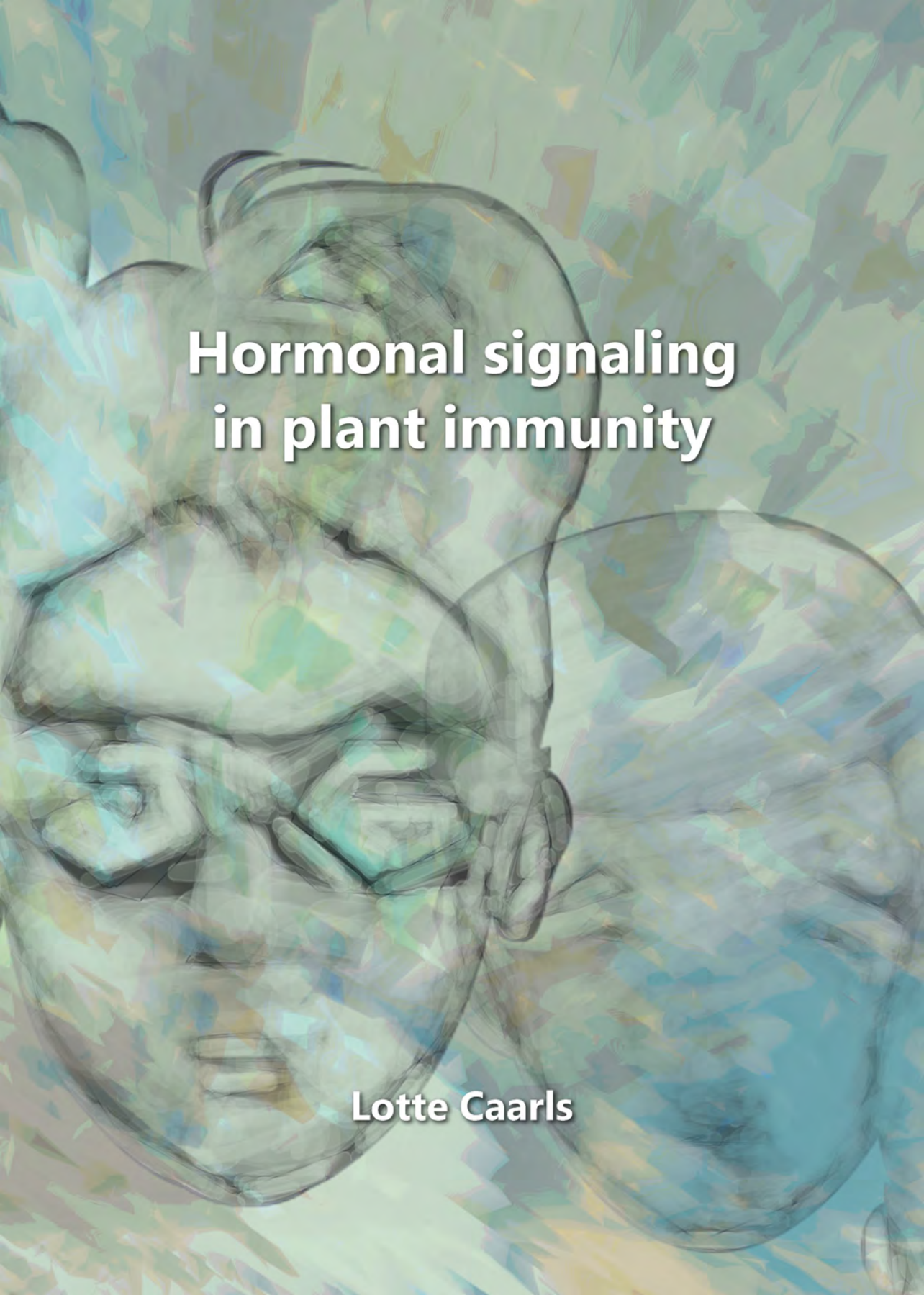

Hormonal signaling in plant immunity

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PhD thesis

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Hormonal signaling in plant immunity

Hormonale regulatie van signaaltransductie in het afweersysteem van planten

(met een samenvatting in het Nederlands)

Proefschrift

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CONTENTS

CHAPTER 1

General introduction

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THE PLANT IMMUNE SYSTEM

In their lifetime, plants intimately interact with a broad range of microbial pathogens and herbivorous insects. These interactions can be advantageous, as is the case with beneficial soil-borne microbes, that aid in the uptake of minerals, stimulate plant growth, or enhance defense (Zamioudis and Pieterse, 2012). However, many interactions of plants with microbes or insects are detrimental for the plant. These bacteria, oomycetes, nematodes, fungi or insects consume or kill tissue or are vectors for plant viruses. Pathogens are divided into different classes dependent on their life style. Biotrophs feed on living tissue, while necrotrophs kill the host tissue and feed on the contents. Other pathogens have both a biotrophic and necrotrophic life stage, and are called hemi-biotrophs (Glazebrook, 2005). To ward off attackers, plants have evolved a sophisticated innate immune system that activates inducible defenses once the attacker is recognized.

The activation of inducible defense responses to infection by pathogens or infestation by insect herbivores relies on the recognition of the attacker. Plants have evolved several means of recognizing attackers. First, microbe-associated molecular patterns (MAMP), such as bacterial flagellin or fungal chitin, are recognized by surface-localized pattern-recognition receptors (PRRs) (Macho and Zipfel, 2014). Molecules from insects, which include different insect-derived proteins, peptides and fatty acids conjugates, are called herbivore-associated molecular patterns (HAMPs) and can also be recognized by the plant (Acevedo *et al.*, 2015). Moreover, damaged, disintegrated cells can release damage-associated molecular patterns (DAMPs) that can also be perceived by PRRs. Examples of plant DAMPs are extracellular ATP and components of the cell wall (Heil and Land, 2014). Finally, pathogens can deliver virulence proteins into the cell (called effectors), aimed to suppress defense responses or acquire nutrients, which in turn can be recognized by plant nucleotide-binding/leucine-rich-repeat (NLR) receptors and trigger defense (Cui *et al.*, 2015).

After recognition of the different danger signals, strikingly common downstream signaling components are elicited in plant cells. These responses entail an influx of Ca^{2+} into the cytosol, the generation of reactive oxygen species (ROS), transient activation of mitogen-activated protein kinases (MAPK) signaling cascades and production of ethylene (Boller and Felix, 2009; Tsuda and Katagiri, 2010; Wu and Baldwin, 2010). All these signals likely converge on the production of plant hormones that act as immune signals in the defense response. Besides ethylene, jasmonic acid (JA) and salicylic acid (SA) emerged as important hormones in plant defense. Ultimately, the hormonal immune signals trigger an extensive transcriptional reprogramming, which results in an efficient defense response (Buscaill and Rivas, 2014). SA triggers the production of antimicrobial pathogenesis-related (PR) proteins and is required

for the onset of systemic acquired resistance (SAR) (Fu and Dong, 2013). Defense responses mediated by JA involve production of several defense-related secondary metabolites and a distinct set of PR proteins (Campos *et al.*, 2014). Moreover, JA is required for the establishment of wound-induced resistance (Howe and Jander, 2008) and rhizobacteria-induced systemic resistance (ISR) (Pieterse *et al.*, 2014b).

HORMONES IN PLANT DEFENSE

SA-dependent defenses are generally effective against (hemi)biotrophic pathogens, whereas JA-mediated defenses are effective against necrotrophic pathogens and herbivorous insects. These conclusions have mainly been based on research with *Arabidopsis thaliana* (Arabidopsis) mutants in the SA or JA signaling pathways, which are generally more susceptible against one type of attacker. For example, a mutant in *NON-EXPRESSOR OF PR GENES1* (*NPR1*), which is blocked in SA signaling, is more susceptible to the biotrophic oomycete *Hyaloperonospora arabidopsidis* and the (hemi)biotroph *Pseudomonas syringae* (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah and Klessig, 1996). Conversely, a mutant in JA-receptor CORONATINE INSENSITIVE1 (COI1) is more susceptible to the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* and to feeding by the Egyptian worm caterpillar (Thomma *et al.*, 1998; Stotz *et al.*, 2002). Moreover, exogenous treatment with SA or SA-analogs has been shown to induce resistance against (hemi)biotrophic pathogens in several plant species (reviewed by Vlot *et al.*, 2009). Treatment with JA can protect the plant against future herbivore attack and reduce the severity of infection by necrotrophic fungi (Baldwin, 1998; Thomma *et al.*, 2000). This reinforces the concept that each hormonal signaling pathway is involved in specific types of defense.

