Members of the family of AP2/ERF transcription factors that regulate JA responses, such as ERF1, ORA59, and ERF4, bind to the GCC-box and have been shown to function in plant defense signaling (Fujimoto et al., 2000; Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004; McGrath et al., 2005; Pré et al., 2008). Several *ERF*s are present in the group of MeJA-induced genes that are suppressed by SA (Supplemental Table S2). Thus, SA

may indirectly suppress MeJA-responsive GCC-box-containing genes by affecting the expression of genes that encode transcription factors that target the GCC-box in JA-responsive genes. To validate our prediction that the antagonistic effect of SA on the JA response is targeted at the GCC-box in JA-responsive genes, we tested the effect of MeJA and SA on the responsiveness of *4XGCC:GUS* reporter lines. We confirmed the findings of Brown et al. (2003) that the GCC-box motif is sufficient for transcriptional activation by MeJA. In addition, we showed that the GCC-box is sufficient for the downregulation of JA-responsive gene expression by SA.

### Mode of action of SA-JA signal interaction

Our results indicate that the antagonistic effect of SA on the JA response functions at the level of gene transcription. Since the GCC-box is sufficient for transcriptional activation by JA and suppression by SA, we hypothesize that the SA pathway interferes with positive transcriptional regulators that bind to the GCC-box, such as ERF1 and ORA59. A plausible scenario may be that SA activates repressors of ERF1 and ORA59 that either inactivate these ERFs by binding to them, or outcompete them through binding to the GCC-box themselves. Our previously finding that over-expression of ORA59 prior to activation of the SA pathway renders plants insensitive to the antagonistic effect of SA (Chapter 5) supports this scenario. Interestingly, *ERF1* and *ORA59* are JA-responsive genes as well and contain a GCC-box in their promoter. Hence, suppression of JA-responsive gene expression via the GCC-box may already function at the level of transcription factor gene expression. A working model for the mode of action of SA-JA signal interaction on GCC-box containing promoters is given in Fig. 9.

Several candidates for the suppression of JA-responsive gene expression have been described, including the WRKY transcription factor WRKY70 (Li et al., 2004) and the glutaredoxin GRX480 (Ndamukong et al., 2007). Overexpression of *WRKY70* caused enhanced expression of SA-responsive *PR* genes and concomitant suppression of JA-responsive *PDF1.2* transcription (Li et al., 2004). GRX480 was identified in a two-hybrid screen for interactors with TGA transcription factors, the latter being important transcriptional regulators of SA-responsive gene expression (Zhang et al., 2003; Ndamukong et al., 2007). Overexpression of *GRX480* completely blocked MeJA-induced *PDF1.2* expression. The suppressive effect of GRX480 on *PDF1.2* induction was abolished in the *tga2 tga5 tga6* triple mutant, indicating that the interaction between GRX480 and TGA transcription factors is essential for the GRX480-dependent cross-talk (Ndamukong et al., 2007). Previously, it was demonstrated that the SA signal transducer NPR1 plays an important role in the SA-mediated downregulation of the JA response (Spoel et al., 2003; Yuan et al., 2007; Koornneef et al., 2008b). The expression of both *WRKY70* and *GRX480* is activated by SA in an NPR1-dependent manner, suggesting that the NPR1 dependency of SA-JA cross-talk may function through the control of downstream transcriptional

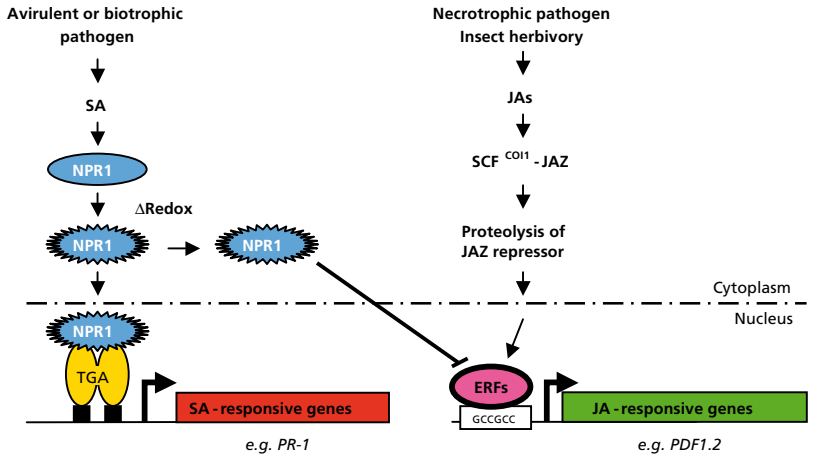



**Figure 9. Model for SA-JA signal interaction on GCC-box containing promoters of JA-responsive genes.**
**Infection by a necrotizing pathogen results in the accumulation of SA and the activation of NPR1. Activated NPR1 (represented by a star-shaped oval) is then translocated into the nucleus where it interacts with TGA transcription factors, ultimately leading to the activation of SA-responsive genes. The activation of NPR1 is controlled by SA-mediated redox changes in the cell. Wounding, such as that caused by insect feeding or infection by a necrotrophic pathogen, results in the accumulation of JAs. Binding of JAs to the $SCF^{COI1}$ ubiquitin-ligase complex leads to degradation of JAZ transcriptional repressor proteins, which results in the activation of the JA response. AP2/ERF transcription factors, such as ERF1 and ORA59, are important transcriptional activators of JA-responsive genes. Binding of ERFs to the GCC-box induces JA-responsive gene expression. The GCC-box is sufficient for SA-mediated suppression of JA-responsive gene expression.**

regulators that on the one hand play a positive role in the activation of SA-responsive genes, and on the other hand suppress JA-responsive genes. However, *grx480* and *wrky70* knockout mutants show wild-type levels of SA-mediated suppression of MeJA-induced *PDF1.2* expression (Ndamukong et al., 2007; Chapter 4), indicating that SA can suppress the JA pathway independently of GRX480 and WRKY70. Hence, the identity of the component(s) of the SA pathway that antagonize JA-dependent activation of GCC-box containing promoters remains to be elucidated. Finally, it should be noted that not all JA-responsive genes that are suppressed by SA contain a GCC-box motif. Hence additional regulatory mechanisms of SA-JA cross-talk must be operative as well.

## Materials and methods

### Plant Material

Seeds of *Arabidopsis thaliana* accessions Col-0 , L*er*-0 and Col-5, and the mutant and transgenic lines *npr1-1* [Col-0] (Cao et al., 1994), *ask1* [L*er*-0] (Yang et al., 2006), *axr6-1* [Col-0] (Hellmann et al., 2003), *arx6-2* [Col-0] (Hellmann et al., 2003), *coi1-1* [Col- 5] (Feys et al., 1994), *coi1-12* [Col-0] (Xiao et al., 2004), *coi1-16* [Col-5] (Ellis and Turner, 2002), *arx1-12* [Col-0] (Lincoln et al., 1990), *axr1-24*

[Col-0] (Tiryaki and Staswick, 2002), *35S:JAZ1-GFP* #2G [Col-0] (Grunewald, 2008), *35S:JAZ1-GFP* #24 [Col-0] (Grunewald, 2008), amiRNA *JAZ1* [Col-0] (Grunewald, 2008), *35S:JAZ10* [Col-0] (originally called At5g13220.1; Yan et al., 2007), *JAZ10* RNAi-7 [Col-0] (originally called At5g13220 RNAi-7; Yan et al., 2007), *JAZ10* RNAi-9 [Col-0] (originally called At5g13220 RNAi-9; Yan et al., 2007), *35S:JAZ10Δ* #OE4A [Col-0] (originally called At5g13220 OE4A; Yan et al., 2007), *35S:JAZ10Δ* #OE4A [Col-0] (originally called At5g13220 OE4B; Yan et al., 2007), *35S:ERF1-1* [Col-0] (Lorenzo et al., 2003), *35S:ERF1-1/coi1-1* [Col-0] (Lorenzo et al., 2003), *PDF1.2:GUS* [Col-0], *4XGCC:GUS* #5 [Col-0], and *4XGCC:GUS* #7 [Col-0] were sown in quartz sand. After two weeks, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min (Pieterse et al., 1998). Plants were cultivated in a growth chamber with an 8-h day (24°C) and 16-h night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10 mM Sequestreen (CIBA-Geigy) once a week. For experiments with *in vitro*-grown plants, seedlings were grown on plates containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), pH 5.7, supplemented with 20 $g{\cdot}L^{-1}$ sucrose and 0.8% (w/v) plant agar.

### Protein extraction and western blot analysis

Entry clones pEN-L4-2-R1 holding the pCaMV *35S* promoter, pEN-R2-GStag-L3 holding a GS-TAP tag and pDONR221-JAZ1 (without stop codon) were recombined by MultiSite Gateway LR reaction using pKCTAP as destination vector. Subsequently, the construct was introduced in the PSB-D culture as described above. Protein extraction and western blot analysis was performed as described by Hemerly (1995). For the detection of JAZ1-CTAP, a 1:2500 dilution of the anti-PAP antibody (Sigma, Steinheim, Germany) was used. As an internal control for loading of the SDS-PAGE gel and transfer of proteins to the membrane, the constitutively accumulating protein CDKA was detected using a primary anti-CDKA antibody (1:2500 dilution) and a secondary peroxidase-conjugated anti-rabbit antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) (1:10000 dilution).

### Construction of transgenic plants

Plasmids for generating transgenic *35S:JAZ1-GFP* and *JAZ1* amiRNA plants were designed and constructed as described (Grunewald, 2008). Col-0 plants were transformed using the floral dip method as described by Clough and Bent (1998). Transformed seedlings were selected as described (Harrison et al., 2006).

For the construction of the I-box knockout line, the 1.2-kb *PDF1.2* promoter fragment was amplified by PCR from genomic DNA of Col-0 plants

using the *PDF1.2* FW2 (5'-GCG AAT TCA TGC ATG CAT CGC CGC ATC G-3') and *PDF1.2* RV2 (5'-CGC TCG AGA TGA TTA TTA CTA TTT TGT TTT C-3') primers. The *PDF1.2* promoter fragment was first cloned into the pCR-Blunt II-TOPO vector for direct insertion of blunt-end PCR products into a plasmid vector (Invitrogen, Breda, the Netherlands). The I-box motif (5'-GATAAG-3') was mutagenized to an *Eco*RI recognition sequence (5'-GAATTC-3') to facilitate identification of mutagenized transformants. Site-directed mutagenesis was carried out by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) according to the manufacturer's protocol. Primers ΔIbox FW (5'-CAA CAA ACA AAA AGC AAG ATG AAT TCT TTT GAT ATT GGC TAC GGG-3') and ΔIbox RV (5'-CCC GTA GCC AAT ATC AAA AGA ATT CAT CTT GCT TTT TGT TTG TTG-3') were designed for the introduction of the desired mutation. Transformants were selected by digestion plasmid DNA with *Eco*RI. After sequence verification, the mutated *PDF1.2ΔIbox* promoter fragment was ligated into the pGREENII 0229-GUS binary vector (Hellens et al., 2000), using the *Spe*I and *Pst*I recognition sites. The plasmid was transformed into *A. tumefaciens* strain C58 (pMP90) (Koncz and Schell, 1986). Col-0 plants were transformed using the floral dip method (Clough and Bent, 1998), and surface-sterilized seeds of transformants were selected on MS medium supplemented with 1% (w/v) sucrose, 0.6% (w/v) plant agar, pH 5.7, and 20 mg.$L^{-1}$ DL-phosphinothicin (Duchefa Biochemie BV, Haarlem, the Netherlands) as a selection.

For the construction of the *4XGCC:GUS* lines, GCC-box monomers of the *PDF1.2* promoter were cloned by annealing the oligonucleotides 5'-ATC CTT AAC CAG CCG CCC ATG TGA-3' and 5'-GAT CTC ACA TGG GCG GCT GGT TAA G-3' and ligating them into the plasmid pIC-20H (Marsh et al., 1984) that was digested with *Bam*HI and *Bgl*II. Monomers were then tetramerized in a head-to-tail configuration using the *Bam*HI and *Bgl*II sites. The tetramers were cloned as *Bam*HI/*Bgl*II fragments in the plasmid GusSH-47 (Pasquali et al., 1994) digested with *Bam*HI such that the orientation of the GCC-boxes relative to the downstream ORF was the same as in the *PDF1.2* promoter. The GUS cassette was transferred to the binary vector pMOG22lambdaCAT using the *Xba*I and *Hin*dIII restriction sites (Pasquali et al., 1994).

### Chemical treatments

Plants were treated with SA and/or MeJA by dipping the leaves into a solution containing 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, the Netherlands) and either 1 mM SA (Mallinckrodt Baker, Deventer, the Netherlands), 0.1 mM MeJA (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), or a combination of these chemicals as described previously (Spoel et al., 2003; Koornneef et al., 2008b). Control treatments were dipped into a solution containing 0.015% (v/v) Silwet L77. Chemical induction of plants grown on MS medium was

performed by transferring 12-day-old seedlings to fresh MS medium supplemented with 0.5 mM SA, 0.02 mM MeJA, or a combination of these chemicals (Spoel et al., 2003). MeJA was added to the solutions from a 1,000-fold concentrated stock in 96% ethanol. To the solutions without MeJA, a similar volume of 96% ethanol was added.

### JAZ degradation assay in cell suspension cultures

For the degradation assays, the plasmid pEN-L4-2-R1 holding the CaMV *35S* promoter, pEN-R2-LUC-L3 and entry clones holding JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ9 (At1g70700) and *MYC2* (At1g32640) open reading frames (ORF) without stop codon were recombined by MultiSite Gateway LR reaction using pKCTAP as destination vector essentially as described (Karimi et al., 2007). The T-DNA in the latter vector additionally expresses *GREEN FLUORESCENT PROTEIN* (*GFP*) under control of the rolD promoter (Van Leene *et al.*, 2007). Plasmids were transfected into *Agrobacterium tumefaciens* strain C58 (pMP90) by electroporation. The Arabidopsis PSB-D cell suspension culture used in this study was maintained and transformed with the plasmids as described previously (Van Leene et al., 2007). Transformed cells were directly selected in liquid medium. Transformed cell cultures were grown for several weeks in the absence of kanamycin before protein degradation assays were performed. Fresh cell cultures were grown for one week after subculturing before use in the JAZ degradation assay. For cross-talk experiments, application of 0.001 mM of JA (Duchefa, Haarlem, the Netherlands) and/or 0.01 mM of SA was added to the cells, which were subsequently harvested at multiple time points by vacuum filtration. Samples were immediately frozen in liquid nitrogen and ground using a Retsch MM300 shaker. Subsequently, proteins were extracted using LUC extraction buffer (100 mM $KPO_4$ pH 7.8, 1 mM EDTA, 7 mM β-mercaptoethanol, 1 mM PMSF and 1 complete protease inhibitor tablet (Roche, Mannheim, Germany) per 10 mL) as described (Salmon et al., 2008). The supernatant was used for subsequent measurements of GFP fluorescence and LUC activity. GFP fluorescence was used to normalize for variations in protein extraction. GFP fluorescence intensity is highly correlated with GFP (Richards et al., 2003) and total protein levels (data not shown). Half-life calculations were performed as described (Dreher et al., 2006) with modifications. For each sample individually, a LUC activity (l)/GFP fluorescence (g) ratio was calculated and divided by an average l/g value for the control samples (i.e. the first time point without JA) to generate a normalized l/g value. For graphic presentation, the natural log of the normalized l/g value was determined and plotted in function of time. For the proteasome-dependency experiment, 60 μM MG132 (Z- Leu-Leu-Leu-H; Boston Biochem, Cambridge, MA, USA) was used.