However, the idea that a single pathway is responsible for defense against a specific group of attackers is an oversimplification of the reality in plants. Hormonal measurements show that in response to each attacker, a hormonal blend is produced in the plant that can contain JA, SA and ethylene. The composition and timing of this hormonal blend depends on attacker and plant species tested (De Vos *et al.*, 2005; Diezel *et al.*, 2009). Furthermore, network studies on wild-type and mutant plants revealed complex interplay between the different hormone-regulated pathways (Sato *et al.*, 2010; Van Verk *et al.*, 2011; Kim *et al.*, 2014). In addition, in response to infection with *P. syringae* pv DC3000*hrpA*-, genes in SA synthesis as well as JA synthesis were upregulated (Lewis *et al.*, 2015). It was also suggested that the different hormone signaling sectors all contribute to immunity to one pathogen, as simultaneous mutation of components of SA-, JA-, and ethylene pathways resulted in higher susceptibly to pathogens than single mutants did (Tsuda *et al.*, 2009). Antagonistic and synergistic interactions between the hormonal signaling pathways

likely enable the plant to fine-tune its defense response against each attacker that it encounters. Here, we review the current knowledge of SA and JA metabolism and signaling and discuss the molecular mechanisms of the antagonistic interactions between the SA and JA pathways.

THE JASMONIC ACID PATHWAY

Jasmonic acid metabolism

JA biosynthesis is initiated by the oxygenation of polyunsaturated fatty acids in chloroplast membranes, such as α-linolenic acid (18:3), by lipoxygenases (LOX), forming fatty acid hydroperoxides. Further dehydration by ALLENE OXIDE SYNTHASE (AOS) and cyclization by ALLENE OXIDE CYCLASE (AOC) results in the formation of 12-oxophytodienoic acid (OPDA). In the peroxisome, OPDA is reduced to OPC-8:0, which is subjected to three cycles of β-oxidation shortening the octanoic acid side chain. This yields the 3R,7S stereoisomer of JA (+)-7-iso-JA) (Schaller and Stintzi, 2009). JA is conjugated to isoleucine by the enzyme JASMONATE RESISTANT 1 (JAR1) to form JA-L-isoleucine (Staswick and Tiryaki, 2004; Suza and Staswick, 2008). (+)-7-iso-JA-L-Ile is considered the bioactive form of JA, as it is most effective in pulling down JA-receptor COI1 and promoting the interaction between COI1 and JA repressors JAZ *in vitro* (Fonseca *et al.*, 2009). JA-Ile presumably diffuses into the nucleus to activate downstream signaling processes.

Besides conjugation to JA-Ile, JA can be modified by numerous other metabolic reactions, forming compounds that are active, partially active or inactive forms of JA. One of these is the methylation of JA resulting in methyl JA (MeJA). Although MeJA is considered active because treatment with MeJA leads to activation of JA-dependent responses, the ability of exogenous MeJA to do this is attributed to its metabolic conversion to JA and subsequently JA-Ile in plants (Tamogami *et al.*, 2008; Wu *et al.*, 2008). Other metabolic conversions of JA produce JA glucosyl ester and cis-jasmone, and conjugations with other amino acids. Finally, hydroxylation and carboxylation of JA and JA-Ile results in inactive forms of JA (Wasternack and Strnad, 2015). Recently, cytochrome P450 hydroxylases have been shown to be involved in ω-oxidation of JA-Ile, which inactivates this bioactive JA molecule. The enzymes CYP94B1 and CYP94B3 hydroxylate JA-Ile, forming 12-OH-JA-Ile (Kitaoka *et al.*, 2011; Koo *et al.*, 2011; Koo *et al.*, 2014), while CYP94C1 further converts 12-OH-JA-Ile to 12-COOH-JA-Ile (Heitz *et al.*, 2012). These conversions are important to keep JA-Ile levels in check and control JA responses.

JA signaling

Under normal growth conditions, when JA levels are low, the activity of transcriptional activators of JA-responsive gene expression is repressed by members of the JASMONATE ZIM-domain (JAZ) family (Chini *et al.*, 2007; Thines *et al.*, 2007). After activation of the JA pathway, the bioactive compound JA-Ile binds to a complex of JAZ and COI1, which is the F-box protein of the E3 ubiquitin ligase SCF^{COI1} (Xie *et al.*, 1998; Katsir *et al.*, 2008; Sheard *et al.*, 2010).The formation of the JAZ-COI1 complex leads to ubiquitination and degradation of JAZ repressors by the proteasome (Chini *et al.*, 2007; Thines *et al.*, 2007). This relieves transcriptional repression of JAZ targets, resulting in activation of gene expression. JAZ proteins repress the action of many transcription factors involved in JA responses. They were initially identified as inhibitors of MYC2, but have now been shown to bind also MYC2-related MYC3 and MYC4 (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011) and several other transcription factors that are involved in JA-responsive processes such as anthocyanin accumulation, plant stamen development, defense against necrotrophic pathogens and insects and delay of flowering time (Qi *et al.*, 2011; Song *et al.*, 2011b; Zhu *et al.*, 2011; Zhai *et al.*, 2015). Moreover, JAZ proteins have also been shown to interact with bHLH transcription factors that act as negative regulators of JA responses (Song *et al.*, 2013), suggesting a negative feedback system where production of JA-Ile also inhibits JA-responsive gene expression.

JAZ proteins use different means to repress transcriptional activation. First, JAZ repress gene expression through an association with co-repressor TOPLESS (TPL), either directly when the JAZ protein in question contains an EAR motif, or via an interaction with adapter protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels *et al.*, 2010; Shyu *et al.*, 2012). JAZ proteins can also recruit HISTONE DEACETYLASE6 (HDA6) to inhibit the activity of EIN3/EIL1 that are positive transcriptional regulators of a subset of JA responses including expression of *ETHYLENE RESPONSE FACTOR1* (*ERF1*), *OCTADECANOID-RESPONSIVE ARABIDOPSIS59* (*ORA59*) and *PLANT DEFENSIN1.2* (*PDF1.2*) (Zhu *et al.*, 2011). Recently, JAZs were shown to compete with a subunit of the Mediator complex, MED25. Mediator is a transcriptional co-activator complex that recruits RNA polymerase II to promoters of JA-responsive genes (Çevik *et al.*, 2012). By interfering with the interaction of MYC3 with MED25, JAZ prevents transcriptional activation of these genes (Zhang *et al.*, 2015).

After degradation of JAZ repressors, transcriptional activators are released and this leads to activation of JA-responsive genes. Two branches are distinguished in JA-responsive gene expression in defense: (i) MYC2 is the master regulator of the MYC branch, which is co-regulated by JA and the hormone abscisic acid (ABA) and activates downstream JA marker genes *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) and *LIPOXYGENASE2* (*LOX2*). The MYC branch is considered to be effective

in defense against herbivorous insects (Lorenzo *et al.*, 2004; Vos *et al.*, 2013b). (ii) EIN3, EIL1 and ERF transcription factors such as ERF1 and ORA59 regulate the ERF branch, which is co-regulated by JA and ethylene, activating the downstream marker gene *PDF1.2,* and is important in defense against necrotrophic pathogens (Zhu *et al.*, 2011; Pieterse *et al.*, 2012; Wasternack and Hause, 2013).

Activation of the JA pathway

How recognition of pathogens or insect attack leads to the JA synthesis or amplification of JA responses is not completely known. Several DAMPs, MAMPS or HAMPS activate JA accumulation and the associated defense responses (reviewed by Campos *et al.*, 2014). In the activation of JA biosynthesis, MAPK signaling cascades as well as CA^{2+} signaling and ROS have been implicated. Once the JA pathway has been activated, positive feedback systems ensure the rapid production of JA and JA-Ile: many genes encoding JA biosynthesis enzymes are upregulated quickly in response to MeJA treatment or JA-stimulating wounding (Reymond *et al.*, 2000; Sasaki *et al.*, 2001; Pauwels *et al.*, 2008). Expression of *JAR1* is also activated by wounding (Suza and Staswick, 2008). However, negative feedback mechanisms are also present in the JA pathway: several enzymes involved in the inactivation of the JA-Ile are upregulated by JA activation (Koo *et al.*, 2011) and expression of *JAZ* and *NINJA* is induced by JA (Chini *et al.*, 2007; Thines *et al.*, 2007; Pauwels *et al.*, 2010). These negative feedback mechanisms dampen the JA response and presumably result in the shutdown of JA processes when the JA-Ile signal is gone. How the different positive and negative feedback systems finally result in an optimal JA-response, is still a major question.

THE SALICYLIC ACID PATHWAY

Salicylic acid metabolism

Salicylic acid in plants is derived from the primary metabolite chorismate and can be generated via two distinct pathways. In the first, chorismate-derived L-phenylalanine is converted into SA via a series of enzymatic reactions initially catalyzed by PHENYLALANINE AMMONIA LYASE (PAL). In the second, chorismate is converted to SA via ISOCHORISMATE SYNTHASE (ICS) (Dempsey *et al.*, 2011). In Arabidopsis, ICS1 is the enzyme responsible for the majority of SA accumulation in response to pathogen attack (Wildermuth *et al.*, 2001; Garcion *et al.*, 2008). SA is transported from the chloroplasts, where it is synthesized, to the cytosol by the MATE-like transporter ENHANCED DISEASE SUSCEPTIBILITY5 (EDS5) (Serrano *et al.*, 2013).

Once synthesized, SA may be subjected to several chemical modifications, including glucosylation, methylation and amino acid conjugation (Dempsey *et al.*, 2011). Two SA glucosyl transferase (SAGT) enzymes in Arabidopsis convert SA into SA 2-*O*-β-D-glucoside (SAG) or salicyloyl glucose ester (SGE) (Dean and Delaney, 2008). SAG is thought to be produced in the cytosol and transported into the vacuole as an non-toxic storage form of SA (Dean *et al.*, 2003). Methylation of SA results in formation of the volatile methyl salicylate ester (MeSA). A conjugated form of MeSA also exists. Conjugations of SA and amino acids have been detected, such as SA-Asp (Dempsey *et al.*, 2011). SA is also hydroxylated to 2,3 dihydroxybenzoic acid (DBHA) and 2,5-DHBA, which are then conjugated to sugars, and are likely inactive forms of SA. 2,3-DHBA and 2,5-DHBA increase in plant leaves with age (Bartsch *et al.*, 2010). Inactivation of SA into 2,3-DHBA occurs both after pathogen infection and during senescence. A mutant in the gene that encodes the enzyme SA 3-HYDROXYLASE (S3H), which hydroxylates SA to 2,3-DHBA, accumulates increased SA, starts to senesce earlier and is more resistant to the pathogen *H. arabidopsidis* (Zhang *et al.*, 2013a; Zeilmaker *et al.*, 2015).

SA signaling

SA causes a transcriptional reprogramming in cells which leads to the activation of many defense-related genes (Wang *et al.*, 2006; Blanco *et al.*, 2009). Transcriptional co-regulator NPR1 controls the expression of many of these genes, and is as such essential for SA-mediated gene expression and disease resistance (Cao *et al.*, 1994; Delaney *et al.*, 1995). By interacting with transcription factors of the TGA family, NPR1 acts as a co-activator of SA-induced gene transcription, activating SA marker genes such as *PR1* (Zhang *et al.*, 1999; Zhou *et al.*, 2000; Després *et al.*, 2003). NPR1 also regulates the expression of several *WRKY* transcription factor genes, which then fine-tune and amplify downstream transcriptional responses (Wang *et al.*, 2006; Eulgem and Somssich, 2007). SA has been shown to bind NPR1 directly, which converts NPR1 from an inactive state into an activated transcriptional co-activator (Wu *et al.*, 2012). NIMIN proteins interact with NPR1 and NIMIN1 and NIMIN3 negatively regulate *PR1* expression (Weigel *et al.*, 2005; Hermann *et al.*, 2013).

Besides the NPR1-dependent pathway, proteins involved in the DNA damage response also play a role in SA-responsive gene expression. BRCA2A, RAD51, RAD51D and SWS1 are recruited to promoters of defense-related genes such as *PR1* after SA treatment, and activate transcription (Durrant *et al.*, 2007; Wang *et al.*, 2010; Song *et al.*, 2011a). SNI1, which was described as a suppressor of NPR1, has now been shown to be part of a structural maintenance of chromosome 5/6 complex, which is required for controlling DNA damage. In response to pathogen infection, the activation of the SA pathway causes DNA damage, and DNA damage sensor proteins RAD17 and ATR are required for the immune response (Yan *et al.*, 2013).

The activity of NPR1 is tightly regulated by several SA-dependent modifications. SA induces a biphasic fluctuation in the cellular redox state that can be sensed by NPR1, which then switches from an oligomer to monomer form by reduction of intermolecular disulfide bonds (Mou *et al.*, 2003). Thioredoxins TRX-h5 and TRX-h3 catalyze the formation of NPR1 monomers, which translocate to the nucleus via nuclear pore proteins (Tada *et al.*, 2008; Cheng *et al.*, 2009). In the nucleus, NPR1 is further modified by sumoylation and phosphorylation of serine residues (Spoel *et al.*, 2009; Saleh *et al.*, 2015). Phosphorylation-mediated degradation of the NPR1 prevents untimely activation of *PR* genes, but turnover of the protein is also required for full-scale transcription of NPR1 targets (Spoel *et al.*, 2009). Sumoylation of NPR1 enhances its interaction with TGA3 and this promotes expression of *PR1* (Saleh *et al.*, 2015). Regulation of NPR1 monomer levels in the nucleus is also dependent on SA. NPR1 and NPR1-homologs NPR3 and NPR4 were described to be SA-receptors (Fu *et al.*, 2012). NPR3 and NPR4 act as CUL3 ligase adapter proteins in proteasome-mediated degradation of NPR1. NPR3 and NPR4 differ in both their binding affinity for SA and binding capacity to NPR1, so that SA levels determine when NPR1 is targeted for degradation. When SA levels are low, NPR4 interacts with NPR1, leading to its degradation, and in this way untimely transcriptional activation in absence of SA is prevented. High SA levels facilitate binding between NPR1 and NPR3, again leading to removal of NPR1 (Fu *et al.*, 2012). This degradation of NPR1 is thought to help activate programmed cell death, of which NPR1 is a negative regulator. When SA levels are intermediate, interaction between NPR1 and NPR3 is prevented, allowing NPR1 to accumulate and activate SA-dependent defenses.

Activation of the SA pathway

The recognition of MAMPs leads to local and systemic accumulation of SA (Mishina and Zeier, 2007). SA accumulation in response to effector-triggered immunity is dependent on EDS1 and its interaction partner PAD4 (Feys *et al.*, 2001). ROS bursts mediated by NAPDH oxidases and extracellular peroxidases precede SA biosynthesis. How ROS bursts trigger SA production is not known (Herrera-Vásquez *et al.*, 2015). The translation of Ca^{2+} signals by calmodulin and calcium dependent protein kinases was also suggested to regulate SA biosynthesis (Seyfferth and Tsuda, 2014). SA biosynthesis is regulated in a spatial and temporal manner, as two different transcription factors were shown to regulate ICS induction in its circadian rhythm and in stomata (Zheng *et al.*, 2015). Negative regulation of ICS transcription by the ethylene transcription factors EIN3/EIL1 and by coronatine-activated ANAC19 are examples of hormonal crosstalk that negatively regulate the SA pathway (Chen *et al.*, 2009; Zheng *et al.*, 2012).

CROSSTALK BETWEEN SALICYLIC ACID/JASMONIC ACID PATHWAYS

The SA- and JA-responsive signaling pathways are interdependent and act in complex networks. Other hormones participate in these defense signaling networks as well and can consequently modulate the outcome of the activated defense arsenal. Abscisic acid (ABA) and ethylene can act synergistically with distinct JA-regulated responses, while they generally antagonize SA responses. Auxin, gibberellins and cytokinins can repress defense-related processes to prioritize growth of the plant, and vice versa their action can be suppressed by SA or JA leading to activation of defense at the expense of plant growth (Pieterse *et al.*, 2012). Antagonistic, synergistic and neutral interactions between SA and JA have been described. The final outcome of the interaction is likely concentration-, timing- and context-dependent (Pieterse *et al.*, 2012). Here, we focus on the antagonistic interactions between the SA and JA signaling pathway (hereafter also referred to as SA/JA crosstalk).

Biological implications of SA/JA crosstalk: trade-offs in disease resistance

One of the earliest reports on antagonistic crosstalk between the SA and JA-signaling pathways was the observation that SA could inhibit the accumulation of proteinase inhibitors that accumulate in response to JA treatment or wounding (Doares *et al.*, 1995). Later, many examples of how this SA/JA crosstalk influences defense have been reported. Here, we focus on model plant Arabidopsis, but the phenomenon has been observed in several species (reviewed by Thaler *et al.*, 2012). SA treatment or activation of SA by an SA-inducing pathogen has been shown to lower resistance against pathogens or insects sensitive to JA-dependent defenses. For example, treatment with SA, or elevated levels of SA in *cpr* mutants, lead to enhanced feeding and growth of caterpillars (Cui *et al.*, 2002; Cipollini *et al.*, 2004). Similarly, overexpression of NPR1 in rice led to increased susceptibility to an insect (Yuan *et al.*, 2007). Infection of plants with *P. syringae* resulted in plants susceptible to the fungus *A. brassicicola* (Spoel *et al.*, 2007) and pre-infection with *H. arabidopsidis* increased susceptibility to *B. cinerea* (Vos *et al.*, 2015). In addition, SA or infection with *H. arabidopsidis* also suppressed attacker-induced expression of JA-responsive genes *PDF1.2* and *VSP2* (Koornneef *et al.*, 2008a). Conversely, mutants deficient in the SA signaling pathway are more resistant to pathogens or insects that are sensitive to JA-dependent defenses, indicating that they normally suppress these defenses. For example, caterpillars feed less on *npr1-1, eds5* and *sid2* mutants (Cui *et al.*, 2002; Stotz *et al.*, 2002).

Furthermore, many pathogens have seemingly used the antagonistic effects between the SA and JA signaling pathways to manipulate the plant immune system to their own good. For example, eggs of the caterpillars *Pieris brassicae* and *Spodoptera*

littoralis induced SA accumulation and expression of SA-dependent genes, likely to suppress JA-dependent defenses that act against the larvae that emerge from the eggs (Bruessow *et al.*, 2010). A *B. cinerea* isolate produces an exopolysaccharide that induced SA accumulation and enhances susceptibility by suppressing JA-dependent *Proteinase Inhibitor* genes (El Oirdi *et al.*, 2011). Finally, whiteflies induce SA responsive gene expression and suppress JA-responsive genes. This suppression is dependent on components of the SA signaling pathway (Zhang *et al.*, 2013b). We discuss the molecular mechanism of how pathogens rewire the hormonal signaling pathways at the end of this chapter.