### RNA extraction and northern blot analysis

RNA extraction and northern blot analysis was performed as described by Van Wees et al. (1999). Northern blots were hybridized with gene-specific probes for *PR-1, PDF1.2* and *VSP2* as described (Van Wees et al., 1999). A probe for *ERF1* was made by PCR amplification of cDNA of MeJA-treated plants using the gene-specific primers 5'-TCC CTT CAA CGA GAA CGA CT-3' and 5'-ACA ACC GGA GAA CAA CCA TC-3'. A probe for the β-glucuronidase-encoding *GUS* gene *uidA* was made by PCR amplification of a plasmid carrying the *GUS* gene using the gene-specific primers 5-AGT GTA CGT ATC ACC GTT TGT GTG TGA AC-3' and 5'-ATC GCC GCT TTG GAC ATA CCA TCC GTA-3' (De Vos et al., 2005; Koornneef et al., 2008a). To check for equal loading, blots were stripped and hybridized with a probe for *18S* ribosomal RNA. The AGI numbers for the genes studied are At2g14610 (*PR-1*), At5g44420 (*PDF1.2*), At5g24770 (*VSP2*), and At3g23240 (*ERF1*). After hybridization with α-$^{32}$P-dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of *PDF1.2* or *VSP2* mRNA on the northern blots were quantified using a BioRad Molecular Imager FX with Quantity One software (BioRad, Veenendaal, the Netherlands). The *PDF1.2* and *VSP2* mRNA levels of the MeJA treatment were set to 100% and compared to *PDF1.2* or *VSP2* mRNA levels of the rest of the treatments. All gene expression analyses have been repeated with similar results.

### GUS assays

In the histochemical GUS assay, GUS activity was assessed by transferring seedlings to a GUS staining solution (1 mM X-Gluc, 100 mM NaPi buffer, pH 7.0, 10mM EDTA, and 0.1% (v/v) Triton X-100). After vacuum infiltration and overnight incubation at 37°C, the seedlings were de-stained by repeated washes in 70% ethanol (Koornneef et al., 2008a).

For the quantitative GUS assays, 2-week-old plate-grown plants were transferred to 6-well plates containing 5 mL MES buffer (5 mM MES, 1mM KCl, pH 5.7). Five seedlings were used per treatment and treatments were performed in triplicate. Twenty-four h after transfer to MES buffer, 1 mL MES buffer supplemented with SA and/or MeJA was added to the seedlings resulting in final concentrations of 0.5 mM SA and 0.1 mM MeJA, respectively. The seedlings were harvested 24 h after induction treatment and immediately frozen in liquid nitrogen. The frozen tissue was used for protein isolation and quantitative GUS activity measurement as described (Pré et al., 2008).

### Sample preparation and microarray data collection

For isolation of RNA, whole rosettes from Col-0 and *npr1-1* plants were mock-treated or treated with SA, MeJA, or a combination of both as described above. Leaf tissue was harvested 28 h after treatment and immediately frozen in liquid

nitrogen. RNA was prepared from three independent biological experiments, each consisting of three to ten plants per treatment, and purified using RNeasy Plant Mini Kit columns (Quiagen Benelux BV, Venlo, the Netherlands). RNA samples were analyzed for quality by capillary electrophoresis, using an Agilent 2100 Bioanalyzer system. Synthesis of cRNA probes, hybridization to ATH1 Affymetrix GeneChips, and collection of data from the hybridized GeneChips were carried out by ServiceXS (Leiden, the Netherlands) and the Affymetrix service station of Leiden University Medical Center, where they passed all internal quality checks. Hybridizations with labeled cRNAs were conducted with Arabidopsis ATH1 full-genome GeneChips (Affymetrix, Santa Clara, USA), containing 22,810 probe sets representing approximately 23,750 Arabidopsis genes (Redman et al., 2004).

### Expression profiling and promoter analysis

GeneChip Operating Software (GCOS; Affymetrix, Santa Clara, USA) was used to globally normalize the expression data on each GeneChip to an average of 200 so that hybridization intensity of all 24 GeneChips was equivalent. In addition, expressed genes were identified by GCOS, which uses statistical criteria to generate a "present" or "absent" call for genes represented by each probe set on the array. For analysis of differentially expressed genes, log2-transformed expression values of three independent biological experiments were compared between treatments using Student's *t* test. Furthermore, the expression level in the induced treatment had to be significantly detectable (present call generated by GCOS) in all three biological replicates. False discovery rate (FDR) correction was applied to account for testing of multiple genes. All *q* values were $<0.06$ and therefore acceptable for inclusion in the analysis. To identify overrepresented promoter elements in clusters of co-regulated genes, the visualization tool of the web-based application Athena was employed (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) (O'Connor et al., 2005) using default settings. In addition, the promoter bootstrapping program POBO was used (Kankainen and Holm, 2004) with the following parameter settings: number of pseudoclusters: 1000, number of promoters in the pseudoclusters: 123.

### Quantitative real-time PCR analysis

Q-RT-PCR analysis was performed as described by Pauwels et al. (2008) to determine the expression of the *AOS* gene (At5g42650) in cultured Arabidopsis cells. Briefly, cell culture samples were taken, immediately frozen in liquid nitrogen and ground using a Retsch MM300 shaker. Total RNA was converted to cDNA with the SuperScript II kit (Invitrogen, Carlsbad, MA, USA). Q-RT-PCRs were run on a Light Cycler 480 instrument, with the TaqMan Master Kit and the Arabidopsis Universal Probe Library according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). The Universal Probe Library

Assay Design Center (www.universalprobelibrary.com) was used to design primers and select Universal Probe Library probes. The following *AOS*-specific primers were used: 5'-CAC CGG CGT TAG TCA AAT CT-3' and 5'-CGG CGG ATT CTA AGA AAA ACT-3'. Delta-$C_T$ relative quantification with multiple reference gene normalization was performed with the qBase program (medgen.ugent.be/qbase). The reference genes used for normalization were At1g69280, At4g17300, At3g25800, and At1g04300 as described (Pauwels et al., 2008).

**Acknowledgements**

We thank Jan Geerinck and Robin vanden Bossche for their excellent technical assistance. This work was supported by VICI grant no. 865.04.002 of the Earth and Life Sciences Foundation, which is subsidized by the Netherlands Organization of Scientific Research.

## Supplementary material

***Supplemental Table S1.*** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected MeJA- and SA-responsive genes.

***Supplemental Table S2.*** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected SA-JA cross-talk genes.

***Supplemental Table S3.*** MS Excel file with normalized expression levels, fold-change information, AGI numbers and TIGR annotation of the selected NPR1-dependent and –independent SA-JA cross-talk genes.

**All supplementary material can be downloaded from:**

http://www.bio.uu.nl/~fytopath/GeneChip_data.htm

CHAPTER 7

# Summarizing discussion

Antonio Leon-Reyes • Utrecht University

## Cross-talk in plant defense signaling to fine tune defense

Plants are abundantly present on Earth and are at the basis of most food webs. They are members of complex communities and interact with a multitude of other organisms. In addition to parasitic interactions, beneficial relationships are also frequent in nature, improving plant nutrition or helping the plant to overcome abiotic and biotic stresses. These interactions involve fungi, such as mycorrhizal symbionts (Pozo and Azcon-Aguilar, 2007), and bacteria, such as plant growth-promoting rhizobacteria (Van Loon et al., 1998; Van Wees et al., 2008). To combat invasion by pathogens and insects on the one hand, and to accommodate beneficial organisms on the other hand, plants have evolved sophisticated strategies to "perceive" biotic interactions and to translate this "perception" into a defensive or conducive response (Kessler and Baldwin, 2002; Dicke and Hilker, 2003; Harrison, 2005; Chisholm et al., 2006; Jones and Dangl, 2006; Van Wees et al., 2008). Recent advances in defense signaling research revealed that the capacity of plants to respond to the enormous diversity of other organisms in their environment is highly flexible (Chapter 1). The signaling networks that are activated by the plant in response to parasitic, herbivorous and beneficial organisms overlap, indicating that the regulation of the adaptive response of the plant is finely balanced between protection against aggressors and acquisition of benefits.

An important question in plant defense signaling research is how plants integrate signals induced by pathogens, beneficial microbes and insects into the most appropriate adaptive response. In *Arabidopsis thaliana* (Arabidopsis), it was shown that SA-, JA-, and ET-dependent pathways regulate defense responses that are differentially effective against specific types of attackers (Thomma et al., 2001a; Glazebrook, 2005). Pathogens with a biotrophic lifestyle are generally more sensitive to SA-dependent responses, whereas necrotrophic pathogens and herbivorous insects are commonly deterred by JA- and/or ET-dependent defenses. In nature, plants often deal with simultaneous or subsequent invasion by multiple aggressors, which can influence the primary induced defense response of the host plant (Poelman et al., 2008a). Moreover, activation of plant defense mechanisms is associated with ecological fitness costs (Heil and Baldwin, 2002; Heidel et al., 2004; Van Hulten et al., 2006). Hence, plants need regulatory mechanisms to adapt to changes in their hostile environment. Cross-talk between defense signaling pathways helps the plant to minimize fitness costs and create a flexible signaling network that allows the plant to finely tune its defense response to the invaders encountered (Reymond and Farmer, 1998; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Pieterse et al., 2009).

The interaction between the SA and the JA response pathway is one of the best studied examples of defense-related signal cross-talk is (Kunkel and Brooks, 2002;

Thaler et al., 2002b; Glazebrook et al., 2003; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Spoel and Dong, 2008). Many studies have demonstrated that endogenously accumulating SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent defenses over JA-dependent ones (Doherty et al., 1988; Peña-Cortés et al., 1993; Gupta et al., 2000; Spoel et al., 2003). As a result of the negative interaction between SA and JA signaling, activation of the SA response should render a plant more susceptible to attackers that are resisted via JA-dependent defenses and *vice versa*. Although trade-offs between SA and JA signaling have been described in both directions, this thesis research mainly focused on the antagonistic effect of SA on the JA response. In Chapter 2, we found that infection of Arabidopsis by the biotrophic pathogen *Hyaloperonospora arabidopsidis* strongly suppressed JA-mediated defenses that were activated upon feeding by caterpillars of the small cabbage white *Pieris rapae*, indicating that Arabidopsis prioritizes the SA response when attacked by SA- and JA-inducing attackers. Similarly, Spoel et al. (2007) showed that SA-mediated defenses that are triggered upon infection by a virulent strain of the hemi-biotrophic pathogen *Pseudomonas syringae* rendered infected tissues more susceptible to infection by the necrotrophic pathogen *Alternaria brassicicola* by suppressing the JA signaling pathway. Trade-offs between SA-dependent pathogen resistance and JA-dependent defense against insect herbivory have been repeatedly reported in several plant species, suggesting that it is a conserved mechanism of defense regulation (Thaler et al., 1999; Felton and Korth, 2000; Pieterse et al., 2001; Bostock, 2005). In Chapter 2, we found that SA-mediated suppression of JA responses was functional in 18 Arabidopsis accessions that were collected from very different geographic locations, confirming that this trait is evolutionary conserved and may play an important role in the ecology of plant defense. Although detectable in all Arabidopsis accessions tested, we also observed that the degree of SA-JA antagonism among Arabidopsis accessions varied between 50% and 96% (Chapters 2, 4, 5, 6), indicating that Arabidopsis possesses intraspecific genetic variability to express this type of pathway cross-talk. This is in agreement with Traw et al. (2003), who demonstrated that the accessions Col-0 and Ws-0 are differentially sensitive to SA-JA cross-talk in the Arabidopsis-*P. syringae* interaction. Hence, it is tempting to speculate that sensitivity to SA-JA cross-talk has been a player in the evolutionary process of adaptation of each accession to its biotic environment.

## Kinetics of SA-JA cross-talk regulation

In Arabidopsis, the production of the defense signals SA, JA, and ET has been shown to vary depending on the type of organism that interacts with the plant (De Vos et al., 2005). The quantity, composition and timing of this signal signature results in the activation of a specific set of genes that eventually shapes the outcome of the defense

response that is triggered by the attacker encountered (De Vos et al., 2005; Mur et al., 2006). However, additional ways of regulation are also important. For example, the bacterial pathogen *P. syringae,* the necrotrophic fungus *A. brassicicola,* the cell-content feeding thrips *Frankliniella occidentalis* and the chewing caterpillar *P. rapae* all stimulate JA biosynthesis and JA-responsive gene expression in Arabidopsis (De Vos et al., 2005). Yet, most JA-responsive genes that were activated by each attacker were specific for the plant–attacker combination. Hence, the signal signature has an important primary role in the orchestration of the defense response of the plant, but other regulatory mechanisms, such as pathway cross-talk, or additional attacker-induced signals, eventually shape the highly complex attacker-specific defense response (De Vos et al., 2005; Pauwels et al., 2009).

Previously, Mur et al. (2006) demonstrated that treatment of Arabidopsis with low concentrations of JA and SA resulted in a synergistic effect on the JA- and SA-responsive genes *PDF1.2* and *PR-1,* respectively. However, at higher concentrations the effects were antagonistic, demonstrating that the outcome of the SA-JA interaction is dependent upon the relative concentration of each hormone. In Chapter 2, we investigated the kinetics of SA and JA signaling in relation to the outcome of the SA-JA signal interaction. It appeared that very low doses of SA are already able to suppress JA-responsive *PDF1.2* transcription, suggesting that this SA-JA cross-talk mechanism is highly sensitive. However, this antagonistic effect was only apparent when the SA pathway was activated *after* the onset of the JA response, or within a time frame of about 30 h prior to the activation of the JA response, indicating that the ability of SA to suppress JA-responsive gene expression is transient. The window of opportunity of SA to down-regulate JA-responsive gene expression coincided with a transient SA-induced change in the cellular redox potential. Moreover, inhibition of glutathione biosynthesis by BSO strongly affected SA-mediated suppression of MeJA-induced *PDF1.2* expression, suggesting that SA-JA cross-talk is redox regulated.

In Chapter 4, we discovered that the final outcome of the interplay between SA, JA and ET signaling is dependent on the sequence in which these plant hormones are produced. If the SA pathway is activated prior, or at the same time as the JA response, then the SA response will suppress the JA pathway. When the JA pathway is activated first in the absence of ET, then SA can still suppress the JA pathway. However, when the JA and ET pathways are activated simultaneously, then the potentiated JA response becomes insensitive to suppression by SA. For example, inoculation of the JA- and ET-inducing pathogen *A. brassicicola* 1 day before inoculation of the SA-inducing pathogen *H. arabidopsidis* abolished SA-JA cross-talk in susceptible *pad3-1* plants (Chapter 4).

Collectively, these findings demonstrate that concentration, timing, and sequence of initiation of the SA, JA, and ET signaling pathways are important for the outcome of the SA-JA signal interaction. The blend of defense signals

produced during a plant-attacker interaction varies greatly and is highly dependent on the type of invader encountered (De Vos et al., 2005). Hence, the kinetics of phytohormone biosynthesis and signaling during the interaction of a plant with its attacker(s) could be highly decisive in the final outcome of the defense response to the attacker encountered. Ecological studies have shown that the induced defense response of a plant to a specific invader can be influenced by the history of the plant in terms of the type of attackers that the plant has encountered in the past (Poelman et al., 2008b; Poelman et al., 2008a). In view of our findings, it is tempting to speculate that when Arabidopsis is first attacked by a SA-inducing pathogen, the SA pathway will suppress JA responses. This will result in enhanced resistance against pathogens that are sensitive to SA-dependent defenses (e.g. biotrophs), but it will also lead to enhanced susceptibility to necrotrophic pathogens and insects that are sensitive to JA-dependent defenses. *Vice versa*, when a plant is first attacked by a pathogen that strongly activates both the JA and the ET response, then these plants develop enhanced resistance to pathogens and insects that are sensitive to JA/ET-dependent defenses. When a secondary, SA-inducing pathogen comes into play, the JA/ET-dependent defenses will remain active as they can not be suppressed by the SA response that is activated by this pathogen. It must be noted that the data we provide in this thesis do not necessarily hold true for other plant species. In other plant species similar molecular mechanisms may operate, but due to the ecological context in which a plant species has evolved, the order of signal prioritization may be different among plant species.