MOLECULAR MECHANISMS OF SALICYLIC ACID/JASMONIC ACID CROSSTALK

Recent work indicates that suppression of the JA-response pathway by SA is predominantly regulated at the level of gene transcription (Van der Does *et al.*, 2013). First, SA/JA crosstalk proved to be independent of downregulation of JA biosynthesis itself, as the SA-mediated suppression of MeJA-induced *PDF1.2* was intact in the JA biosynthesis mutant *aos/dde2* (Leon-Reyes *et al.*, 2010b). Using the JA-receptor mutant *coi1-1* ectopically expressing *ERF1* to constitutively express downstream JA-responsive genes, Van der Does and colleagues (2013) further demonstrated that SA can suppress ERF1-activated *PDF1.2* independently of COI1. Moreover, using GCC:GUS reporter lines, the GCC-box, which is a crucial *cis*-element in the regulation of *PDF1.2* expression, was shown to be sufficient for SA/JA crosstalk. This indicates that SA antagonizes JA signaling downstream of COI1, possibly by interfering with JA-regulated transcription factors. The ERF transcription factor ORA59 was then demonstrated to be degraded by SA. At the SA signaling side, using mutant *npr1-1*, master regulator NPR1 was previously shown to be essential for suppression of JA-responsive gene expression (Spoel *et al.*, 2003). Further, several WRKY and TGA transcription factors have been shown to be important for SA/JA crosstalk (Pieterse *et al.*, 2012; Gimenez-Ibanez and Solano, 2013). However, the ways by which these transcriptional regulators down-regulate JA signaling in the presence of SA are largely unknown. Here, we discuss the regulatory mechanisms that SA employs to repress JA-regulated transcriptional activity. Where relevant, examples of how other hormones interfere with hormone-dependent transcriptional regulation will be given.

SA-mediated effects on activity or localization of transcription factors

SA-induced modification of transcriptional regulators via redox signaling

The activation of the immune response in plants is associated with rapid production of reactive oxygen intermediates (ROI) and increased levels of nitric oxide (NO). Redox-sensing small-molecule couples, such as reduced and oxidized glutathione, can limit damage from these redox active molecules. Moreover, these redox sensors transduce changes in ROI and NO levels into posttranslational modifications by reduction or oxidation of cysteine residues of transcriptional regulators, causing changes in transcriptional activity (Frederickson and Loake, 2014). Redox signaling is important in SA signaling and moreover, SA-induced redox changes are associated with the suppression of JA responses as well.

Role of reduction of transcriptional regulators in SA signaling

In SA signaling, master regulator NPR1 is subject to several redox-dependent modifications. It sequesters in the cytoplasm as an oligomer, formed by intermolecular disulfide bonds, which are facilitated by S-nitrosylation of cysteine residues via NO donor S-nitrosoglutathione (GSNO) (Fig. 1B). SA triggers cycles of cellular reduction and oxidation, measurable for example by enhanced total glutathione levels and a higher ratio of reduced to oxidized glutathione after SA treatment (Spoel and Loake, 2011). In response to activation of the SA pathway, thioredoxins catalyze the reduction of intermolecular disulphide bonds, causing a conformational change of NPR1 to its monomeric form. As a monomer, NPR1 is able to translocate from the cytosol to the nucleus and activate downstream signaling (Mou *et al.*, 2003; Tada *et al.*, 2008). Other transcriptional regulators functioning in the SA pathway are also redox controlled. TGA1 contains intramolecular disulfide bonds that prevent its interaction with NPR1. Only after reduction of these bonds under high SA conditions, TGA1 is able to interact with NPR1. Further S-nitrosylation and S-glutathionylation of the cysteine residues of TGA1 result in enhanced binding to DNA and activation of transcription (Fig. 1A) (Després *et al.*, 2003; Lindermayr *et al.*, 2010). Recently, it was suggested that redox also reinforces the circadian rhythm of defense-related genes in an NPR1-dependent manner (Zhou *et al.*, 2015).

Role of the redox state in SA/JA crosstalk signaling

Redox-mediated reduction of transcriptional regulators is not only essential for SA signaling, but is also implicated in SA/JA crosstalk. The enhancement in glutathione levels after SA treatment was shown to coincide exactly with the window of opportunity in which SA could suppress JA-induced *PDF1.2* expression, i.e. within 30 hours after application of SA. In addition, treatment with glutathione synthesis

inhibitor BSO blocked SA-mediated antagonism of *PDF1.2* expression (Koornneef *et al.*, 2008a). Interestingly, JA can also influence the redox state of cells, but, in contrast to SA, it decreases the total amount of glutathione, and shifts the ratio between reduced and oxidized glutathione towards the oxidized state (Spoel and Loake, 2011). When SA and JA were applied simultaneously, the pattern of glutathione increase was the same as after treatment with SA alone, suggesting a role for redox regulation in prioritization of the SA pathway over the JA pathway (Koornneef *et al.*, 2008a). So far, it is unclear how the SA-induced cellular reduction can influence JA-inducible responses.

Master regulator NPR1 is essential for SA/JA crosstalk and, therefore, the importance of SA-induced redox changes in SA/JA crosstalk could be related to reduction and translocation of NPR1 to the nucleus. However, the nuclear localization of NPR1 that follows SA-induced monomerization is, although essential for SA-responsive gene expression, not needed for SA-mediated suppression of JA-dependent genes (Spoel *et al.*, 2003; Leon-Reyes *et al.*, 2009). This was shown with Arabidopsis plants that overexpress a fusion protein of NPR1 that was retained in the cytosol: stimulation of the SA pathway in these plants resulted in a wild-type level of suppression of JA-induced *PDF1.2* (Spoel *et al.*, 2003). The role of NPR1 in the cytoplasm for SA/JA crosstalk was confirmed in rice (*Oryza sativa*), where overexpression of OsNPR1 suppressed JA-responsive gene expression and defense against insects. However, when a mutated form of OsNPR1 was overexpressed that was constitutively present in the nucleus, herbivore resistance and expression of a JA-responsive gene were not affected (Yuan *et al.*, 2007). Although NPR1 is exclusively needed in the cytosol for SA/JA crosstalk, it is still possible that redox-mediated modification of NPR1 is important in SA/JA crosstalk, for example if there is a role for the monomeric form of NPR1 in the cytosol to suppress JA signaling (Spoel *et al.*, 2003; Beckers and Spoel, 2006). Alternatively, redox signaling may be important for post-translational modification of other factors with a role in SA/JA crosstalk, as described below.

The importance of redox regulation in SA/JA crosstalk is supported by the role of glutaredoxins (GRX) in this phenomenon. GRX are small ubiquitous redox enzymes that use glutathione to reduce their targets (Ndamukong *et al.*, 2007; Ströher and Millar, 2012). SA is known to induce the expression of at least two glutaredoxins, namely GRX480 and GRXS13, which are members of the group III class of glutaredoxins in Arabidopsis. Overexpression of glutaredoxin GRX480 blocks the induction of *PDF1.2* by JA, and overexpression of GRXS13 makes plants more susceptible to the necrotrophic fungus *B. cinerea*, suggesting a role for both glutaredoxins in suppression of JA signaling (Ndamukong *et al.*, 2007; La Camera *et al.*, 2011). In fact, 10 more group III glutaredoxins, which are also called ROXYs, are able to suppress activation of the *ORA59* promoter and are thus potentially

involved in suppression of the JA pathway (Zander *et al.*, 2012). Their antagonistic action on JA responses is likely downstream of NPR1, because expression of *GRX480* is reduced in the *npr1-1* mutant and overexpression of GRX480 in the *npr1-1* background still results in suppression of *PDF1.2* expression (Zander *et al.*, 2012; Herrera-Vásquez *et al.*, 2014). TGA transcription factors that are implicated in different hormonal signaling pathways and in SA/JA crosstalk are possible targets of group III glutaredoxins, as they are shown to interact with each other (Fig. 1C). Moreover, JA-induced *PDF1.2* expression is not impaired when GRX480 is overexpressed in the triple mutant *tga2tga5tga6* background, showing that the function of this glutaredoxin in suppression of JA-responses is dependent on these TGA transcription factors (Ndamukong *et al.*, 2007; Zander *et al.*, 2012).

Sequestration and degradation of transcription factors by SA

SA could antagonize JA signaling by preventing accessibility of JA-responsive transcriptional regulators to their target genes. This could be achieved by sequestering transcription factors in inactive complexes or by degradation of positive regulators.

Sequestering transcriptional regulators by complexation

By directing transcription factors to the cytosol, the possibility to activate transcription is obviously obstructed. In addition, transcription factors can be kept in check in the nuclear compartment as well, by inducing complex formation with other proteins that inhibit binding to the DNA, resulting in reduced transcription. There are no examples yet of SA-mediated sequestration of transcription factors leading to antagonism of JA signaling. However, some other plant hormone signaling interactions have been reported to be partly regulated via this mechanism, of which an example is the interaction between the SA and the ABA signaling pathways. The transcription factor WRKY40 is induced by SA and suppresses expression of the ABA-responsive genes *ABI4* and *ABI5*. After ABA treatment, the ABA receptor ABAR interacts with WRKY40, which is then recruited to the cytosol. By this recruitment, binding of WRKY40 to ABA responsive promoters is inhibited and repression of ABA responsive genes is lifted (Shang *et al.*, 2010; Liu *et al.*, 2012).

In animal cells, cytosolic sequestration of a transcriptional regulator was shown to control the antagonistic interaction between SA and prostaglandin signaling, which shares several aspects with SA/JA crosstalk in plants. SA and aspirin block the formation of prostaglandins in animal cells, which are considered structural analogues of JA in plants. SA induces retention of transcription factor NF-κB in the cytoplasm by enforcing its interaction with IκB. In response to stress, IκB kinase is activated and degrades IκB, leading to nuclear localization of NF-κB, which then

activates gene expression, necessary for the production of prostaglandins. In cells that are exposed to SA, degradation of IκB is inhibited, which prevents the nuclear translocation of NF-κB. Interestingly, IκB in animals has structural similarity with NPR1 (reviewed by Spoel and Dong, 2012). In plants, the cytosolic location of NPR1 is important for SA-mediated antagonism of JA-responsive gene expression (Spoel *et al.*, 2003; Stein *et al.*, 2008). One possible function for cytosolic NPR1 is that it may sequester JA-regulated transcriptional activators in the cytoplasm, thereby preventing them from moving to the nucleus and activating transcription. However, whether SA can interfere with translocation of JA-responsive transcription factors to the nucleus remains to be demonstrated.

In the nucleus, transcription factors can be prevented from binding DNA and thus activating gene expression by interacting with repressor proteins, which have been reported to function as important regulators in several hormone signaling pathways (Robert-Seilaniantz *et al.*, 2011). JAZ proteins in the JA pathway are examples of such repressors. JA-induced ubiquitination of JAZ proteins mediates their degradation via the 26S proteasome, which releases their repressive effect on positive transcriptional regulators. By increasing the stability of repressor proteins, hormones can antagonize another hormone's action. An example of this crosstalk mechanism is found in the SA-auxin interaction. Parallel to JAZ repressor proteins in the JA pathway, AUX-IAA proteins are the negative regulators that bind and inactivate activators of auxin signaling. Binding of auxin to F-box proteins TIR1 and TIR1-related proteins, which act as auxin receptors, leads to degradation of AUX-IAA repressors. SA was shown to inhibit the auxin signaling pathway through stabilization of AUX/IAA repressor proteins, probably indirectly through repression of TIR1. In this way, SA could lift the disease promoting effect of auxin in the infection of Arabidopsis by *Pseudomonas syringae* (Wang *et al.*, 2007). Also crosstalk between JA and GA pathways is regulated through interaction with their key repressor proteins, JAZs and DELLAs, respectively. In the absence of GA, stabilized DELLA can interact with JAZ proteins, thus reducing the repressive effect of JAZ on JA-responsive gene expression. DELLAs are degraded when GA levels rise, leading to enhanced suppression of JA signaling by JAZs (Hou *et al.*, 2010; Pieterse *et al.*, 2014a). On the other hand, JA delays GA-mediated degradation of DELLAs, which is associated with a reduction in growth, suggesting that the trade-off between JA-dependent defense and GA-dependent growth can be regulated by the DELLA-JAZ signaling module (Yang *et al.*, 2012). There is no evidence however, that SA interferes with the stability of JAZs to antagonize JA signaling. First, JAZ1 and JAZ9, two of the most important JAZ proteins, are still degraded in JA-treated Arabidopsis when plants are additionally treated with SA. Second, SA was shown to antagonize the JA signaling pathway downstream of COI1, the F-box protein that interacts with JAZ repressor proteins to target them for ubiquitination (Van der Does *et al.*, 2013).

SA-mediated degradation of JA-regulated transcription factors

SA-induced degradation of activating transcription factors of JA signaling could contribute to the repression of JA-responsive genes. SA was shown to lead to degradation of ORA59, a positive regulator in the ERF branch of the JA pathway. A whole-genome expression profiling analysis showed that the GCC-box was overrepresented in MeJA-induced genes that were antagonized by SA at 24h after treatment with a combination of the hormones. The GCC-box was subsequently shown to be sufficient for suppression by SA (Van der Does *et al.*, 2013). Similarly, the GCC-box was enriched in promoters of ethylene-induced genes that were suppressed by SA (Zander *et al.*, 2014). The GCC-box is an essential promoter element for activation of *PDF1.2* expression and ERF transcription factor ORA59 is an important regulator in this activation (Zarei *et al.*, 2011). Van der Does and co-workers suggested that downregulation of transcription of *ORA59* is not essential for SA/JA crosstalk, but showed that protein levels of ORA59 diminished after SA treatment (Van der Does *et al.*, 2013), suggesting that SA could target positive regulators in the JA pathway for degradation. Recently, application of egg extract of *Pieris brassicae*, which induces the SA pathway in Arabidopsis plants, was shown to lead to a reduction of protein levels of MYC2, MYC3 and MYC4 (Schmiesing *et al.*, 2016). This indicates that SA could target these transcription factors for degradation as well.

Phosphorylation of transcription factors influences transcription

Perception of pathogenic microbes by the plant leads to activation of mitogen-activated protein kinases (MPKs) that can subsequently phosphorylate transcriptional regulators. Phosphorylation of transcription factors influences gene transcription by changing the binding strength to DNA, or affecting sequestration or stability (Tena *et al.*, 2011). In particular MPK3, MPK4 and MPK6, which act at the last step of MAPK signaling cascades, are known to phosphorylate transcription factors and are implicated in immune signaling (Meng and Zhang, 2013). For example, upon phosphorylation of WRKY33 by MPK3 and MPK6, WRKY33 is able to activate expression of *WRKY33* itself, and can activate expression of camalexin biosynthesis genes, such as *PAD3* (Mao *et al.*, 2011). It has also been suggested that WRKY33 is controlled by sequestration in a complex with MKS1 and MPK4. Upon bacterial pathogen attack the activated MAPK signaling cascade phosphorylates MKS1, which leads to disassociation from MPK4 so that WRKY33 could bind to the promoter of *PAD3* (Qiu *et al.*, 2008).