## The role of ET in the SA-JA cross-talk

Since ET is an important modulator of plant defense and a major constituent of the blend of defense signals that is produced during many plant-attacker interactions (Broekaert et al., 2006; Van Loon et al., 2006a; Adie et al., 2007a; Von Dahl and Baldwin, 2007), we investigated the effect of ET on the SA-JA signal interaction in Chapter 3. Previously, it was demonstrated that SA-activated NPR1 is required for the suppression of JA-responsive gene expression by SA (Spoel et al., 2003) and that activation of NPR1 is redox regulated (Mou et al., 2003). In uninduced cells, NPR1 is present as an oligomer that is formed through intermolecular disulfide bonds. SA mediates a change in the cellular redox potential, resulting in the reduction of the NPR1 oligomer to its active monomeric form. Monomeric NPR1 is then translocated into the nucleus where it functions as a transcriptional co-activator of SA-responsive genes such as *PR-1* (Dong, 2004). For the suppression of JA-responsive gene expression, translocation of SA-activated NPR1 into the nucleus is not required, as has been demonstrated in both Arabidopsis and rice (Spoel et al., 2003; Yuan et al., 2007), suggesting an important role for cytosolic

NPR1 in SA-JA cross-talk. Thus, although the role of NPR1 in SA-JA cross-talk and SA-induced *PR-1* gene expression seems to be dissimilar, it is plausible that both defense responses are controlled by active NPR1 monomers that are produced upon changes in the redox state. Especially, since we demonstrated in Chapter 2 that SA-JA cross-talk is also redox regulated.

In Chapter 3, we added another layer of complexity on the role of NPR1 in SA-JA cross-talk, since we demonstrated that ET signaling strongly affects the requirement of NPR1 in the antagonistic effect of SA on JA-dependent defenses. These findings indicate that the final outcome of the SA-JA signal interaction during the complex interaction of plants with their attackers can be shaped by ET. This was supported by our observation that the antagonistic effect of SA on MeJA-induced resistance against feeding by ET-noninducing thrips *F. occidentalis* was controlled by NPR1. By contrast, suppression of JA-dependent resistance against the JA- and ET-inducing necrotroph *A. brassicicola* by SA functioned independently of NPR1. Our results in Chapter 3, strengthened the notion that NPR1 plays a dual role in regulating SA-JA cross-talk on the one hand, and activation of SA-responsive *PR* gene expression on the other hand. We proposed a model in which the cytosolic function of NPR1 plays a role in SA-JA cross-talk and can be bypassed by ET, and in which the nuclear function of NPR1 plays a role in the activation of SA-responsive genes and can be stimulated by ET.

In chapter 4, we continued analyzing the effect of ET on SA-JA cross-talk. In earlier studies, we activated the SA response either at the same time, or prior to the activation of the JA/ET response. In Chapter 4, we provided evidence that stimulation of the JA and ET response prior to the activation of the SA response can render the plant insensitive to SA-mediated suppression of JA signaling. JA and ET often act synergistically on the activation of defense-related gene expression (Penninckx et al., 1998; Broekaert et al., 2006; Adie et al., 2007a). The AP2-domain transcription factors, ERF1 and ORA59 have been described as important integrators of the JA and ET pathways (Lorenzo et al., 2003; Lorenzo and Solano, 2005; Pré et al., 2008). In Chapter 4, we show that besides its role in the integration of the JA and ET pathway, ORA59 also plays an important role in counteracting SA-mediated suppression of *PDF1.2* expression. Overexpression of *ORA59* activated *PDF1.2* transcription, which subsequently could not be suppressed by SA. Overexpression of *ERF1* had a much smaller effect on counteracting the antagonistic function of SA (Chapter 6), suggesting that ORA59 plays a dominant role in this phenomenon. Based on our findings we concluded that potentiation of the JA response by simultaneous activation of the JA and ET pathway prior to induction of the SA pathway, blocks the ability of SA to inhibit the JA pathway. A plausible scenario for the molecular mechanism behind this observation is that SA-induced repressors of the JA response our outcompeted by the enhanced levels of ORA59.

## Site of action of SA-JA signal interaction

The JA signaling pathway is well-studied and in recent years exciting new insights into its regulation have been uncovered (Chico et al., 2008; Katsir et al., 2008; Staswick, 2008). This provided plenty of opportunities to investigate the site of action by which SA exerts its antagonistic effect on the JA signaling pathway. In Chapter 5, we tested the hypothesis that SA-mediated suppression of JA signaling is caused by the down regulation of the expression of JA biosynthesis genes. The JA-responsive JA biosynthesis genes *LOX2, AOS, OAC3* and *OPR3* were downregulated by SA. Hence, we hypothesized that JA biosynthesis may be a potential target of the antagonistic effect of SA on JA signaling. However, induction of the JA response in the JA biosynthesis mutant *aos/dde2* could be suppressed by SA to wild-type levels, indicating that downregulation of JA biosynthesis is not required for SA-mediated suppression of JA signaling. Spoel et al. (2007) and Adie et al. (2007b) have demonstrated that JA levels in Arabidopsis are not negatively affected by SA accumulation, which supports our conclusion that the antagonistic effect of SA on JA signaling acts downstream of JA biosynthesis.

The E3 ubiquitin-ligase $SCF^{COI1}$-complex plays a crucial role in the regulation of the JA response as it targets JAZ transcriptional repressor proteins for degradation upon perception of JAs (Chico et al., 2008; Katsir et al., 2008; Staswick, 2008). In Chapter 6, we tested whether $SCF^{COI1}$ is a potential target of SA during the suppression of JA signaling. However, mutants with defects in proteins from the $SCF^{COI1}$-complex showed near wild-type levels of SA-JA cross-talk, suggesting that it is unlikely that SA inhibits the JA signaling pathway via $SCF^{COI1}$.

In the auxin signaling pathway, auxins activate auxin-responsive genes through direct physical interaction with TIR1-like F-box proteins in the $SCF^{TIR1}$-complex, which in turn target the Aux/IAA family of transcriptional repressors for degradation via the proteasome (Gray et al., 2001; Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). Since Wang et al. (2007) demonstrated that SA inhibits the auxin signaling pathway through the stabilization of members of the Aux/IAA family of transcriptional repressor proteins, we postulated that the antagonistic effect of SA on JA signaling might similarly function via the stabilization of JAZ transcriptional respressor proteins. However, we found that SA had no effect on the stability of the JAZ proteins, suggesting that SA-mediated suppression of the JA response is not targeted at the $SCF^{COI1}$-JAZ machinery (Chapter 6). To check whether SA suppresses the JA pathway downstream of the $SCF^{COI1}$-JAZ machinery (Chapter 6), we activated *PDF1.2* expression in the JA-insensitive *coi1-1* mutant background through ectopic expression of *35S:ERF1*. Exogenous application of SA strongly suppressed ERF1-mediated *PDF1.2* transcription, indicating that SA exerts its suppressive effect on the JA pathway in an COI1-independent manner.

Analysis of the SA-JA cross-talk transcriptome showed that the GCC-box is significantly overrepresented in the promoters of JA-responsive genes that are sensitive to suppression by SA. Using transgenic plants in which the expression of the *GUS* reporter gene was driven by 4 copies of the GCC-box, we were able to show that the GCC-box is not only sufficient for induction by MeJA (as previously shown by Brown et al. (2003)), it is also sufficient for suppression by SA. Since the GCC-box is sufficient for transcriptional activation by JA and suppression by SA, we hypothesized that the SA pathway interferes with positive transcriptional regulators that bind to the GCC-box, such as ERF1 and ORA59, and which have been shown to activate JA-dependent plant defenses (Fujimoto et al., 2000; Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004; McGrath et al., 2005; Pré et al., 2008). We proposed a model for SA-JA cross-talk in which SA activate a repressor of ERF1 and ORA59 that either inactivate these ERFs by binding to them, or outcompete them through binding to the GCC-box. Our observation in Chapter 5 that over-expression of ORA59 prior to activation of the SA pathway renders plants insensitive to the antagonistic effect of SA supports this scenario. *ERF1* and *ORA59* are JA-responsive genes as well and contain a GCC-box in their promoter, suggesting that SA-mediated suppression of JA-responsive gene expression via the GCC-box may also function at the level of transcription factor gene expression.

## Molecular integrators of SA-JA-ET cross-talk: a model

Recent advances in plant defense signaling research provided a wealth of new insights into the molecular players that are involved in the regulation of the induced defense signaling network. Several candidates for the suppression of JA-responsive gene expression have been described (Chapter 1). A central player is the redox-sensitive protein NPR1, which is able to transduce the SA signal, resulting in the suppression of the JA pathway (Spoel et al., 2003). ET can bypass the need of NPR1 (Chapter 3) in SA-JA cross-talk. Hence, the JA pathway can be suppressed in an NPR1-dependent and an NPR1-independent manner (Chapter 3 and 6). NPR1 is also an important regulator of the expression of genes that have previously been identified as important regulators of SA-JA cross-talk. Both the WRKY transcription factor gene *WRKY70* (Li et al., 2004) and the glutaredoxin gene *GRX480* (Ndamukong et al., 2007) are activated by SA in an NPR1-dependent manner. Subsequently, they have a positive effect on the expression of SA-responsive genes and a negative effect on JA-responsive genes. GRX480 was shown to interact with TGA transcription factors, which are important transcriptional regulators of SA-responsive gene expression (Zhang et al., 2003; Ndamukong et al., 2007). Ectopic expression of *GRX480* completely blocked MeJA-induced *PDF1.2* expression, but

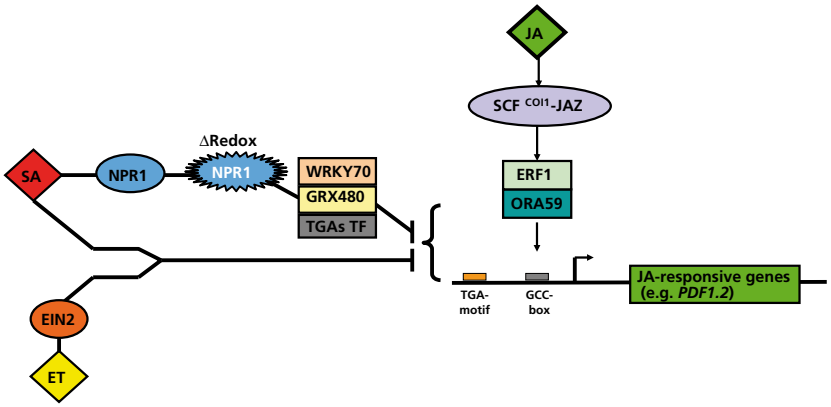



**Figure 1. Molecular players involved in cross-talk between SA, JA and ET signaling pathways in Arabidopsis.** **JA-responsive genes, such as *PDF1.2,* are activated upon induction of the JA signaling pathway. The AP2/ERF transcription factors ERF1 and ORA59 are important regulators of *PDF1.2* expression. Induction of the SA pathway leads to a change in the redox status of plant cells, which activates NPR1. Activated NPR1 induces the expression of WRKY70 and GRX480/TGAs, resulting in the activation of SA-responsive genes and the suppression of JA-responsive genes, such as *PDF1.2.* In combination with ET, SA is able to suppress the JA pathway in an NPR1-indepenent manner. However, when the JA and ET pathways are simultaneously activated prior to stimulation of the SA pathway, then the JA pathway is insensitive to suppression by SA. ORA59 plays an important role in this respect. Suppression of the JA pathway acts downstream of the SCF$^{COI1}$-JAZ complex and is likely directly targeted at the level of gene transcription. The GCC-box in JA-responsive promoters was identified as an important target for suppression by SA.**

this suppressive effect was abolished in the *tga2 tga5 tga6* triple mutant, indicating that the interaction between GRX480 and TGA transcription factors is essential for the GRX480-dependent cross-talk (Ndamukong et al., 2007). However, knockout mutants *grx480* and *wrky70* show wild-type levels of SA-JA cross-talk (Ndamukong et al., 2007; Chapter 4), indicating that SA can suppress the JA pathway independently of GRX480 and WRKY70. In Chapter 6 we provided evidence that the suppressive effect of SA on JA-responsive gene expression is targeted at the GCC-box in JA-responsive promoters. The identity of the component(s) of the SA pathway that antagonize JA-dependent activation of GCC-box containing promoters is still not known and must be subject of future research. Finally, it should be noted that not all JA-responsive genes that are suppressed by SA contain a GCC-box in their promoter. A nice example is *VSP2,* which is highly sensitive to SA-mediated suppression. *VSP2* gene expression is regulated by the transcription factor MYC2 (Lorenzo et al., 2004). Whether MYC2-mediated expression of JA-responsive gene expression is directly targeted by SA is currently unknown.

In summary, the main goal of the research described in this thesis was to unravel molecular mechanisms underlying cross-communication between the defense-related signaling pathways that are regulated by the phytohormones SA, JA and ET. A working model for the mode of action of SA-JA-ET signal interaction from the research performed in this thesis is described in Fig. 1.

## References

**Abe, H., Ohnishi, J., Narusaka, M., Seo, S., Narusaka, Y., Tsuda, S., and Kobayashi, M.** (2008). Function of jasmonate in response and tolerance of Arabidopsis to thrips feeding. Plant Cell Physiol. **49,** 68-80.

**Abramoff, M.D., Magelhaes, P.J., and Ram, S.J.** (2004). Image processing with ImageJ. Biophotonics International **11,** 36-42.

**Adie, B., Chico, J.M., Rubio-Somoza, I., and Solano, R.** (2007a). Modulation of plant defenses by ethylene. J. Plant Growth Regul. **26,** 160-177.

**Adie, B.A.T., Perez-Perez, J., Perez-Perez, M.M., Godoy, M., Sanchez-Serrano, J.J., Schmelz, E.A., and Solano, R.** (2007b). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. Plant Cell **19,** 1665-1681.

**Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R.** (1999). *EIN2*, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284,** 2148-2152.

**Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C., Maclean, D.J., Ebert, P.R., and 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,** 3460-3479.

**Asselbergh, B., De Vleesschauwer, D., and Höfte, M.** (2008). Global switches and fine-tuning - ABA modulates plant pathogen defense. Mol. Plant-Microbe Interact. **21,** 709-719.

**Assmann, S.M., Snyder, J.A., and Lee, Y.R.J.** (2000). ABA-deficient (*aba1*) and ABA-insensitive (*abi1-1, abi2-1*) mutants of Arabidopsis have a wild-type stomatal response to humidity. Plant Cell Environ. **23,** 387-395.

**Bakker, P.A.H.M., Pieterse, C.M.J., and Van Loon, L.C.** (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology **97,** 239-243.

**Beckers, G.J.M., and Spoel, S.H.** (2006). Fine-tuning plant defence signalling: salicylate versus jasmonate. Plant Biol. **8,** 1-10.

**Belkhadir, Y., and Chory, J.** (2006). Brassinosteroid signaling: A paradigm for steroid hormone signaling from the cell surface. Science **314,** 1410-1411.

**Bell, E., and Mullet, J.E.** (1993). Characterization of an Arabidopsis-lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol. **103,** 1133-1137.

**Benjamins, R., and Scheres, B.** (2008). Auxin: The looping star in plant development. Annu. Rev. Plant Biol. **59,** 443-465.

**Berrocal-Lobo, M., and Molina, A.** (2004). Ethylene response factor 1 mediates Arabidopsis resistance to the soilborne fungus *Fusarium oxysporum*. Mol. Plant-Microbe Interact. **17,** 763-770.

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

**Bezemer, T.M., and Van Dam, N.M.** (2005). Linking aboveground and belowground interactions via induced plant defenses. Trends Ecol. Evol. **20,** 617-624.

**Bielawski, W., and Joy, K.W.** (1986). Reduced and oxidised glutathione and glutathione-reductase activity in tissues of Pisum sativum. Planta **169,** 267-272.

**Bleecker, A.B., Estelle, M.A., Sommerville, C., and Kende, H.** (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241,** 1086-1089.

**Bodenhausen, N., and Reymond, P.** (2007). Signaling pathways controlling induced resistance to insect herbivores in Arabidopsis. Mol. Plant-Microbe Interact. **20,** 1406-1420.

**Borello, U., Ceccarelli, E., and Giuliano, G.** (1993). Constitutive, light-responsive and circadian clock-responsive factors compete for the different I box elements in plant light-regulated promoters. Plant J. **4,** 611-619.

**Bostock, R.M.** (1999). Signal conflicts and synergies in induced resistance to multiple attackers. Physiol. Mol. Plant Pathol. **55,** 99-109.

**Bostock, R.M.** (2005). Signal crosstalk and induced resistance: straddling the line between cost and benefit. Annu. Rev. Phytopathol. **43,** 545-580.

**Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X.** (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell **6,** 1845-1857.

**Brodersen, P., Petersen, M., Bjorn Nielsen, H., Zhu, S., Newman, M.-A., Shokat, K.M., Rietz, S., Parker, J., and Mundy, J.** (2006). Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. Plant J. **47,** 532-546.

**Broekaert, W.F., Terras, F.R.G., Cammue, B.P.A., and Vanderleyden, J.** (1990). An automated quantitative assay for fungal growth. FEMS Microbiol. Lett. **69,** 55-60.

**Broekaert, W.F., Delaure, S.L., De Bolle, M.F.C., and Cammuel, B.P.A.** (2006). The role of ethylene in host-pathogen interactions. Annu. Rev. Phytopathol. **44,** 393-416.

**Brooks, D.M., Bender, C.L., and Kunkel, B.N.** (2005). The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. Mol. Plant Pathol. **6,** 629-639.

**Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J., and Manners, J.M.** (2003). A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of Arabidopsis. Plant Physiol. **132,** 1020-1032.

**Buchanan, B.B., Gruissem, W., and Jones, R.L.** (2000). Biochemistry & Molecular Biology of Plants. (Rockville, Maryland: American Society of Plant Physiologists).

**Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X.** (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583-1592.

**Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X.** (1997). The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88,** 57-63.

**Chen, Z.Y., Agnew, J.L., Cohen, J.D., He, P., Shan, L.B., Sheen, J., and Kunkel, B.N.** (2007). *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. Proc. Natl. Acad. Sci. USA **104,** 20131-20136.

**Cheng, H., Qin, L., Lee, S., Fu, X., Richards, D.E., Cao, D., Luo, D., Harberd, N.P., and Peng, J.** (2004). Gibberellin regulates Arabidopsis floral development via suppression of DELLA protein function. Development **131,** 1055-1064.

**Chico, J.M., Chini, A., Fonseca, S., and Solano, R.** (2008). JAZ repressors set the rhythm in jasmonate signaling. Curr. Opin. Plant Biol. **11,** 486-494.

**Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D.G., Felix, G., and Boller, T.** (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448,** 497-500.

**Chini, A., Fonseca, S., Chico, J.M., Fernández-Calvo, P., and Solano, R.** (2009). The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. Plant J. **in press**.

**Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L., and Solano, R.** (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448,** 666-U664.

**Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J.** (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell **124,** 803-814.

**Chung, H.S., and Howe, G.A.** (2009). A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. Plant Cell **21,** 131-145.

**Cipollini, D., Enright, S., Traw, M.B., and Bergelson, J.** (2004). Salicylic acid inhibits jasmonic acid-induced resistance of *Arabidopsis thaliana* to *Spodoptera exigua*. Mol. Ecol. **13,** 1643-1653.

**Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X.** (1998). Uncoupling *PR* gene expression from NPR1 and bacterial resistance: characterization of the dominant Arabidopsis *cpr6-1* mutant. Plant Cell **10,** 557-569.

**Clarke, J.D., Volko, S.M., Ledford, H., Ausubel, F.M., and Dong, X.** (2000). Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in Arabidopsis. Plant Cell **12,** 2175-2190.

**Clough, S.J., and Bent, A.F.** (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735-743.

**Conklin, P.L., Pallanca, J.E., Last, R.L., and Smirnoff, N.** (1997). L-ascorbic acid metabolism in the ascorbate-deficient Arabidopsis mutant *vtc1*. Plant Physiol. **115,** 1277-1285.

**Conrath, U., Beckers, G.J.M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.-A., Pieterse, C.M.J., Poinssot, B., Pozo, M.J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, W., Zimmerli, L., and Mauch-Mani, B.** (2006). Priming: getting ready for battle. Mol. Plant-Microbe Interact. **19,** 1062-1071.

**Creelman, R.A., and Mulpuri, R.** (2002). The oxylipin pathway in Arabidopsis. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville: American Society of Plant Biologists), pp. 1-24.

**Cristescu, S.M., De Martinis, D., Hekkert, S.T., Parker, D.H., and Harren, F.J.M.** (2002). Ethylene production by *Botrytis cinerea* in vitro and in tomatoes. Appl. Environ. Microbiol. **68,** 5342-5350.

**Cui, J., Jander, G., Racki, L.R., Kim, P.D., Pierce, N.E., and Ausubel, F.M.** (2002). Signals involved in Arabidopsis resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. Plant Physiol. **129,** 551-564.

**Cui, J., Bahrami, A.K., Pringle, E.G., Hernandez-Guzman, G., Bender, C.L., Pierce, N.E., and Ausubel, F.M.** (2005). *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. Proc. Natl. Acad. Sci. USA **102,** 1791-1796.

**Czechowski, T., Bari, R.P., Stitt, M., Scheible, W.R., and Udvardi, M.K.** (2004). Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant J. **38,** 366-379.

**De Laat, A.M.M., and Van Loon, L.C.** (1982). Regulation of ethylene biosynthesis in virus-infected tobacco leaves. II. Time course of levels of intermediates and *in vivo* conversion rates. Plant Physiol. **69,** 240-245.

**De Sutter, V., Vanderhaegen, R., Tilleman, S., Lammertyn, F., Vanhoutte, I., Karimi, M., Inze, D., Goossens, A., and Hilson, P.** (2005). Exploration of jasmonate signalling via automated ans standarized transient expression assays in tobacco cells. Plant J **44,** 1065-1076.

**de Torres-Zabala, M., Truman, W., Bennett, M.H., Lafforgue, G., Mansfield, J.W., Egea, P.R., Bogre, L., and Grant, M.** (2007). *Pseudomonas syringae* pv. *tomato* hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. EMBO J. **26,** 1434-1443.

**De Vos, M.** (2006). Signal signature, transcriptomics and effectiveness of induced pathogen and insect resistance in Arabidopsis (Ph.D. Thesis, Utrecht Univeristy, the Netherlands), pp. 145.

**De Vos, M., Van Zaanen, W., Koornneef, A., Korzelius, J.P., Dicke, M., Van Loon, L.C., and Pieterse, C.M.J.** (2006). Herbivore-induced resistance against microbial pathogens in Arabidopsis. Plant Physiol. **142,** 352-363.

**De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Métraux, J.P., Van Loon, L.C., Dicke, M., and Pieterse, C.M.J.** (2005). Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. Mol. Plant-Microbe Interact. **18,** 923-937.

**De Wit, P.J.G.M.** (1997). Pathogen avirulence and plant resistance: a key role for recognition. Trends Plant Sci. **2,** 452–458.

**Delaney, T.P., Friedrich, L., and Ryals, J.A.** (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. USA **92,** 6602-6606.

**Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J.** (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247-1250.

**Delker, C., Stenzel, I., Hause, B., Miersch, O., Feussner, I., and Wasternack, C.** (2006). Jasmonate biosynthesis in *Arabidopsis thaliana* - Enzymes, products, regulation. Plant Biol. **8,** 297-306.

**Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P.R.** (2000). The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12,** 279–290.

**Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., and Fobert, P.R.** (2003). The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell **15,** 2181-2191.

**Devoto, A., and Turner, J.G.** (2003). Regulation of jasmonate-mediated plant responses in Arabidopsis. Ann. Bot. **92,** 329-337.

**Devoto, A., Muskett, P.R., and Shirasu, K.** (2003). Role of ubiquitination in the regulation of plant defence against pathogens. Curr. Opinion Plant Biol. **6,** 307-311.

**Devoto, A., Nieto-Rostro, M., Xie, D.X., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G.** (2002). COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. Plant J. **32,** 457-466.

**Dharmasiri, N., Dharmasiri, S., and Estelle, M.** (2005a). The F-box protein TIR1 is an auxin receptor. Nature **435,** 441-445.

**Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jurgens, G., and Estelle, M.** (2005b). Plant development is regulated by a family of auxin receptor F box proteins. Dev. Cell **9,** 109-119.

**Dicke, M., and Hilker, M.** (2003). Induced plant defences: from molecular biology to evolutionary ecology. Basic Appl. Ecol. **4,** 3-14.

**Doares, S.H., Narváez-Vásquez, J., Conconi, A., and Ryan, C.A.** (1995). Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol. **108,** 1741-1746.

**Doherty, H.M., Selvendran, R.R., and Bowles, D.J.** (1988). The wound response of tomato plants can be inhibited by aspirin and related hydroxy-benzoic acids. Physiol. Mol. Plant Pathol. **33,** 377-384.

**Dong, X.** (1998). SA, JA, ethylene, and disease resistance in plants. Curr. Opin. Plant Biol. **1,** 316-323.

**Dong, X.** (2004). NPR1, all things considered. Curr. Opin. Plant Biol. **7,** 547-552.

**Dreher, K.A., Brown, J., Saw, R.E., and Callis, J.** (2006). The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. Plant Cell **18,** 699-714.

**Durrant, W.E., and Dong, X.** (2004). Systemic acquired resistance. Annu. Rev. Phytopathol. **42,** 185-209.

**Durrant, W.E., Wang, S., and Dong, X.** (2007). Arabidopsis SNI1 and RAD51D regulate both gene transcription and DNA recombination during the defense response. Proc. Natl. Acad. Sci. USA **104,** 4223-4227.

**Ellis, C., and Turner, J.G.** (2001). The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell **13,** 1025-1033.

**Ellis, C., and Turner, J.G.** (2002). A conditionally fertile *coi1* allele indicates cross-talk between plant hormone signaling pathways in *Arabidopsis thaliana* seeds and young seedlings. Planta **215,** 549-556.

**Ellis, C., Karafyllidis, L., and Turner, J.G.** (2002a). Constitutive activation of jasmonate signaling in an Arabidopsis mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*. Mol. Plant-Microbe Interact. **15,** 1025-1030.

**Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J.G.** (2002b). The Arabidopsis mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. Plant Cell **14,** 1557-1566.

**Eulgem, T., Weigman, V.J., Chang, H.S., McDowell, J.M., Holub, E.B., Glazebrook, J., Zhu, T., and Dangl, J.L.** (2004). Gene expression signatures from three genetically separable resistance gene signaling pathways for downy mildew resistance. Plant Physiol. **135,** 1129-1144.

**Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J., and Parker, J.E.** (1999). EDS1, an essential component of *R* gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. USA **96,** 3292-3297.

**Felton, G.W., and Korth, K.L.** (2000). Trade-offs between pathogen and herbivore resistance. Curr. Opin. Plant Biol. **3,** 309-314.

**Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N., and Deng, X.W.** (2003). The COP9 signalosome interacts physically with $SCF^{COI1}$ and modulates jasmonate responses. Plant Cell **15,** 1083-1094.

**Feussner, I., and Wasternack, C.** (2002). The lipoxygenase pathway. Annu. Rev. Plant Biol. **53,** 275-297.

**Feys, B.J.F., Benedetti, C.E., Penfold, C.N., and Turner, J.G.** (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,** 751-759.

**Flors, V., Ton, J., van Doorn, R., Jakab, G., Garcia-Agustin, P., and Mauch-Mani, B.** (2008). Interplay between JA, SA and ABA signalling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. Plant J. **54,** 81-92.

**Fodor, J., Gullner, G., Adam, A.L., Barna, B., Komives, T., and Kiraly, Z.** (1997). Local and systemic responses of antioxidants to tobacco mosaic virus infection and to salicylic acid in tobacco (role in systemic acquired resistance). Plant Physiol. **114,** 1443-1451.

**Fonseca, S., Chini, A., Hamberg, M., Adie, B., Porzel, A., Kramell, R., Miersch, O., Wasternack, C., and Solano, R.** (2009). (+)-7-*iso*-Jasmonyl-L-isoleucine is the endogenous bioactive jasmonate. Nat. Chem. Biol. **5,** 344-350.

**Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M.** (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12,** 393-404.

**Glazebrook, J.** (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43,** 205-227.

**Glazebrook, J., and Ausubel, F.M.** (1994). Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc. Natl. Acad. Sci. USA **91,** 8955-8959.

**Glazebrook, J., Rogers, E.E., and Ausubel, F.M.** (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics **143,** 973-982.

**Glazebrook, J., Chen, W., Estes, B., Chang, H.-S., Nawrath, C., Métraux, J.-P., Zhu, T., and Katagiri, F.** (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J. **34,** 217-228.

**Göhre, V., and Robatzek, S.** (2008). Breaking the barriers: Microbial effector molecules subvert plant immunity. Annu. Rev. Phytopathol. **46,** 189-215.

**Grant, M., and Lamb, C.** (2006a). Systemic immunity. Curr. Opin. Plant Biol. **9,** 414-420.

**Grant, M.R., and Lamb, C.** (2006b). Systemic immunity. Curr. Opin. Plant Biol. **9,** 414-420.

**Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M.** (2001). Auxin regulates $SCF^{TIR1}$-dependent degradation of AUX/IAA proteins. Nature **414,** 271-276.

**Griffith, O.W., and Meister, A.** (1979). Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S-n*-butyl homocysteine sulfoximine). J. Biol. Chem. **254,** 7558-7560.

**Grunewald, W.** (2008). Functional analysis of *Arabidopsis thaliana* genes expressed during feeding site establishment of plant-parasitic nematodes (Ph.D. Thesis, Ghent University, Belgium).

**Guo, H.W., and Ecker, J.R.** (2003). Plant responses to ethylene gas are mediated by SCF (EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115,** 667-677.

**Gupta, V., Willits, M.G., and Glazebrook, J.** (2000). *Arabidopsis thaliana* EDS4 contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. Mol. Plant-Microbe Interact. **13,** 503-511.

**Guzmán, P., and Ecker, J.R.** (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2,** 513-523.

**Harms, K., Ramirez, I., and Peña-Cortés, H.** (1998). Inhibition of wound-induced accumulation of allene oxide synthase transcripts in flax leaves by aspirin and salicylic acid. Plant Physiol. **118,** 1057-1065.

**Harrison, M.J.** (2005). Signaling in the arbuscular mycorrhizal symbiosis. Annu. Rev. Microbiol. **59,** 19-42.

**Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C., and Cottage, A.** (2006). A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. Plant Methods **2,** 19.

**Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M.E., He, K., Li, J., Schroeder, J.I., Peck, S.C., and Rathjen, J.P.** (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proc. Natl. Acad. Sci. USA **104,** 12217-12222.

**Heidel, A.J., Clarke, J.D., Antonovics, J., and Dong, X.** (2004). Fitness costs of mutations affecting the systemic acquired resistance pathway in *Arabidopsis thaliana*. Genetics **168,** 2197-2206.

**Heil, M., and Baldwin, I.T.** (2002). Fitness costs of induced resistance: emerging experimental support for a slippery concept. Trends Plant Sci. **7,** 61-67.

**Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., and Mullineaux, P.M.** (2000). pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol. Biol. **42,** 819-832.

**Hellmann, H., Hobbie, L., Chapman, A., Dharmasiri, S., Dharmasiri, N., del Pozo, C., Reinhardt, D., and Estelle, M.** (2003). Arabidopsis *AXR6* encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis. EMBO J. **22,** 3314-3325.

**Hemerly, A., Engler, J.D., Bergounioux, C., Van Montagu, M., Engler, G., Inzé, D., and Ferreira, P.** (1995). Dominant-negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. EMBO J. **14,** 3925-3936.

**Hoagland, D.R., and Arnon, D.I.** (1938). The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Bull. **347,** 36-39.