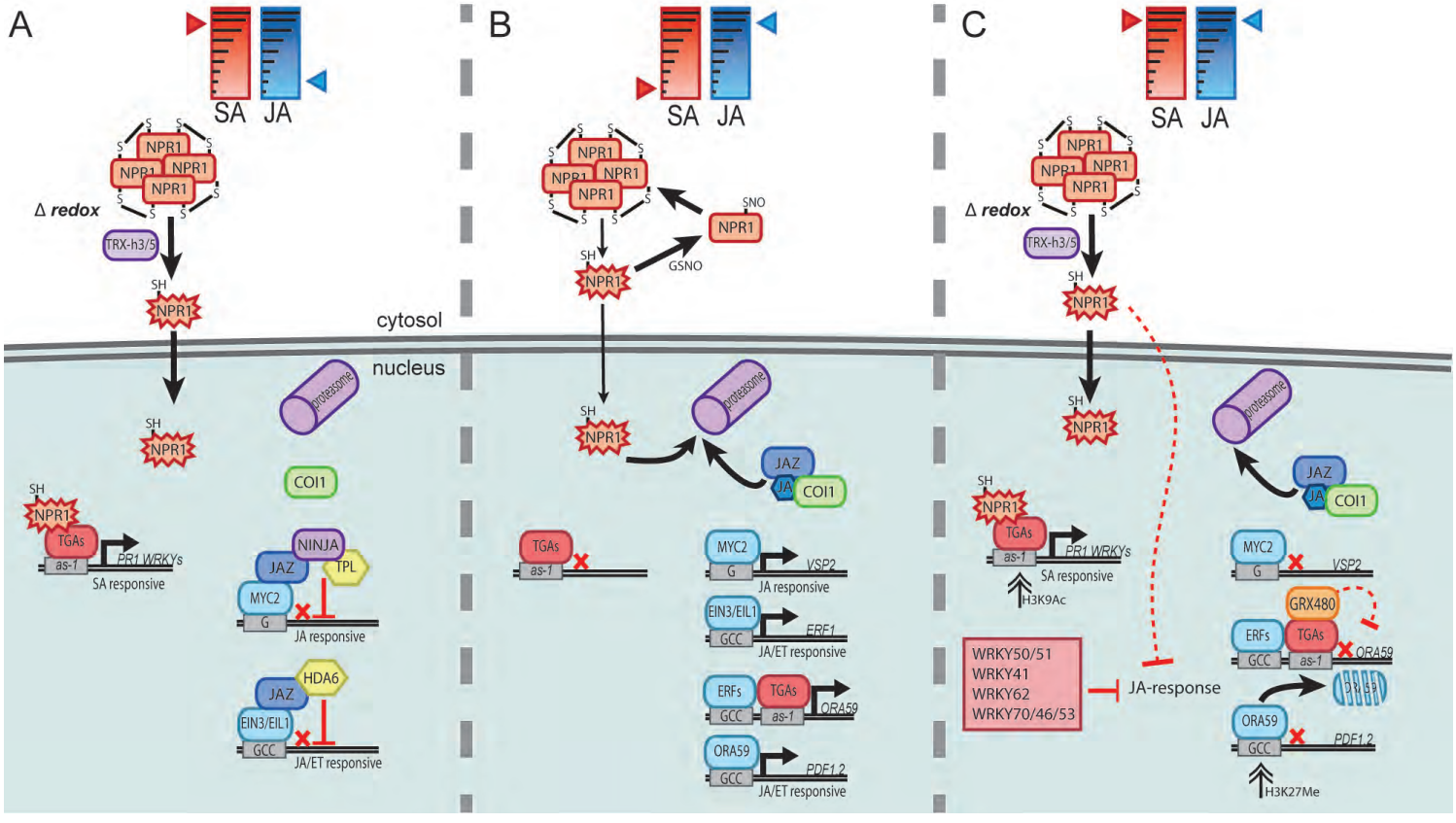

Figure 1: Simplified model of the molecular machinery involved in the transcriptional regulation of the SA signaling pathway (**A**), the JA signaling pathway (**B**), or the antagonism of SA on the JA signaling pathway (**C**). By inducing reduction and monomerization of NPR1, SA activates NPR1 (star-shaped), which then triggers gene expression in the nucleus. JA-responsive genes are kept in check by JAZ repressors in the absence of JA. MYC or ERF transcription factors activate JA-responsive genes in the presence of JA, but only if SA is absent. Activation of both the SA and JA signaling pathways leads to antagonism of JA-responsive gene expression by SA. There are indications for roles in SA/JA crosstalk for cytosolic NPR1, and nuclear localized TGAs, GRX480, and WRKYs. See text for details on the molecular processes underlying the transcriptional control, like redox signaling, sequestration, degradation, phosphorylation and chromatin modification. Solid lines indicate established (in)activities and dashed lines hypothesized (in)activities, where black arrows specify activation and red blocks suppression. Red crosses indicate that gene transcription is hampered.

There is not much known about the role of MAPK cascades in the interplay between different hormone pathways. MAPK cascades are important in the JA pathway, so inhibition of MAPK cascades by SA could be an effective way to antagonize JA signaling. For example, JA activates MPK6 and many AP2/ERF transcription factors are phosphorylated and activated by MPK6, among which positive regulators ERF6 and ERF104 (Takahashi *et al.*, 2007; Bethke *et al.*, 2009; Popescu *et al.*, 2009; Meng *et al.*, 2013). It is not known if SA can prevent this phosphorylation to inhibit activation of the JA-regulated AP2/ERF transcription factors. MPK4 was thought to function as an integrator of SA and JA signaling as the mutant *mpk4* constitutively expresses SA-inducible *PR* genes and fails to express *PDF1.2*, which correlates with enhanced resistance to biotrophic pathogens and increased susceptibility to necrotrophic pathogens (Petersen *et al.*, 2000; Brodersen *et al.*, 2006). However, recently it was suggested that MPK4 is guarded by the R protein SUMM2. Reduction of the kinase activity of MPK4 by the bacterial effector HopAI1 is monitored by SUMM2, and leads to activation of SA-dependent defense responses (Zhang *et al.*, 2012c). The effects of MPK4 on SA signaling are thus indirect, and this makes a role for MPK4 as an integrator of SA and JA signaling unlikely. However, whether MPK4's role in JA signaling is a direct or indirect one needs to be studied further.

SA-inducible expression of transcription factor genes that suppress JA responses

SA may also antagonize JA-inducible gene transcription by inducing the expression of genes encoding transcriptional regulators that interfere with JA signaling. These SA-induced regulators could inhibit a positive regulator of JA-inducible gene expression by interacting with it, as was described for the GRX480-TGA interaction above. Alternatively, SA could induce transcription of suppressive transcription factors that directly bind to the promoter of JA responsive genes to repress their expression. Examples of TGA, ERF, WRKY and bHLH transcription factors that are induced by SA and inhibit JA-dependent transcription are reviewed below.

TGA transcription factor family

TGA transcription factors have a role in various hormone-regulated transcriptional responses. They can generally activate SA-dependent gene expression, but are also known to have both positive and negative effects on JA/ethylene-dependent responses. TGA transcription factors are a class of bZIP transcription factors that bind to the *as-1* element (TGACG) in promoters. In Arabidopsis, ten TGAs exist of which several have been shown to interact with NPR1 (reviewed by Gatz, 2013). The *PR1* promoter contains an *as-1* element, and the triple mutant *tga2tga5tga6* is, like *npr1*, compromised in SAR and does not express *PR1* upon treatment with the

SA-mimic INA (Zhang *et al.*, 2003). In response to SA, a ternary complex of TGA, NPR1 and DNA is formed that can activate transcription of *PR1* (Fig. 1A). In non-induced conditions, suppression of *PR1* by TGAs has also been reported (Rochon *et al.*, 2006; Pape *et al.*, 2010). TGAs are important for activation of JA/ethylene-dependent genes as well. Although mutant *tga2tga5tga6* adult plants responded with *PDF1.2* induction upon treatment with JA, they did not express *PDF1.2* in response to ethylene or *B. cinerea* infection (Zander *et al.*, 2010).

In addition, TGAs can be essential for suppression of JA responsive genes by SA, as JA-induced *PDF1.2* is not suppressed after a combination treatment with SA in mutant *tga2tga3tga5tga6* (Leon-Reyes *et al.*, 2010a). Microarray analysis comparing wild-type and *tga2tga5tga6* mutant plants showed that after treatment with ethylene precursor ACC, 374 genes were induced in wild-type plants, of which 136 were dependent on TGA2/TGA5/TGA6. Half of these ACC-inducible TGA-dependent genes were, in wild-type plants, suppressed by SA after a combination treatment of ACC with SA. This suggests a role for TGAs in both activation of ethylene-responsive genes and SA-mediated repression of these genes (Zander *et al.*, 2014). The *PDF1.2* promoter contains an *as-1* element, but this was shown not to be important for the antagonistic effect on JA-induced *PDF1.2* expression by SA (Spoel *et al.*, 2003). However, Zander *et al.* (2014) showed that the TGAs directly target the *as-1* element in the promoter of *ORA59* and could regulate both induction of *ORA59* by ACC treatment and suppression of *ORA59* by SA (Fig. 1C). Transcriptional regulation of *ORA59* by TGAs is in line with the observation that the GCC-box is enriched in the promoter elements of ACC-induced, SA-suppressed genes. How can TGA factors act as both activators and repressors in different hormone signaling pathways? Possibly, different co-factors can be recruited to TGA factors depending on both the promoter context and the hormonal context. In the case of activation of transcription by SA, TGAs have been shown to interact with transcriptional activators NPR1 and GRAS protein SLC14 (Rochon *et al.*, 2006; Fode *et al.*, 2008). Upon JA accumulation, TGAs may interact with so-far unknown JA signaling regulators to promote JA responsive gene expression. When SA/JA crosstalk is activated, SA induces glutaredoxins, which could interact with TGAs on the *ORA59* promoter leading to repression of JA-inducible genes (Fig. 1C). Glutaredoxins were shown to down-regulate *ORA59* expression in a TGA-dependent manner, as discussed above (Zander *et al.*, 2012).

Both Zander *et al.* (2014) and Van der Does *et al.* (2013) point to ORA59 as a major target of antagonism by SA. However, while the first show that SA targets expression of *ORA59*, the protein levels of ORA59 were shown to be influenced by SA by the latter. The apparent discrepancy between these two studies could partly be explained by the different combination of hormones that both groups studied, SA-ethylene or SA-JA, respectively. Support for differences in crosstalk

mechanisms depending on hormonal context comes from the observation that in an ethylene-rich environment the SA-antagonized expression of JA-inducible *PDF1.2* became independent of NPR1 (Leon-Reyes *et al.*, 2009) or was even completely impaired when plant tissue was exposed to high levels of ethylene prior to treatment with SA (Leon-Reyes *et al.*, 2010a). However, it is very well possible that ORA59 is regulated by SA at both the transcriptional and post-translational level, and that both mechanisms complement each other (Fig. 1C).

ERF transcription factor family

Transcription factors of the ERF subfamily of AP2/ERF family of transcription factors can bind the GCC-box. Several ERFs have been implicated in plant defense signaling (Huang *et al.*, 2015). They can act as activators of transcription, such as ORA59, but also as repressors. Fourteen of the 122 ERFs in Arabidopsis contain an EAR domain, which is an active repressor domain that interacts with the general co-repressor TPL (Nakano *et al.*, 2006). EAR-domain-containing ERF4 and ERF9 were shown to be able to suppress *PDF1.2* expression (McGrath *et al.*, 2005; Maruyama *et al.*, 2009). Because of the importance of the GCC-box in SA/JA crosstalk, the suppression of JA-responsive genes may, besides through negative regulation of ORA59 by SA as described before, in part be regulated by suppressive SA-induced ERFs. This hypothesis has up to now not been tested.

WRKY transcription factor family

WRKY transcription factors are foremost known for their inducibility by SA and pathogens, and their role in regulating SA-dependent gene expression. There are, however, also examples of WRKYs that positively regulate other hormone-regulated genes, including JA-responsive defense genes (Journot-Catalino *et al.*, 2006; Xu *et al.*, 2006; Birkenbihl *et al.*, 2012). The W-box (C/TTGACC/T) is a DNA element that is bound by WRKY transcription factors (Eulgem and Somssich, 2007). Importantly, the W-box motif was reported to be enriched in JA-responsive genes that were antagonized by SA (Van der Does *et al.*, 2013), suggesting the involvement of WRKYs in SA/JA crosstalk as well. Indeed, several WRKYs have been implicated in suppression of JA-induced *PDF1.2* expression (Fig. 1C). Overexpression of SA-induced WRKY70 suppressed MeJA-induced *PDF1.2* expression (Li *et al.*, 2004; Li *et al.*, 2006). However, in a *wrky70* mutant, JA-dependent genes were induced by JA and suppressed by the combination treatment, indicating that WRKY70 is sufficient but not required for SA/JA crosstalk (Ren *et al.*, 2008; Leon-Reyes *et al.*, 2010a). Redundancy of different WRKYs could possibly explain the lack of a crosstalk phenotype of the single *wrky70* mutant, as double and triple mutants of *wrky70* with *wrky46* and *wrky53* did show enhanced *PDF1.2* expression after MeJA treatment

(Hu *et al.*, 2012). Overexpression of the transcription factor MYB44 also led to suppression of the JA marker genes *VSP1* and *PDF1.2*, which is likely established through activation of *WRKY70*. MYB44 is inducible by SA and binds to the *WRKY70* promoter leading to its expression (Shim *et al.*, 2013; Zou *et al.*, 2013). Furthermore, WRKY62 was suggested to function in suppression of JA responses, because a *wrky62* mutant displayed enhanced expression of JA responsive genes, while an overexpressor exhibited reduced expression. *WRKY62* is induced by SA and was suggested to act downstream of cytosolic NPR1 (Mao *et al.*, 2007). To end, WRKY41 has been implicated in suppression of JA responsiveness, since overexpression of WRKY41 led to increased *PR5* and reduced *PDF1.2* expression. However, in contrast to the aforementioned *WRKY* genes, *WRKY41* is likely not a direct target of NPR1 and SA only slightly induces *WRKY41* expression (Higashi *et al.*, 2008).

Studies on the *ssi2* mutant revealed two other WRKYs that are involved in SA/JA crosstalk. The *ssi2* mutant was initially identified in a screen for *npr1* suppressors and displays high SA responses while JA responses are repressed (Shah *et al.*, 2001). The increased SA levels were not needed for the repression of JA responses, but instead lowered levels of 18:1 fatty acids appeared to regulate the repression of JA signaling (Kachroo *et al.*, 2001; Kachroo *et al.*, 2003; Nandi *et al.*, 2005). In *ssi2* mutants, 19 *WRKYs* were induced, of which five in a SA-independent manner. Double mutants of *ssi2* with *wrky50* or *wrky51* restored the induction of *PDF1.2* and resistance against *B. cinerea* without altering the 18:1 fatty acid levels. WRKY50 and WRKY51 thus negatively regulate JA responses under low 18:1 conditions. Single and double mutants of *wrky50* and *wrky51* also failed to suppress *PDF1.2* and *VSP2* after a combination treatment with SA and JA (Gao *et al.*, 2011). Therefore, these two WRKYs seem to play important roles in the suppression of JA responses.

How can WRKY transcription factors repress JA responses? After their induction by SA, they could bind to W-boxes in JA-responsive genes to inhibit their expression directly (Van der Does *et al.*, 2013), or they could repress JA-responsive genes indirectly. There is no experimental proof of either repressive mechanism under the influence of SA yet, but recently WRKY51 has been reported to interact with JAV1, a VQ-motif containing protein that negatively regulates JA responses and acts in the nucleus (Hu *et al.*, 2013).

bHLH transcription factor family

Transcription factors of the bHLH family, including MYC2, play crucial roles in the JA signaling pathway. MYC2 is a positive master regulator of JA responses (reviewed by Kazan and Manners, 2013). Recent years have witnessed an boost in bHLHs that function as negative regulators in the JA signaling pathway (Nakata *et al.*, 2013; Sasaki-Sekimoto *et al.*, 2013; Song *et al.*, 2013; Fonseca *et al.*, 2014). However, it

is unlikely that these repressive bHLHs are manipulated by SA to establish SA/JA crosstalk. First, they are not obviously regulated at the transcription level by SA (BAR public database), and recently, wound-induced *VSP2* expression was shown to be still suppressed by SA-inducing egg extract in the quadruple mutant of four repressive bHLH transcription factors (Schmiesing *et al.*, 2016).

SA/JA crosstalk could be enforced by chromatin modification at target genes

SA can further control gene expression by remodeling of chromatin around target genes. Chromatin is the complex of DNA and histones and its condensed structure can reduce accessibility of DNA and thus inhibit transcription. Modifications of chromatin can result in local loosening of this structure, which creates access for transcriptional machinery and regulatory proteins to the DNA. Chromatin modifications include methylation, acetylation, phosphorylation, ubiquitination or sumoylation of histones (Iwasaki and Paszkowski, 2014). Acetylation of histones is associated with activation of genes, while deacetylation of histones is correlated with gene repression. Enzymes called histone acyltransferases and histone deacetylases (HDA) can carry out these respective histone modifications (Liu *et al.*, 2014). Both HDA6 and HDA19 were described to interfere with JA signaling. HDA6 interacts with JAZ1, JAZ3 and JAZ9 and is recruited to repress EIN3/EIL1-dependent transcription (Zhu *et al.*, 2011). In contrast, HDA19 was reported to have a positive role in the ERF branch and in defense against *A. brassicicola* (Zhou *et al.*, 2005). HDA19 also targets SA signaling by binding to the *PR1* and *PR2* promoters leading to their repression (Choi *et al.*, 2012), and by reducing transcriptional activity of WRKY38 and WRKY62 (Kim *et al.*, 2008). Since chromatin remodeling plays an important role in SA and JA signaling, it could also well be manipulated by SA to antagonize JA signaling. However, Koornneef and colleagues (2008b) showed that at the *PDF1.2* promoter there was no change in acetylation of histones after exogenous application of a combination of SA and MeJA.

Chromatin modifications are also described to be an important mechanism to prime plants for enhanced defense (Conrath, 2011). Interestingly, it was suggested that priming and SA/JA crosstalk could be carried over to offspring through acetylation and methylation of histones at different promoter sites as well. Luna *et al.* (2012) showed that Arabidopsis plants that were inoculated with the bacterial pathogen *P. syringae* in the first generation, were more resistant to *P. syringae* and the oomycete pathogen *H. arabidopsidis* in the next generation, and more susceptible to the necrotrophic pathogen *A. brassicicola*. This correlated with increased *PR1* expression and reduced *VSP2* and *PDF1.2* expression in the second generation and was dependent on NPR1. Acetylation of histone H3 at Lys-9 (H3K9) at the *PR1* promoter, which is associated with increased transcription, was enhanced in these plants. Conversely,

tri-methylation of H3K27, which is associated with transcriptional silencing, was enriched at the *PDF1.2* promoter (Fig. 1C), suggesting that histone modifications were responsible for the observed increased or decreased transcription (Luna *et al.*, 2012). It is not clear yet how these changes can be transmitted to offspring, since there is no evidence that histone modifications are inherited. DNA methylation, which is often associated with histone modifications, is a possible modification that could be passed on to next generations. DNA methylation was shown to have an effect on SA- and JA-regulated responses: epiRIL lines, which are identical at the DNA sequence level but highly variable at the level of DNA methylation, showed differences in responsiveness to both treatments (Latzel *et al.*, 2012).

Rewiring of hormone-regulated transcription by pathogens

In the evolutionary arms race, pathogens have evolved effectors that are secreted into plant cells upon infection to reduce disease resistance or increase plant susceptibility (reviewed by Kazan and Lyons, 2014). Interestingly, several pathogen effectors can highjack a plant's intricate hormonal crosstalk mechanism for their own good, resulting in lower induction of effective defenses. Some effectors are hormones themselves or are hormone-mimics that disturb the hormone balance in plants. The most famous example of such an effector is the JA-mimic coronatine, that is secreted by *Pseudomonas* pathogens and suppresses SA signaling (Zheng *et al.*, 2012). More recently, effectors that interfere with signaling hubs in transcriptional regulation of JA signaling, such as JAZs, have been discovered. Effectors HopZ1a and HopX1 of two different *Pseudomonas* pathogen strains bind to and degrade JAZ repressor proteins, leading to activation of JA signaling and concomitant suppression of SA-regulated defense signaling (Jiang *et al.*, 2013; Gimenez-Ibanez *et al.*, 2014).