**Howden, R., Andersen, C.R., Goldsbrough, P.B., and Cobbett, C.S.** (1995). A cadmium-sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. Plant Physiol. **107,** 1067-1073.

**Howe, G.A.** (2004). Jasmonates as signals in the wound response. J. Plant Growth Regul. **23,** 223-237.

**Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M.** (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10,** 1321-1332.

**Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K.** (2001). The *DEFECTIVE IN ANTHER DEHISCENCE1* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell **13,** 2191-2209.

**Jelenska, J., Yao, N., Vinatzer, B.A., Wright, C.M., Brodsky, J.L., and Greenberg, J.T.** (2007). A J domain virulence effector of *Pseudomonas syringae* remodels host chloroplasts and suppresses defenses. Curr. Biol. **17,** 499-508.

**Jensen, A.B., Raventos, D., and Mundy, J.** (2002). Fusion genetic analysis of jasmonate-signalling mutants in Arabidopsis. Plant J. **29,** 595-606.

**Johansson, A., Staal, J., and Dixelius, C.** (2006). Early responses in the Arabidopsis-*Verticillium longisporum* pathosystem are dependent on NDR1, JA- and ET-associated signals via cytosolic NPR1 and RFO1. Mol. Plant-Microbe Interact. **19,** 958-969.

**Jones, J.D.G., and Dangl, J.L.** (2006). The plant immune system. Nature **444,** 323-329.

**Kachroo, P., Shanklin, J., Shah, J., Whittle, E.J., and Klessig, D.F.** (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proc. Natl. Acad. Sci. USA **98,** 9448-9453.

**Kachroo, P., Kachroo, A., Lapchyk, L., Hildebrand, D., and Klessig, D.F.** (2003). Restoration of defective cross talk in *ssi2* mutants: Role of salicylic acid, jasmonic acid, and fatty acids in SSI2-mediated signaling. Mol. Plant-Microbe Interact. **16,** 1022-1029.

**Kankainen, M., and Holm, L.** (2004). POBO, transcription factor binding site verification with bootstrapping. Nucl. Acids Res. **32,** 222-229.

**Karimi, M., Depicker, A., and Hilson, P.** (2007). Recombinational cloning with plant gateway vectors. Plant Physiol. **145,** 1144-1154.

**Karpinski, S., Escobar, C., Karpinska, B., Creissen, G., and Mullineaux, P.M.** (1997). Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell **9,** 627-640.

**Katagiri, F.** (2004). A global view of defense gene expression regulation - a highly interconnected signaling network. Curr. Opin. Plant Biol. **7,** 506-511.

**Katsir, L., Chung, H.S., Koo, A.J.K., and Howe, G.A.** (2008). Jasmonate signaling: a conserved mechanism of hormone sensing. Curr. Opin. Plant Biol. **11,** 428-435.

**Kazan, K., and Manners, J.M.** (2008). Jasmonate signaling: Toward an integrated view. Plant Physiol. **146,** 1459-1468.

**Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Abu Qamar, S., Mengiste, T., Betsuyaku, S., Parker, J.E., Mussig, C., Thomma, B.P.H.J., Albrecht, C., De Vries, S.C., Hirt, H., and Nürnberger, T.** (2007). The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. Curr. Biol. **17,** 1116-1122.

**Kempema, L.A., Cui, X., Holzer, F.M., and Walling, L.L.** (2007). Arabidopsis transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs. Similarities and distinctions in responses to aphids. Plant Physiol. **143,** 849-865.

**Kendrick, M.D., and Chang, C.** (2008). Ethylene signaling: new levels of complexity and regulation. Curr. Opin. Plant Biol. **11,** 479-485.

**Kepinski, S., and Leyser, O.** (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature **435,** 446-451.

**Kesarwani, M., Yoo, J., and Dong, X.** (2007). Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant Physiol. **144,** 336-346.

**Kessler, A., and Baldwin, I.T.** (2002). Plant responses to insect herbivory: The emerging molecular analysis. Annu. Rev. Plant Biol. **53,** 299-328.

**Kessler, A., Halitschke, R., and Baldwin, I.T.** (2004). Silencing the jasmonate cascade: induced plant defenses and insect populations. Science **305,** 665-668.

**Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R.** (1993). CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell **72,** 427-441.

**Kinkema, M., Fan, W., and Dong, X.** (2000). Nuclear localization of NPR1 is required for activation of *PR* gene expression. Plant Cell **12,** 2339-2350.

**Kliebenstein, D.J., Figuth, A., and Mitchell-Olds, T.** (2002). Genetic architecture of plastic methyl jasmonate responses in *Arabidopsis thaliana*. Genetics **161,** 1685-1696.

**Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F., and Kunkel, B.N.** (2001). Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. Plant J. **26,** 509-522.

**Koike, S., and Patterson, B.D.** (1988). Diurnal variation of glutathione levels in tomato seedlings. HortScience **23,** 713-714.

**Koncz, C., and Schell, J.** (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. **204,** 383-396.

**Koo, A.J.K., Chung, H.S., Kobayashi, Y., and Howe, G.A.** (2006). Identification of a peroxisomal acyl-activating enzyme involved in the biosynthesis of jasmonic acid in arabidopsis. J. Biol. Chem. **281,** 33511-33520.

**Koornneef, A., and Pieterse, C.M.J.** (2008). Cross-talk in defense signaling. Plant Physiol. **146,** 839-844.

**Koornneef, A., Verhage, A., Leon-Reyes, A., Snetselaar, R., Van Loon, L.C., and Pieterse, C.M.J.** (2008a). Towards a reporter system to identify regulators of cross-talk between salicylate and jasmonate signaling pathways in Arabidopsis. Plant Signaling Behavior **3,** 543-546.

**Koornneef, A., Leon-Reyes, A., Ritsema, T., Verhage, A., Den Otter, F.C., Van Loon, L.C., and Pieterse, C.M.J.** (2008b). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. Plant Physiol. **147,** 1358-1368.

**Koornneef, M., and Van der Veen, J.H.** (1980). Induction and analysis of gibberellin-sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. Theor. Appl. Genet. **58,** 257-263.

**Koornneef, M., Alonso-Blanco, C., and Vreugdenhil, D.** (2004). Naturally occurring genetic variation in *Arabidopsis thaliana*. Annu. Rev. Plant Biol. **55,** 141-172.

**Koornneef, M., Jorna, M.L., Brinkhorst-Van der Swan, D.L.C., and Karssen, C.M.** (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. Theor. Appl. Gen. **61,** 385-393.

**Kubigsteltig, I.I., and Weiler, E.W.** (2003). Arabidopsis mutants affected in the transcriptional control of allene oxide synthase, the enzyme catalyzing the entrance step in octadecanoid biosynthesis. Planta **217,** 748-757.

**Kunkel, B.N., and Brooks, D.M.** (2002). Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. **5,** 325-331.

**Laudert, D., and Weiler, E.W.** (1998). Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. Plant J. **15,** 675-684.

**Laurie-Berry, N., Joardar, V., Street, I.H., and Kunkel, B.N.** (2006). The *Arabidopsis thaliana JASMONATE INSENSITIVE 1* gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. Mol. Plant-Microbe Interact. **19,** 789-800.

**Lawton, K.A., Potter, S.L., Uknes, S., and Ryals, J.** (1994). Acquired resistance signal transduction in Arabidopsis is ethylene independent. Plant Cell **6,** 581-588.

**Lee, S.H., and Cho, H.T.** (2006). PINOID positively regulates auxin efflux in Arabidopsis root hair cells and tobacco cells. Plant Cell **18,** 1604-1616.

**Leon-Reyes, A., Spoel, S.H., De Lange, E.S., Abe, H., Kobayashi, M., Tsuda, S., Millenaar, F.F., Welschen, R.A.M., Ritsema, T., and Pieterse, C.M.J.** (2009). Ethylene modulates the role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in cross talk between salicylate and jasmonate signaling. Plant Physiol. **149,** 1797-1809.

**Li, J., Brader, G., and Palva, E.T.** (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell **16,** 319-331.

**Li, J., Brader, G., Kariola, T., and Palva, E.T.** (2006). WRKY70 modulates the selection of signaling pathways in plant defense. Plant J. **46,** 477-491.

**Li, X., Zhang, Y., Clarke, J.D., Li, Y., and Dong, X.** (1999). Identification and cloning of a negative regulator of systemic acquired resistance, SNI1, through a screen for suppressors of *npr1-1*. Cell **98,** 329-339.

**Lincoln, C., Britton, J.H., and Estelle, M.** (1990). Growth and development of the *axr1* mutants of Arabidopsis. Plant Cell **2,** 1071-1080.

**Liu, J., and Wang, X.-J.** (2006). An integrative analysis of the effects of auxin on jasmonic acid biosynthesis in *Arabidopsis thaliana*. J. Integr. Plant Biol. **48,** 99-103.

**Loake, G., and Grant, M.** (2007). Salicylic acid in plant defence-the players and protagonists. Curr. Opin. Plant Biol. **10,** 466-472.

**López, M.A., Bannenberg, G., and Castresana, C.** (2008). Controlling hormone signaling is a plant and pathogen challenge for growth and survival. Curr. Opin. Plant Biol. **11,** 420-427.

**Lorenzo, O., and Solano, R.** (2005). Molecular players regulating the jasmonate signalling network. Curr. Opin. Plant Biol. **8,** 532-540.

**Lorenzo, O., Piqueras, R., Sánchez-Serrano, J.J., and Solano, R.** (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15,** 165-178.

**Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R.** (2004). *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell **16,** 1938-1950.

**Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J., and Cameron, R.K.** (2002). A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. Nature **419,** 399-403.

**Marsh, J.L., Erfle, M., and Wykes, E.J.** (1984). The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32,** 481-485.

**Mateo, A., Funck, D., Muhlenbock, P., Kular, B., Mullineaux, P.M., and Karpinski, S.** (2006). Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. J. Exp. Bot. **57,** 1795-1807.

**Mauch-Mani, B., and Mauch, F.** (2005). The role of abscisic acid in plant-pathogen interactions. Curr. Opin. Plant Biol. **8,** 409-414.

**May, M.J., and Leaver, C.J.** (1993). Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. Plant Physiol. **103,** 621-627.

**McGrath, K.C., Dombrecht, B., Manners, J.M., Schenk, P.M., Edgar, C.I., Maclean, D.J., Scheible, W.-R., Udvardi, M.K., and Kazan, K.** (2005). Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. Plant Physiol. **139,** 949-959.

**Métraux, J.P.** (2002). Recent breakthroughs in the study of salicylic acid biosynthesis. Trends Plant Sci. **7,** 332-334.

**Millenaar, F.F., Cox, M.C.H., de Jong van Berkel, Y.E.M., Welschen, R.A.M., Pierik, R., Voesenek, L.A.C.J., and Peeters, A.J.M.** (2005). Ethylene-induced differential growth of petioles in Arabidopsis. Analyzing natural variation, response kinetics, and regulation. Plant Physiol. **137,** 998-1008.

**Mishina, T.E., and 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 J. **50,** 500-513.

**Mohr, P.G., and Cahill, D.M.** (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with *Pseudomonas syringae* pv. *tomato*. Funct. Integr. Genomics **7,** 181-191.

**Moran, P.** (1998). Plant-mediated interactions between insects and fungal plant pathogen and the role of chemical responses to infection. Oecologia **115,** 523-530.

**Mou, Z., Fan, W.H., and Dong, X.N.** (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113,** 935-944.

**Mur, L.A.J., Kenton, P., Atzorn, R., Miersch, O., and 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 Physiol. **140,** 249-262.

**Murashige, T., and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15** 473-497.

**Musser, R.O., Hum-Musser, S.M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J.B., and Felton, G.W.** (2002). Herbivory: Caterpillar saliva beats plant defences - A new weapon emerges in the evolutionary arms race between plants and herbivores. Nature **416,** 599-600.

**Nagpal, P., Ellis, C.M., Weber, H., Ploense, S.E., Barkawi, L.S., Guilfoyle, T.J., Hagen, G., Alonso, J.M., Cohen, J.D., Farmer, E.E., Ecker, J.R., and Reed, J.W.** (2005). Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development **132,** 4107-4118.

**Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I., and Yoshida, S.** (2003). Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. Plant J. **33,** 887-898.

**Navarro, L., Bari, R., Achard, P., Lison, P., Nemri, A., Harberd, N.P., and Jones, J.D.G.** (2008). DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. Curr. Biol. **18,** 650-655.

**Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D.G.** (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science **312,** 436-439.

**Nawrath, C., and Métraux, J.-P.** (1999). Salicylic acid induction-deficient mutants of Arabidopsis express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11,** 1393-1404.

**Ndamukong, I., Abdallat, A.A., Thurow, C., Fode, B., Zander, M., Weigel, R., and Gatz, C.** (2007). SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive *PDF1.2* transcription. Plant J. **50,** 128-139.

**Nickstadt, A., Thomma, B.P.H.J., Feussner, I., Kangasjarvi, J., Zeier, J., Loeffler, C., Scheel, D., and Berger, S.** (2004). The jasmonate-insensitive mutant *jin1* shows increased resistance to biotrophic as well as necrotrophic pathogens. Mol. Plant Pathol. **5,** 425-434.

**Noctor, G., and Foyer, C.H.** (1998). Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. **49,** 249-279.

**Noctor, G., Arisi, A.-C.M., Jouanin, L., Valadier, M.-H., Roux, Y., and Foyer, C.H.** (1997). Light-dependent modulation of foliar glutathione synthesis and associated amino acid metabolism in poplar overexpressing γ-glutamylcysteine synthetase. Planta **202,** 357-369.

**Nomura, K., Melotto, M., and He, S.-Y.** (2005). Suppression of host defense in compatible plant-*Pseudomonas syringae* interactions. Curr. Opin. Plant Biol. **8,** 361-368.

**Norton, G., Pappusamy, A., Yusof, F., Pujade-Renaud, V., Perkins, M., Griffiths, D., and Jones, H.** (2007). Characterisation of recombinant *Hevea brasiliensis* allene oxide synthase: Effects of cycloxygenase inhibitors, lipoxygenase inhibitors and salicylates on enzyme activity. Plant Physiol. Biochem. **45,** 129-138.

**Nürnberger, T., and Kemmerling, B.** (2009). Pathogen-associated molecular patterns (PAMP) and PAMP-triggered immunity. Annu. Plant Rev. **34,** 16-47.

**O'Connor, T.R., Dyreson, C., and Wyrick, J.J.** (2005). Athena: a resource for rapid visualization and systematic analysis of Arabidopsis promoter sequences. Bioinformatics **21,** 4411-4413.

**Pan, Z., Camara, B., Gardner, H.W., and Backhaus, R.A.** (1998). Aspirin inhibition and acetylation of the plant cytochrome P450, allene oxide synthase, resembles that of animal prostaglandin endoperoxide H synthase **273,** 18139-18145.

**Park, J.-H., Halitschke, R., Kim, H.B., Baldwin, I.T., Feldmann, K.A., and Feyereisen, R.** (2002). A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J. **31,** 1-12.

**Pasquali, G., Ouwerkerk, P.B.F., and Memelink, J.** (1994). Versatile transformation vectors to assay the promoter activity of DNA elements in plants. Gene **149,** 373-374.

**Pauwels, L., Inzé, D., and Goossens, A.** (2009). Jasmonate-inducible gene: what does it mean? Trends Plant Sci. **14,** 87-91.

**Pauwels, L., Morreel, K., De Witte, E., Lammertyn, F., Van Montagu, M., Boerjan, W., Inzé, D., and Goossens, A.** (2008). Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. Proc. Natl. Acad. Sci. USA **105,** 1380-1385.

**Peña-Cortés, H., Albrecht, T., Prat, S., Weiler, E.W., and Willmitzer, L.** (1993). Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta **191,** 123-128.

**Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Métraux, J.-P., and Broekaert, W.F.** (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

**Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J.** (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell **103,** 1111-1120.

**Pierpoint, W.S.** (1997). The natural history of salicylic acid - plant product and mammalian medicine. Interdiscip. Sci. Rev. **22,** 45-52.

**Pieterse, C.M.J., and Van Loon, L.C.** (1999). Salicylic acid-independent plant defence pathways. Trends Plant Sci. **4,** 52-58.

**Pieterse, C.M.J., and Van Loon, L.C.** (2004). NPR1: the spider in the web of induced resistance signaling pathways. Curr. Opin. Plant Biol. **7,** 456-464.

**Pieterse, C.M.J., and Dicke, M.** (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends Plant Sci **12,** 564-569.

**Pieterse, C.M.J., Ton, J., and Van Loon, L.C.** (2001). Cross-talk between plant defence signalling pathways: boost or burden? AgBiotechNet **3,** ABN 068.

**Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S.C.M.** (2009). Networking by small-molecules hormones in plant immunity. Nature Chem. Biol. **5,** 308-316.

**Pieterse, C.M.J., Van Wees, S.C.M., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., and Van Loon, L.C.** (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell **10,** 1571-1580.

**Poelman, E.H., van Loon, J.J.A., and Dicke, M.** (2008a). Consequences of variation in plant defense for biodiversity at higher trophic levels. Trends Plant Sci. **13,** 534-541.

**Poelman, E.H., Broekgaarden, C., Van Loon, J.J.A., and Dicke, M.** (2008b). Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. Mol. Ecol. **17,** 3352-3365.

**Pozo, M.J., and Azcon-Aguilar, C.** (2007). Unraveling mycorrhiza-induced resistance. Curr. Opion Plant Biol. **10,** 393-398.

**Pozo, M.J., Van Loon, L.C., and Pieterse, C.M.J.** (2004). Jasmonates - signals in plant-microbe interactions. J. Plant Growth Regul. **23,** 211-222.

**Pozo, M.J., Van der Ent, S., Van Loon, L.C., and Pieterse, C.M.J.** (2008). Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. New Phytologist **180,** 511-523.

**Pré, M., Atallah, M., Champion, A., De Vos, M., Pieterse, C.M.J., and Memelink, J.** (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. Plant Physiol. **147,** 1347-1357.

**Pruitt, R.E., and Meyerowitz, E.M.** (1986). Characterization of the genome of *Arabidopsis thaliana*. J. Mol. Biol. **187,** 169-183.

**Rao, M.V., Lee, H.-I., Creelman, R.A., Mullet, J.E., and Davis, K.R.** (2000). Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. Plant Cell **12,** 1633-1646.

**Redman, J.C., Haas, B.J., Tanimoto, G., and Town, C.D.** (2004). Development and evaluation of an Arabidopsis whole genome Affymetrix probe array. Plant J. **38,** 545-561.

**Reymond, P., and Farmer, E.E.** (1998). Jasmonate and salicylate as global signals for defense gene expression. Curr. Opin. Plant Biol. **1,** 404-411.

**Reymond, P., Bodenhausen, N., Van Poecke, R.M.P., Krishnamurthy, V., Dicke, M., and Farmer, E.E.** (2004). A conserved transcriptional pattern in response to a specialist and a generalist herbivore. Plant Cell **16,** 3132-3147.

**Richards, H.A., Halfhill, M.D., Millwood, R.J., and Stewart, C.N.** (2003). Quantitative GFP fluorescence as an indicator of recombinant protein synthesis in transgenic plants. Plant Cell Reports **22,** 117-121.

**Richmond, T.A., and Bleecker, A.B.** (1999). A defect in γ-oxidation causes abnormal inflorescence development in Arabidopsis. Plant Cell **11,** 1911-1923.

**Robert-Seilaniantz, A., Navarro, L., Bari, R., and Jones, J.D.G.** (2007). Pathological hormone imbalances. Curr. Opin. Plant Biol. **10,** 372-379.

**Rochon, A., Boyle, P., Wignes, T., Fobert, P.R., and Després, C.** (2006). The coactivator function of Arabidopsis NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. Plant Cell **18,** 3670-3685.

**Rojo, E., Solano, R., and Sanchez-Serrano, J.J.** (2003). Interactions between signaling compounds involved in plant defense. J. Plant Growth Regul. **22,** 82-98.

**Rombauts, S., Florquin, K., Lescot, M., Marchal, K., Rouze, P., and Van de Peer, Y.** (2003). Computational approaches to identify promoters and cis-regulatory elements in plant genomes. Plant Physiol. **132,** 1162-1176.

**Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J., and Estelle, M.** (1998). The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. Genes Dev. **12,** 198-207.

**Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., and Quackenbush, J.** (2003). TM4: A free, open-source system for microarray data management and analysis. Biotechniques **34,** 374-378.

**Salmon, J., Ramos, J., and Callis, J.** (2008). Degradation of the auxin response factor ARF1. Plant J. **54,** 118-128.

**Salzman, R.A., Brady, J.A., Finlayson, S.A., Buchanan, C.D., Summer, E.J., Sun, F., Klein, P.E., Klein, R.R., Pratt, L.H., Cordonnier-Pratt, M.M., and Mullet, J.E.** (2005). Transcriptional profiling of sorghum induced by methyl jasmonate, salicylic acid, and aminocyclopropane carboxylic acid reveals cooperative regulation and novel gene responses. Plant Physiol. **138,** 352-368.

**Sambrook, J., Fritsch, E.F., and Maniatis, T.** (1989). Molecular Cloning: A Laboratory Manual, 2nd edition. (Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press).

**Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takamiya, X., Ohta, H., and Tabata, S.** (2001). Monitoring of methyl jasmonate-responsive genes in Arabidopsis by cDNA macroarray: Self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. DNA Res. **8,** 153-161.

**Schafer, F.Q., and Buettner, G.R.** (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. **30,** 1191-1212.

**Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M.** (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc. Natl. Acad. Sci. USA **97,** 11655-11660.

**Schoonhoven, L.M., Van Loon, J.J.A., and Dicke, M.** (2005). Insect-Plant Biology. (Oxford: Oxford University Press).

**Schupp, R., and Rennenberg, H.** (1988). Diurnal changes in the glutathione content of spruce needles (*Picea Abies* L.). Plant Sci. **57,** 113-117.

**Schwessinger, B., and Zipfel, C.** (2008). News from the frontline: recent insights into PAMP-triggered immunity in plants. Curr. Opin. Plant Biol. **11,** 389-395.

**Seo, H.S., Song, J.T., Cheong, J.-J., Lee, Y.-H., Lee, Y.-W., Hwang, I., Lee, J.S., and Choi, Y.D.** (2001). Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. USA **98,** 4788-4793.

**Shah, J., Tsui, F., and Klessig, D.F.** (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol. Plant-Microbe Interact. **10,** 69-78.

**Shah, J., Kachroo, P., and Klessig, D.F.** (1999). The Arabidopsis *ssi1* mutation restores pathogenesis-related gene expression in *npr1* plants and renders defensin gene expression salicylic acid dependent. Plant Cell **11,** 191-206.

**Shan, L.B., He, P., Li, J.M., Heese, A., Peck, S.C., Nürnberger, T., Martin, G.B., and Sheen, J.** (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. Cell Host Microbe **4,** 17-27.

**Siemens, J., Keller, I., Sarx, J., Kunz, S., Schuller, A., Nagel, W., Schmulling, T., Parniske, M., and Ludwig-Muller, J.** (2006). Transcriptome analysis of Arabidopsis clubroots indicate a key role for cytokinins in disease development. Mol. Plant-Microbe Interact. **19,** 480-494.

**Song, W.-C., Funk, C.D., and Brash, A.R.** (1993). Molecular-cloning of an allene oxide synthase - a cytochrome-P450 specialized for the metabolism of fatty-acid hydroperoxides. Proc. Natl. Acad. Sci. USA **90,** 8519-8523.

**Spaepen, S., Vanderleyden, J., and Remans, R.** (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. Fems Microbiol. Rev. **31,** 425-448.

**Spoel, S.H., and Dong, X.** (2008). Making sense of hormone crosstalk during plant immune responses. Cell Host & Microbe **3,** 348-351.

**Spoel, S.H., Johnson, J.S., and Dong, X.** (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. Proc. Natl. Acad. Sci. USA **104,** 18842-18847.

**Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., and Pieterse, C.M.J.** (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15,** 760-770.

**Staswick, P.E.** (2008). JAZing up jasmonate signaling. Trends Plant Sci. **13,** 66-71.

**Staswick, P.E., and Tiryaki, I.** (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell **16,** 2117-2127.

**Staswick, P.E., Yuen, G.Y., and Lehman, C.C.** (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Natl. Acad. Sci. USA **89,** 6837-6840.

**Stintzi, A., and Browse, J.** (2000). The Arabidopsis male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc. Natl. Acad. Sci. USA **97,** 10625-10630.

**Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E.E.** (2001). Plant defense in the absence of jasmonic acid: The role of cyclopentenones. Proc. Natl. Acad. Sci. USA **98,** 12837-12842.

**Stotz, H.U., Koch, T., Biedermann, A., Weniger, K., Boland, W., and Mitchell-Olds, T.** (2002). Evidence for regulation of resistance in Arabidopsis to Egyptian cotton worm by salicylic and jasmonic acid signaling pathways. Planta **214,** 648-652.

**Stout, M.J., Thaler, J.S., and Thomma, B.P.H.J.** (2006). Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. Annu. Rev. Entomol. **51,** 663-689.

**Straus, D.S., and Glass, C.K.** (2001). Cyclopentenone prostaglandins: new insights on biological activities and cellular targets. Med. Res. Rev. **21,** 185-210.

**Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X.** (2008). Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. Science **321,** 952-956.

**Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.-S., Han, B., Zhu, T., Zou, G.Z., and Katagiri, F.** (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. Plant Cell **15,** 317-330.

**Thaler, J.S., Fidantsef, A.L., and Bostock, R.M.** (2002a). Antagonism between jasmonate- and salicylate-mediated induced plant resistance: effects of concentration and timing of elicitation on defense-related proteins, herbivore, and pathogen performance in tomato. J. Chem. Ecol. **28,** 1131-1159.

**Thaler, J.S., Fidantsef, A.L., Duffey, S.S., and Bostock, R.M.** (1999). Trade-offs in plant defense against pathogens and herbivores: a field demonstration of chemical elicitors of induced resistance. J. Chem. Ecol. **25,** 1597-1609.

**Thaler, J.S., Karban, R., Ullman, D.E., Boege, K., and Bostock, R.M.** (2002b). Cross-talk between jasmonate and salicylate plant defense pathways: effects on several plant parasites. Oecologia **131,** 227-235.

**Thatcher, L.F., Anderson, J.P., and Singh, K.B.** (2005). Plant defence responses: what have we learnt from Arabidopsis? Functional Plant Biol. **32,** 1-19.

**Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G.H., Nomura, K., He, S.Y., Howe, G.A., and Browse, J.** (2007). JAZ repressor proteins are targets of the $SCF^{COI1}$ complex during jasmonate signalling. Nature **448,** 661-U662.

**Thomma, B.P.H.J., Nelissen, I., Eggermont, K., and Broekaert, W.F.** (1999). Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J. **19,** 163-171.

**Thomma, B.P.H.J., Eggermont, K., Broekaert, W.F., and Cammue, B.P.A.** (2000). Disease development of several fungi on Arabidopsis can be reduced by treatment with methyl jasmonate. Plant Physiol. Biochem. **38,** 421-427.

**Thomma, B.P.H.J., Penninckx, I.A.M.A., Broekaert, W.F., and Cammue, B.P.A.** (2001a). The complexity of disease signaling in *Arabidopsis*. Curr. Opin. Immunol. **13,** 63-68.

**Thomma, B.P.H.J., Tierens, K.F.M., Penninckx, I.A.M.A., Mauch-Mani, B., Broekaert, W.F., and Cammue, B.P.A.** (2001b). Different micro-organisms differentially induce Arabidopsis disease response pathways. Plant Physiol. Biochem. **39,** 673-680.

**Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F.** (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA **95,** 15107-15111.

**Tiryaki, I., and Staswick, P.E.** (2002). An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant *axr1*. Plant Physiol. **130,** 887-894.

**Ton, J., Van Pelt, J.A., Van Loon, L.C., and Pieterse, C.M.J.** (2002). Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. **15,** 27-34.

**Traw, M.B., Kim, J., Enright, S., Cipollini, D.F., and Bergelson, J.** (2003). Negative cross-talk between salicylate- and jasmonate-mediated pathways in the Wassilewskija ecotype of *Arabidopsis thaliana*. Mol. Ecol. **12,** 1125-1135.

**Tsuda, K., Sato, M., Glazebrook, J., Cohen, J.D., and Katagiri, F.** (2008). Interplay between MAMP-triggered and SA-mediated defense responses. Plant J. **53,** 763-775.

**Tzfira, T., and Citovsky, V.** (2006). *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr. Opin. Biotechnol. **17,** 147-154.

**Uppalapati, S.R., Ishiga, Y., Wangdi, T., Kunkel, B.N., Anand, A., Mysore, K.S., and Bender, C.L.** (2007). The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. Mol. Plant-Microbe Interact. **20,** 955-965.

**Van der Ent, S., Verhagen, B.W.M., Van Doorn, R., Bakker, D., Verlaan, M.G., Pel, M.J.C., Joosten, R.G., Proveniers, M.C.G., Van Loon, L.C., Ton, J., and Pieterse, C.M.J.** (2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. Plant Physiol. **146,** 1293-1304.

**Van der Ouderaa, F.J., Buytenhek, M., Nugteren, D.H., and Van Dorp, D.A.** (1980). Acetylation of prostaglandin endoperoxide synthase with acetylsalicylic acid. Eur. J. Biochem. **109,** 1-8.

**Van der Putten, W.H., Vet, L.E.M., Harvey, J.A., and Wäckers, F.L.** (2001). Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. Trends Ecol. Evol. **16,** 547-554.

**Van Hulten, M., Pelser, M., Van Loon, L.C., Pieterse, C.M.J., and Ton, J.** (2006). Costs and benefits of priming for defense in Arabidopsis. Proc. Natl. Acad. Sci. USA **103,** 5602-5607.

**Van Leene, J., Stals, H., Eeckhout, D., Persiau, G., De Slijke, E.V., Van Isterdael, G., De Clercq, A., Bonnet, E., Laukens, K., Remmerie, N., Henderickx, K., De Vijlder, T., Abdelkrim, A., Pharazyn, A., Van Onckelen, H., Inzé, D., Witters, E., and De Jaeger, G.** (2007). A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis thaliana*. Mol. Cell. Proteomics **6,** 1226-1238.

**Van Leeuwen, H., Kliebenstein, D.J., West, M.A.L., Kim, K., Van Poecke, R., Katagiri, F., Michelmore, R.W., Doerge, R.W., and St.Clair, D.A.** (2007). Natural variation among *Arabidopsis thaliana* accessions for transcriptome response to exogenous salicylic acid. Plant Cell **19,** 2099-2110.

**Van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J.** (1998). Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. **36,** 453-483.

**Van Loon, L.C., Geraats, B.P.J., and Linthorst, H.J.M.** (2006a). Ethylene as a modulator of disease resistance in plants. Trends Plant Sci. **11,** 184-191.

**Van Loon, L.C., Rep, M., and Pieterse, C.M.J.** (2006b). Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. **44,** 135-162.

**Van Oosten, V.R., Bodenhausen, N., Reymond, P., Van Pelt, J.A., Van Loon, L.C., Dicke, M., and Pieterse, C.M.J.** (2008). Differential effectiveness of microbially induced resistance against herbivorous insects in Arabidopsis. Mol. Plant-Microbe Interact. **21,** 919-930.

**Van Poecke, R.M.P., and Dicke, M.** (2004). Indirect defence of plants against herbivores: using *Arabidopsis thaliana* as a model plant. Plant Biol. **6,** 387-401.

**Van Wees, S.C.M., Van der Ent, S., and Pieterse, C.M.J.** (2008). Plant immune responses triggered by beneficial microbes. Curr. Opin. Plant Biol. **11,** 443-448.

**Van Wees, S.C.M., Luijendijk, M., Smoorenburg, I., Van Loon, L.C., and Pieterse, C.M.J.** (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 Mol. Biol. **41,** 537-549.

**Van Wees, S.C.M., De Swart, E.A.M., Van Pelt, J.A., Van Loon, L.C., and Pieterse, C.M.J.** (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **97,** 8711-8716.

**Vanacker, H., Lu, H., Rate, D.N., and Greenberg, J.T.** (2001). A role for salicylic acid and NPR1 in regulating cell growth in Arabidopsis. Plant J. **28,** 209-216.

**Verberne, M.C., Hoekstra, J., Bol, J.F., and Linthorst, H.J.M.** (2003). Signaling of systemic acquired resistance in tobacco depends on ethylene perception. Plant J. **35,** 27-32.

**Vlot, A.C., Klessig, D.F., and Park, S.-W.** (2008). Systemic acquired resistance: the elusive signal(s). Curr. Opin. Plant Biol. **11,** 436-442.

**Von Dahl, C.C., and Baldwin, I.T.** (2007). Deciphering the role of ethylene in plant-herbivore interactions. J. Plant Growth Regul. **26,** 201-209.

**Von Malek, B., Van der Graaff, E., Schneitz, K., and Keller, B.** (2002). The Arabidopsis male-sterile mutant *dde2-2* is defective in the *ALLENE OXIDE SYNTHASE* gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta **216,** 187-192.

**Walling, L.L.** (2008). Avoiding effective defenses: Strategies employed by phloem-feeding insects. Plant Physiol. **146,** 859-866.

**Walters, D., and Heil, M.** (2007). Costs and trade-offs associated with induced resistance. Physiol. Mol. Plant Pathol. **71,** 3-17.

**Walters, D., Newton, A., and Lyon, G.** (2007). Induced Resistance for Plant Defence: A Sustainable Approach to Crop Protection. (Oxford: Blackwell).

**Walters, D.R., and McRoberts, N.** (2006). Plants and biotrophs: a pivotal role for cytokinins? Trends Plant Sci. **11,** 581-586.

**Wang, D., Weaver, N.D., Kesarwani, M., and Dong, X.** (2005). Induction of protein secretory pathway is required for systemic acquired resistance. Science **308,** 1036-1040.

**Wang, D., Pajerowska-Mukhtar, K., Hendrickson Culler, A., and Dong, X.** (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr. Biol. **17,** 1784-1790.

**Wang, K.L.C., Yoshida, H., Lurin, C., and Ecker, J.R.** (2004). Regulation of ethylene gas biosynthesis by the Arabidopsis ETO1 protein. Nature **428,** 945-950.

**Wasternack, C.** (2007). Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. **100,** 681-697.

**Weigel, R.R., Pfitzner, U.M., and Gatz, C.** (2005). Interaction of NIMIN1 with NPR1 modulates *PR* gene expression in Arabidopsis. Plant Cell **17,** 1279-1291.

**Wiermer, M., Feys, B.J., and Parker, J.E.** (2005). Plant immunity: the EDS1 regulatory node. Curr. Opin. Plant Biol. **8,** 383-389.

**Woeste, K.E., Ye, C., and Kieber, J.J.** (1999). Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. Plant Physiol. **119,** 521-529.

**Xiao, S., Dai, L., Liu, F., Wang, Z., Peng, W., and Xie, D.** (2004). COS1: An Arabidopsis *coronatine insensitive1* suppressor essential for regulation of jasmonate-mediated plant defense and senescence. Plant Cell **16,** 1132-1142.

**Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G.** (1998). *COI1*: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280,** 1091-1094.

**Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D.** (2002). The $SCF^{COI1}$ ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell **14,** 1919-1935.

**Yan, Y., Stolz, S., Chetelat, A., Reymond, P., Pagni, M., Dubugnon, L., and Farmer, E.E.** (2007). A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell **19,** 2470-2483.

**Yang, X., Timofejeva, L., Ma, H., and Makaroff, C.A.** (2006). The Arabidopsis SKP1 homolog ASK1 controls meiotic chromosome remodeling and release of chromatin from the nuclear membrane and nucleolus. J. Cell Sci. **119,** 3754-3763.

**Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., Maruyama-Nakashita, A., Kudo, T., Shinozaki, K., Yoshida, S., and Nakashita, H.** (2008). Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in Arabidopsis. Plant Cell **20,** 1678-1692.

**Yoo, S.-D., Cho, Y.-H., and Sheen, J.** (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. **2,** 1565-1572.

**Yuan, Y., Zhong, S., Li, Q., Zhu, Z., Lou, Y., Wang, L., Wang, J., Wang, M., Li, Q., Yang, D., and He, Z.** (2007). Functional analysis of rice *NPR1*-like genes reveals that *OsNPR1/NH1* is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. Plant Biotechnol. J. **5,** 313-324.

**Zarate, S.I., Kempema, L.A., and Walling, L.L.** (2007). Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiol. **143,** 866-875.

**Zarei, A.** (2007). Funtional analysis of jasmonate-responsive transcription factors in *Arabidopsis thaliana* (Ph.D. Thesis, Leiden University).

**Zhang, Y.L., Tessaro, M.J., Lassner, M., and Li, X.** (2003). Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. Plant Cell **15,** 2647-2653.

**Zhao, Y., Thilmony, R., Bender, C.L., Schaller, A., He, S.-Y., and Howe, G.A.** (2003). Virulence systems of *Pseudomonas syringae* pv. *tomato* promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. Plant J. **36,** 485-499.

**Zhao, Y., Hull, A.K., Gupta, N.R., Goss, K.A., Alonso, J., Ecker, J.R., Normanly, J., Chory, J., and Celenza, J.L.** (2002). Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev. **16,** 3100-3112.

## Summary

Plants intimately communicate with their environment. They read signals coming from the "outside", such as signals produced upon feeding by herbivorous insects or infection by microbial pathogens, and translated them to the "inside" to respond appropriately to the attacker encountered. The efficiency of responding to different signals determines the survival of the plant under attack. For my thesis research, I tried to understand how plants defend themselves against a wide variety of pathogens and insects, with special emphasis on understanding how plants fine-tune their defense response upon attack by multiple threats through a mechanism called cross-talk. The current knowledge on hormonal pathways and cross-talk is reviewed in Chapter 1.

During my PhD research we focused on the molecular mechanism of antagonism between salicylic acid (SA) and jasmonic acid (JA). In the model plant species *Arabidopsis thaliana,* the SA response is effective against pathogens with a biotrophic life style, whereas the JA response is more effective against necrotrophic pathogens and insect herbivores. In Chapter 2, we showed the JA response in Arabidopsis is highly sensitive to suppression by SA, indicating that the SA pathway can be prioritized over the JA pathway. This SA-JA cross-talk appeared to be highly conserved among Arabidopsis accessions that were collected from very different geographic locations. In addition we showed that the kinetics of signal production is very important for the final outcome of the defense response.

In Chapter 3, we demonstrated that ethylene (ET), another plant hormone, plays an important role in the modulation of SA-mediated suppression of the JA response. In the absence of ET, SA-mediated suppression of JA signaling is mediated via the defense regulatory protein NPR1. However, production of ET, such as upon infection by the necrotrophic pathogen *Alternaria brassicicola*, rendered SA-JA cross-talk independent of NPR1. This finding uncovered yet another layer of complexity in signaling during the plant immune response.

In Chapter 4, we demonstrated that ET can make plants insensitive to SA-mediated suppression of JA signaling. When the JA and ET pathways are simultaneously induced prior to activation of the SA signaling, then Arabidopsis plants were insensitive to SA-JA cross-talk. We discovered that the ERF/AP2

transcription factor ORA59, is responsible for blocking SA-mediated suppression of JA responses. Together, we provide evidence that the final outcome of the interaction between the defense-related signals SA and JA is dependent on the kinetics of their production and the context in which their signaling pathways are activated. In addition ET can have an important role in shaping the final outcome of the plant defense signaling network that is activated upon pathogen or insect attack.

In Chapters 5 and 6 we investigated the site of action of SA on the JA signaling pathway. In Chapter 5, we demonstrated that JA biosynthesis is not a major target of SA in the suppression of JA signaling. In Chapter 6 we demonstrated that SA targets the JA signaling pathway downstream of the $SCF^{COI1}$-complex and the JAZ repressor proteins. Using a molecular genetic approach, we provided evidence that SA suppresses the JA response at the level of gene transcription and that the GCC-box motif in JA-responsive promoters is sufficient for SA-mediated suppression.

Collectively, this work provides novel insight into how plants regulate their defense response upon attack by multiple attackers. It appeared that the context in which the defense response is triggered plays an important role in the final outcome of the defense response. Moreover, we made important progress in uncovering the molecular basis of SA-JA cross-talk. Hence, the results presented in this thesis may be valuable for the development of novel strategies for crop protection.

## Resumen

Los organismos vegetales o plantas tienen una comunicación muy estrecha con el medio ambiente que los rodea. Ellos detectan las señales provenientes del exterior como por ejemplo las producidas por insectos herbívoros o patógenos, y las trasladan a su interior para dar una respuesta adecuada. La eficiente respuesta a las diferentes señales percibidas determina la sobrevivencia de las plantas en un ambiente hostil. Durante mi tesis de PhD, investigué como las plantas se defienden por sí solas contra una amplia variedad de patógenos e insectos, enfatizando en cómo las plantas controlan sus defensas cuando se encuentran atacadas por más de una plaga o enfermedad. El mecanismo analizado en esta tesis se llama "comunicación cruzada" o *cross-talk*. Me concentré en estudiar cómo funciona el mecanismo molecular antagónico presente entre el ácido salicílico (SA) y el ácido jasmónico (JA). En la planta modelo *Arabidopsis thaliana,* la respuesta del SA funciona adecuadamente contra patógenos que tienen una forma de vida biotrófica, mientras que la respuesta comandada por el JA es radicalmente más efectiva contra los patógenos necrotróficos e insectos herbívoros. En el Capítulo 1 se describe los modelos de comunicación cruzada de las hormonas de defensa en plantas que se conocen hasta el momento. En el Capítulo 2 se demostró que la respuesta del JA en Arabidopsis es muy sensible a la supresión del SA, indicando que la ruta de defensa del SA se prioriza mayoritariamente sobre la ruta de defensa del JA. Este mecanismo de comunicación cruzada entre el SA-JA es muy conservada, ya que encontré que este se encuentra en varias accesiones de Arabidopsis colectadas en diferentes regiones geográficas. Además se demostró que la kinética durante la producción de las hormonas es fundamental para determinar la defensa final de la planta.

En el Capítulo 3 se comprobó que el etileno (ET), otra hormona de gran importancia en las plantas, juega un papel modulador para la supresión de respuesta del JA mediada por el SA. En la ausencia de ET, la supresión del SA sobre el JA depende de la proteína de defensa llamada NPR1. Al contrario, el aumento en la producción de ET, como por ejemplo después del ataque del patógeno necrotrófico *Alternaria brassicicola*, tiende ha establecer el antagonismo del SA-JA pero independientemente de la proteína NPR1. Este descubrimiento muestra la

complejidad de la señalización (interacción) que ocurre durante la respuesta inmune de la planta.

En el Capítulo 4 se logró demostrar que el ET puede crear plantas no sensibles al antagonismo entre el SA y JA. Cuando las rutas del JA y ET se activan simultanemente antes de la respuesta del SA, las plantas de Arabidopsis no responden a la comunicación cruzada entre el SA y JA. Además, por el bloqueo mecionado anteriormente, se descubrió que el factor de transcripción ERF/AP2 llamado ORA59 juega un rol fundamental durante esta interacción. En resumen, se ha presentado evidencias de que la respuesta final de las plantas después de la activación del SA y JA depende de la kinética de las hormonas producidas y la señalización presente. Además, el ET juega un papel importante en la modulación de la tan compleja ruta de las defensas contra el ataque de insectos y patógenos.

En el Capítulo 5 y 6, se investigó el sitio en dónde ocurre la comunicación cruzada entre el SA y JA. En el Capítulo 5, se demostró que la biosíntesis de JA no es el punto crítico en donde SA antagoniza al JA. En el Capítulo 6, se comprobó que el SA suprime la respuesta del JA más abajo del complejo $SCF^{COI1}$ y las proteínas represoras JAZ en la ruta metabólica. Usando técnicas genéticas moleculares, se descubrió que la supresión del SA a la respuesta JA ocurre a nivel de la transcripción de genes y que el motif GCC-BOX en el promotor de los genes JA es un factor determinante para el establecimiento de la supresión con SA.

Resumiendo, esta investigación espera aportar con conocimientos de cómo las plantas regulan su respuesta a múltiples estreses bióticos. Aparentemente el contexto en dónde la defensa de la planta es activada depende de la reacción final. Además, este es un aporte para ampliar el entendimiento molecular de la comunicación cruzada entre el SA y JA. Considero que los resultados obtenidos en esta tesis serán una valiosa contribución para el desarrollo de nuevas estrategías para la protección de plantas.

## Samenvatting

Planten kunnen net als mensen en dieren ziek worden. Belangrijke veroorzakers van plantenziekten zijn schimmels, bacteriën, en virussen. Ook herbivore insecten zoals rupsen en luizen kunnen het leven van een plant behoorlijk zuur maken. Tesamen vormen schadelijke micro-organismen en insecten een belangrijke oorzaak van opbrengst- en kwaliteitsverlies bij de productie van landbouwgewassen. Om ziekten en plagen tegen te gaan worden er jaarlijks miljarden euro's uitgegeven aan chemische bestrijdingsmiddelen. Tijdens de evolutie hebben planten afweermechanismen ontwikkeld die er voor zorgen dat ze zich efficiënt kunnen verdedigen. Resistentie kan worden bewerkstelligd door verdedigingsmechanismen die continu aanwezig zijn, of door mechanismen die pas geïnduceerd worden na een aanval. Het aanschakelen van afweergeschut vergt veel energie van de plant en kan ten koste gaan van groei en reproductie. Daarom is het van belang dat er gecoördineerde activering van afweer plaatsvindt die specifiek gericht is tegen het type belager waarmee de plant geconfronteerd wordt. Een belangrijke vraag in het onderzoek naar signaal-transductie tijdens de verdediging van planten is dan ook: hoe zijn planten in staat om de signalen die geïnduceerd worden na aanval door ziekteverwekkers en insecten dusdanig te integreren dat dit leidt tot een verdedigingsrespons die specifiek werkzaam is tegen de betrokken aanvaller?

De plantenhormonen salicylzuur (SA), jasmonzuur (JA) en ethyleen (ET) spelen een belangrijke rol bij de coördinatie van geïnduceerde afweer tegen verschillende typen aanvallers. Zodra de plant een belager herkent verhoogt hij de productie van één of meer van deze signaalmoleculen. De signatuur van de hormoonproductie is sterk afhankelijk van het type belager en is bepalend voor de uiteindelijke de afweerreactie. Verhoogde productie van SA is veelal effectief tegen biotrofe pathogenen die zich voeden op levend plantenweefsel. Necrotrofe pathogenen, die leven op dood weefsel, en insectenvraat worden onderdrukt door verdedigingsmechanismen die worden geactiveerd door verhoogde productie van JA en ET. Onderzoek naar de rol van SA, JA en ET heeft aangetoond dat deze drie signaalmoleculen interacteren in een complex netwerk van signaal-transductieroutes die gezamenlijk bepalend zijn voor de inductie van de immuunrespons tegen het

pathogeen of insect dat de plant belaagt. De interactie tussen de verschillende signaal-transductieroutes heet "cross-talk" (hoofdstuk 1).

Onderzoek aan de modelplant *Arabidopsis thaliana* (zandraket) heeft aangetoond dat SA een antagonistisch effect heeft op genen die worden geïnduceerd door JA, zoals *PLANT DEFENSIN* (*PDF1.2*). Het vermogen van SA om de JA response te onderdrukken is echter wel afhankelijk van de kinetiek van de productie van de signaalmoleculen (hoofdstuk 2). Wanneer de SA response vlak voor of gelijk met de JA response wordt geactiveerd, dan worden door JA gereguleerde afweermechanismen onderdrukt en krijgt de door SA gereguleerde afweer de overhand. Echter, wanneer de JA response tegelijk met de ET response is geactiveerd, dan wordt de JA response ongevoelig voor SA/JA cross-talk (hoofdstuk 4). De transcriptiefactor ORA59 speelt hierin een belangrijke regulerende rol (hoofdstuk 4).

Het eiwit NPR1 speelt een belangrijke rol in de regulatie van SA/JA cross-talk. Activering van NPR1 geschiedt door een redox verandering na accumulatie van SA (hoofdstuk 2). Geactiveerd NPR1 is cruciaal voor het remmen van de JA signaal-transductieroute. Echter, wanneer naast SA en JA ook ET wordt geproduceerd, dan wordt de rol van NPR1 omzeild en is SA in staat om de JA response op een NPR1-onafhankelijke manier te remmen (hoofdstuk 3).

Onderzoek naar het moleculaire mechanisme van SA/JA cross-talk liet zien dat SA geen effect heeft op de biosynthese route van JA (hoofdstuk 5). Ook belangrijke spelers in de JA signaal-transductieroute, zoals $SCF^{COI1}$ en de JAZ repressor eiwitten, bleken niet de target te zijn van SA in SA/JA cross-talk. Met behulp van microarrays is het genexpressie profiel van Arabidopsis bestudeerd na behandeling met de plantenhormonen SA en methyl JA (MeJA). Analyse van de regulerende elementen in de promoters van de SA/JA cross-talk genen toonde een overrepresentatie aan van twee motieven: de I box en de GCC box. Mutatie van de I box in de promoter van het *PDF1.2* gen had geen effect op de onderdrukking van *PDF1.2* door SA, wat erop duidt dat dit element niet essentieel is voor de regulatie van SA/JA cross-talk (hoofdstuk 6). De GCC box zorgt ervoor dat de promoter van het *PDF1.2* gen kan reageren op MeJA. Ons onderzoek heeft aangetoond dat de GCC box voldoende is voor SA/JA cross-talk en dat de antagonistische werking van SA op de expressie van door JA gereguleerde genen werkt op het niveau van genexpressie (hoofdstuk 6).

Het werk beschreven in dit proefschrift heeft belangrijke nieuwe inzichten opgeleverd in de moleculaire mechanismen die ten grondslag liggen aan de interacties tussen verschillende hormonale signaal-transductieroutes die de natuurlijke afweerreakties van planten reguleren. De interacties tussen de hormonale signaal-transductieroutes helpen de plant bij het in stelling brengen van het juiste afweergeschut voor de meest effectieve verdediging.

## Agradecimientos/Acknowledgements

Qué pena! Es lo único que puedo pensar en este momento. Se acabo este maravilloso período de mi vida. Después de 6 años en Holanda y de muchas experiencias, no me queda más que decir: GRACIAS! DANK U VEEL! THANKS!

Gracias a ti Lorena, que buscaste en tu poca paciencia (como dices tú) acompañarme en este largo viaje, a un lugar fuera de tu medio ambiente y lejos de tu familia. Se que para ti ha sido muy dificil este período, por eso gracias, por tu lealtad, compañerismo y amor. A mi hija Isabella, gracias por tus momentos de alegría, fue lo más maravilloso tu llegada al mundo en un país extraño, que él cual, te recibió con los brazos abiertos. Gracias mija por ser como tú eres.

A mi querida madre Clara Elena, que en paz descanse, gracias por todas tus enseñanzas y cariño. Te digo una cosa: Acabé madre! Un sueño no solo mío sino tuyo también, gracias por darme fuerza todo este tiempo. A mi padre Héctor y hermano Andrés, por su apoyo en la culminación de este gran proyecto. Muchas gracias por creer en mi y darme el valor y empuje a realizar mi Ph.D. A mis suegros Mireya y Mario y a mi cuñada Mireya (hija), sin Uds no se habría podido realizar este sueño, muchas gracias por su apoyo y por sus visitas a Holanda, pasamos momentos inolvidables y disfrutando de su compañía en Wageningen/Utrecht. Muchas gracias a mi querida familia, a abuelita Beatriz y a todos los Reyes, que me ayudaron desde el principio en este proyecto y me dieron todo el apoyo para continuarlo, muchas gracias por su cariño.

Prof. Dr. Ir. Cornelus Marinus Joseph Pieterse, thanks a lot for being who you are as a person and as a scientist. You have been an incredible boss during this period. You gave us a lot of support especially to Lorena and Isabella, we are so grateful for that because without your support, I would not be able to finish my PhD in Holland. I really enjoyed working with you since your dirty sense of humor correlates perfectly with my disgusting imagination, sometimes too much! We have so much fun in Australia, a trip that I will never forget. Thx Corné.

Dr. Tita Ritsema, thank you for your understanding and giving me a lot of time to discuss about SA-JA cross-talk (a topic that we both did not know). At the beginning of my PhD student period, you were my ears listening to my crazy hypotheses and you were my eyes to guide me to the broad highway of science. Many thanks Tita.

Thank you very much to all people of the PMI-group, your interesting discussions during the half year reports, helped me a lot to fill up the blanks and complete the really nice stories that I included in this thesis. Annemart, many thanks for introducing me to the world of cross-talk and Arabidopsis, many thx. Adriaan, thx for having been a good lab partner and friend. You fed me once a week with pizza together with some green stuff, and once you almost killed me with a pink drink! Thx I will never forget that! Hans, Ientse and Ruth many thanks for your support in this project. I am sorry for the many times I drove you crazy with all those samples (-20 and -80), filling up greenhouses and cells with my plants, pictures, new chemicals, eppys, plates, seeds, etc. Many thanks to the "foreigners" working in the lab with me, Christos, Silvia, and Hanka. I was so cool to share the lab with you till very late at night/morning. I appreciated your input in my project and giving me an extra hand when I really needed (especially to join me with the protoplast dance o el baile del protoplasto! many thx my friends!) To Kees, Guido, Sjoerd, Saskia, Peter, Dieuwertje, Rogier, Vivian, Martin, Marieke (finally I know that you are really called Maria Helena Adriana), Roeland, María, Mohammad, Jurriaan, Chiel, Ido, Joost, Tieme, Annemiek, Joyce and Nico many thanks for your support and suggestions that brought this project alive. To Dr. ir. Steven Spoel, many thanks for having been a great support for this project and share many ideas, data and even your shorts! I really had a great time drinking beer at your place and visiting Xinnian's lab at Duke University.

All my gratitude and thanks to Alex (my dear paranimph), Rashmi, Karst and Herma. You were there with us in the goods and the bad times, thanks a lot my friends! We really enjoyed having barbecues at your homes and had trips together. Besides, you showed us what really is Dutch culture (bierje?), which I really liked and appreciated ☺ It was great!

Many thanks to Prof. Dr. Johan Memelink for accepting me in his lab and have interesting scientific discussions together. The period that I spent there, I realized that you pipetted (physically) more in my project than actually Corné hahaha. You are an example of a passionate scientist which I respect a lot. Thanks to Ana and Marcel, for introducing me to the protoplast world! I had a great time working together with you and also enjoyed being in another lab and city atmosphere. Besides, thanks for showing me the rainbow!

Many thanks to Dr. Alain Goossens for accepting me in his lab. Since we discussed in China, I knew we could make a nice collaboration and work, and we did it! I really enjoyed working at the VIB and lived for 2 months in Ghent. Laurens, it was pleasure to work with you. It was so much fun to talk about science and have so many coffee breaks meanwhile the robot will do all the work for us! Great! Many thanks to Robin for showing me the wonderful nightlife of Ghent, thx for that! Decadance! Thx to Lander and Jan for their technical assistance, it was great to have you around: and for the last time, I am not gay, you are!

To my dear M.Sc. students that were stocked under my supervision for a period of a year: Du Yujuan alias Jasper and Elvira de Lange. Thank you very much for being great students, very enthusiastic and skilled. Because of your help many successful experiments are presented in this work, so many thanks for your effort and following me during these crazy and hectic times. Also, thanks to Tamara van Molken for showing me that ecology and using clover as a model can be more interesting than Arabidopsis. Sure!

Thank you very much to our Dutch friends Rob, Els and Arafan for their great friendship and inviting us to their home to enjoy great dinners together. Els, many thanks for giving all the support that Lorena needed in those lonely times. And Rob, many thanks for helping me to make the garden in the back and cut the grass of our house at Maarten Schrijverlaan 6. Great times!

Muchas gracias amigos de Wageningen, yo creo que Uds nos hicieron sentir como en casa. A Luis, muchas gracias por tu amistad y hospitalidad, tu nos diste la mano cuando más necesitabamos, gracias mi hermano. A Katarina muchas gracias por tu amistad y por mover todas las cosas desde Holanda a Suiza, que viaje más loco. A Jenny, Mark, y Danny, gracias por ser buenos amigos y disfrutar juntos gratos momentos y por supuesto de la comida ecuatoriana. To my friends of the master's program: Jaap van Tuyl, Regis, Wen, Ronald, Karel, Linke, Santiago, Pablo, Alvarito and Bernita. We always keep in touch during this period and I always keep you in mind for all the good memories that we had together.

A mis profesores de la Universidad San Francisco de Quito que me alentaron y me motivaron a seguirles los pasos. Al Dr. Washington Padilla, Dr. Eduardo Uzcategui, Dr. Carlos Fabara y al Dr. Leonardo Corral gracias por su apoyo antes y durante este período.

Thanks you very much to my friends from Ecophysiology of Utrecht University. Rens, thank you very much because you brought me to Utrecht after I finished my masters in Wageningen. That opportunity changed my life, many thx. Thank you Ton for being a good supervisor and showing me that science is so much fun when you know what to do! Also you showed me that the next day after a party it can be possible to precipitate DNA with my breath! Many thanks to Rob, Frank and Ronald for their interesting discussions and helping me in many experiments during this thesis.

A mis panas que nos fueron a visitar a las Holandas. Esteban, Fedor y Chibule que bien que se pasaron por esas tierras y nos pudieron visitar. Además conocer algo de lo que hice alla en Holanda. Chévere no? Por que solo en Holanda se hace investigación de la buena jajajaja.

Thanks a lot of all the Dutch people, who helps us during all the moving from here to there, all the free stuff that gave us after Isabella was born, and their kindness and friendship to help us go around a foreign country for the period of 6 years. Many thanks and see you later.

Work hard and play hard!

**Antonio Leon-Reyes**

# List of publications

## Published Research papers

Pieterse CMJ, **Leon-Reyes A,** Van der Ent S and Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* 5: 308-316

Bargmann BOR, Laxalt AM, Riet B, Testerink C, Merquiol E, Mosblech A, **Leon-Reyes A,** Pieterse CMJ, Haring MA, Heilmann I, Bartels D and Munnik T (2009) Reassessing the role of phospholipase D in the *Arabidopsis* wounding response. *Plant, Cell & Environment* In press.

**Leon-Reyes A**, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, Millenaar FF, Welschen RAM, Ritsema T and Pieterse CMJ (2009) Ethylene Modulates the Role of NPR1 in Cross-Talk Between Salicylate and Jasmonate Signaling. *Plant Physiology* 149: 1797-1809

Koornneef A, Verhage A, **Leon-Reyes A**, Snetselaar R, Van Loon LC and Pieterse CMJ (2008) Towards a reporter system to identify regulators of cross-talk between salicylate and jasmonate signaling pathways in Arabidopsis. *Plant Signaling & Behavior* 3: 543-546.

Koornneef A, **Leon-Reyes A**, Ritsema T, Verhage A, Den Otter FC, Van Loon LC and Pieterse CMJ (2008) Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant Physiology* 147: 1358-1368.

## Published book chapters

Pieterse CMJ, Koornneef A, **Leon-Reyes A**, Ritsema T, Verhage A, Joosten R, De Vos M, Van Oosten V and Dicke M (2008). Cross-talk between signaling pathways leading to defense against pathogens and insects. In: *Biology of Molecular Plant-Microbe Interactions*, Vol. 6 (M. Lorito, S. Woo and F. Scala, eds), The International Society for Molecular Plant-Microbe Interactions, St. Paul, MN., pp. 000-000.

**Leon-Reyes A,** Prins TP, Van Empel JP and van Tuyl JM (2004) Differences in epicuticular wax layer in tulip can influence resistance to *Botrytis tulipae,* *ISHS Acta Horticulturae 673*: IX International Symposium on Flower Bulbs.

## Submitted research papers

**Leon-Reyes A,** Yujuan D, Koornneef A, Proietti S, Korbes A, Memelink J, Pieterse CMJ and Ritsema T. Potentiation of jasmonate signaling by ethylene counteracts salicylate-mediated suppression of the jasmonate response via the AP2/ERF transcription factor ORA59. *Submitted*

**Leon-Reyes A,** Koornneef A, van der Does D, Pauwels L, Goossens A, De Lange ES, Rodenburg N, Návarová H, van Wees SCM, Memelink J, Ritsema T and Pieterse CMJ. Suppression of jasmonate signaling by salicylic acid acts downstream JA biosynthesis and $SCF^{COI1}$-JAZ complex and targets GCC-box promoter elements. *Submitted*

Ritsema T, van Zanten M, **Leon-Reyes A**, Voesenek LACJ. Peeters AJM, Millenaar FF and Pieterse CMJ. Kinome profiling of pathogen defense signals reveals a role for jasmonate and salicylic acid in light control of differential petiole growth in *Arabidopsis thaliana***.** *Submitted*

Van Molken T, De Caluwe H, Van Dam NM, Hordijk C, **Leon-Reyes A**, Snoeren TAL and Stuefer JF. Volatile emissions by virus-infected plants repel herbivores. *Submitted*

Proietti S, Bertini L, Van der Ent S, **Leon-Reyes A**, Pieterse CMJ, Tucci M, Caporale C and Caruso C. Isolation and functional characterization of two WRKY orthologs transcription factors from wheat and Arabidopsis. *Submitted*

## Curriculum vitae

Antonio Leon-Reyes was born on 25 February 1976 in Quito, Ecuador. In the year 1995, before finishing High School in Ecuador, he studied his last year at Melrose-Mindoro High School in Wisconsin, USA. After retuning to Ecuador, he studied a major in Agribusiness and a minor in Chemistry at Universidad San Francisco de Quito. He finished his B.Sc. with Magna Cum Laude in the year 1999. From the year 1999 to 2002, he worked as a production manager of cut-flower farms in Latitud 0 and Newman Flowers located close to Quito. In 2002, he decided to continue his studies at Wageningen University in the Netherlands where he followed the master program Plant Breeding and Genetic Resources. His thesis research was performed at Plant Research International in the group of Dr. Jaap van Tuyl under the supervision of Dr. Theo Prins. His master thesis was called "In planta screening for resistance to *Botrytis tulipae* in tulip and molecular screening for Lily Mottle Virus (LMoV) resistance in lily". After finishing his masters in the beginning of 2004, he performed a 10-month internship at Utrecht University in the laboratory of Prof. Rens Voesenek under the supervision of Dr. Ton Peeters. Here, he worked on the project entitled "Molecular regulation of *Rumex palustris* genes under submergence conditions". After that period, he returned to Ecuador where he worked as a breeder of *Zantedeschia* flowers for the Dutch company SANDE. In April 2005, he returned to the Netherlands to continue his Ph.D. studies under the supervision of Prof. Corné Pieterse and Dr. Tita Ritsema of the Plant-Microbe Interactions group at Utrecht University. He worked on a project called "Plant innate immunity: cross-talk between signaling pathways to fine-tune defense". In April 2009 he finished his PhD studies and his thesis is entitled "Making sense out of signaling during plant defense". After his Ph.D. research, he returned to Quito, Ecuador to perform research and teach in the field of agro-biotechnology at Agrobiolab, Escuela Politécnica del Ejército (ESPE) y Universidad San Francisco de Quito (USFQ). More information: http://sites.google.com/site/antonioleonreyessite/