Other effectors can establish antagonism of SA signaling by manipulating the plant transcriptional machinery via interference with Mediator subunits. Mediator is a multi-protein transcriptional co-activator complex, which functions as a bridge between transcription factors and RNA polymerase II. Mediator recruits RNA polymerase II to promoters in response to different signals and controls the polymerase activity during transcription initiation and elongation (Conaway and Conaway, 2011). Several Mediator subunits have been implicated in SA- and/or JA-dependent gene expression. Mediator subunit MED16 was shown was shown to be important in defense against both biotrophic and necrotrophic pathogens by regulating SA- and JA/ethylene-responsive transcription and could therefore be viewed as a node of convergence between SA- and JA/ethylene-dependent pathways (Wathugala *et al.*, 2012; Zhang *et al.*, 2012b). Subunit MED25 was shown to be important for activation of JA-dependent genes, and likely acts through interaction with JA-responsive transcription factors, including ERF1, ORA59 and

MYC2 (Çevik *et al.*, 2012). The subunit MED19 positively regulates SA-dependent resistance that is effective against *H. arabidopsidis.* MED19 was shown to be targeted for degradation by the *H. arabidopsidis* effector HaRxL44. Expression of HaRxL44 in plants led to induction of JA-responsive genes, a response that is observed in *med19* plants as well (Caillaud *et al.*, 2013). These data suggest that HaRxL44 induces degradation of MED19 to rewire transcription from SA-responsive to JA-responsive, leading to enhanced infection by *H. arabidopsidis*. This example illustrates the highly sophisticated manner in which effectors manipulate the plant transcriptional machinery to influence hormonal signaling.

OUTLINE OF THE THESIS

Regulation of hormone homeostasis and crosstalk between hormonal signaling pathways are essential for the plant to control trade-offs between growth and defense and fine-tune its defenses (Vos *et al.*, 2013a). In this review we focused on the molecular mechanisms (potentially) underlying antagonistic effects of SA on JA-mediated transcriptional responses and highlighted several transcriptional regulators (such as NPR1, TGA, WRKY and ORA59) as signal integrators. Although knowledge on the molecular players in both SA and JA-signaling pathways is increasing, our understanding of the molecular mechanisms of SA/JA crosstalk is still limited. Important outstanding questions are: what is the role of redox signaling in suppression of JA responses and how does SA-activated NPR1 suppress JA-responsive gene expression? Furthermore, how does SA target positive transcription factors in the JA-pathway, and do SA-induced transcriptional repressors also play a role? The main goal of the research described in this thesis was to unravel the molecular mechanism(s) underlying SA-mediated suppression of JA-responsive gene expression. Furthermore, we investigated another mechanism of suppression of the JA pathway by identifying novel JA-inactivating enzymes.

In Chapter 2, we show that SA-mediated suppression of *PDF1.2* expression requires *de novo* protein synthesis. As the GCC-box was described before to be a central target of SA/JA crosstalk, we searched for these novel synthesized factors in the family of GCC-box binding ERF transcription factors. We selected SA-induced *ERF*s and ERFs that contain an EAR repression domain for further investigation. In total, seventeen *erf* mutants were selected and were tested for SA-mediated suppression of JA-responsive genes. Knock-outs in several *ERFs* result in increased *PDF1.2* and *VSP2* expression, showing that we identify ERFs that have a role in suppression of JA-dependent signaling. However, SA-mediated suppression of *PDF1.2* and *VSP2* was unaffected in all mutants. We also show that co-repressor TPL, which represses JA-responsive genes in conditions with low JA, is not required for suppression of

PDF1.2 after SA treatment. These results together show that it is unlikely that ERF repressor proteins or co-repressor TPL play a role in SA-mediated suppression of JA-responsive gene expression, thereby invalidating an important hypothesis in SA/JA crosstalk research.

In Chapter 3, we investigate the role of NPR1 in SA-mediated suppression of JA-induced gene expression. Suppression by SA of a subset of SA-antagonized JA-inducible genes was shown to be dependent on NPR1, and the GCC-box was enriched in the promoters of these genes. Next, natural variation of the NPR1 protein was studied in a set of Arabidopsis accessions that differed in their ability to display SA/JA crosstalk. Several mutants in redox transmission and mutants that change the location, activity or stability of NPR1 were then tested for suppression of JA-induced gene expression by SA. In this way, we show that redox regulation of the NPR1 protein and its degradation by an interaction with NPR3 and NPR4 are likely not essential for SA/JA crosstalk. In contrast, nuclear localization of NPR1 is required for SA-mediated suppression of *PDF1.2* and *VSP2* expression in adult plants. We further demonstrate that two lines overexpressing cysteine mutated (Cys^{82} or Cys^{216}) versions of the NPR1 protein, C82A and C216A, in the *npr1-1* background, are not impaired in SA-induced *PR1* expression, but are compromised in the SA-mediated suppression of JA marker genes. This allowed us to dissect the function of NPR1 in SA-responsive *PR1* gene expression from that in SA-mediated suppression of *PDF1.2* and *VSP2*. We hypothesized that the cysteine-mutated NPR1 was disrupted in part of the NPR1-regulated SA-induced transcriptome that is important for SA/JA crosstalk. By performing RNA-seq, we identify 32 genes that were induced by SA in Col-0 in an NPR1-dependent manner and lower expressed in C82A compared to Col-0. These genes, which included four *WRKY* genes that are direct targets of NPR1, may be investigated for a role in SA/JA crosstalk. Our data thus suggest that to suppress JA-responsive gene expression in adult plants, NPR1 is required to localize to the nucleus and activate WRKY targets, which may function in the suppression of JA-responsive genes.

In Chapter 4, we describe the identification of four 2-oxoglutarate/Fe(II)-dependent oxygenases that negatively regulate JA responses by hydroxylation and inactivation of the JA molecule. An inactive form of JA, 12-OH-JA, has been described in several species, and accumulates after activation of the JA pathway. However, the enzyme converting JA into this molecule was so far not known. As SA, GA and auxin have been shown to be hydroxylated by members of the 2OG-oxygenase family, we investigate the JA-inducibility of 50 members of this family and found that there is a clade of four proteins whose genes are induced by JA, which we named JASMONATE-INDUCED OXYGENASEs (JOXs). Knockdown of these four genes in the quadruple mutant *jox1jox2jox3jox4* resulted in phenotypes indicative

of enhanced JA responses, including increased resistance to *B. cinerea* and *Mamestra brassicae*, higher expression of JA-dependent defense-related genes, and inhibition of root and shoot growth. We next showed that this quadruple mutant accumulates JA, and is unable to turn over JA into 12-OH-JA when plants were treated with JA. In contrast, the overexpression line of *JOX1* accumulated more 12-OH-JA after JA treatment. The research described in this chapter identifies the enzymes responsible for JA hydroxylation and shows that inactivation of JA is important to keep a good balance of defense and growth.

In Chapter 5, the results presented in this thesis are discussed in the context of the current knowledge of defense hormone signaling networks in Arabidopsis.

CHAPTER 2

Assessing the role of ERF transcriptional repressors in salicylic acid-mediated suppression of jasmonic acid-responsive genes

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Submitted.

ABSTRACT

Salicylic acid (SA) and jasmonic acid (JA) cross-communicate in the plant immune signaling network to precisely regulate induced defenses against microbial pathogens and insect herbivores. In Arabidopsis, SA antagonizes a large set of JA-responsive genes, partly by targeting the ERF-type transcriptional activator ORA59. Members of the ERF transcription factor family typically bind to GCC-box motifs in the promoters of JA- and ethylene-responsive genes, thereby positively or negatively regulating their expression. The GCC-box motif is sufficient for SA-mediated suppression of JA-responsive gene expression. Suppressors of transcriptional activity have been shown to play central roles in attuning hormonal signaling. Here, we investigated whether SA-induced ERF-type transcriptional repressors, which may compete with JA-induced ERF-type activators for binding at the GCC-box, play a role in the SA/JA antagonism. We identified a group of SA-inducible ERFs, some of which possess an EAR transcriptional repressor motif, as putative negative regulators of JA-responsive gene expression. We identify several ERFs that have a role in suppression of JA-dependent signaling, as revealed by a higher JA-induced *PDF1.2* or *VSP2* expression in the corresponding *erf* mutant. However, SA-mediated suppression of these genes was not impaired in any of the mutants, showing the transcription factors are not required for SA-mediated suppression of JA-induced gene expression. Moreover, a mutant in the general co-repressor TOPLESS, which interacts with EAR-domains, also was not affected in SA-mediated antagonism of *PDF1.2* and *VSP2*. Collectively, these results suggest that SA-regulated ERF transcriptional repressors are not required for antagonism of JA-responsive gene expression by SA. We further show that *de novo* SA-induced protein synthesis is required for suppression of JA-induced *PDF1.2*, pointing to a SA-stimulated production of an as yet unknown protein that suppresses JA-induced transcription.

INTRODUCTION

Plants intimately interact with a broad range of microbial pathogens and insect herbivores. To respond to this diversity of enemies, plants possess a highly sophisticated defense system in which the plant hormones salicylic acid (SA) and jasmonic acid (JA) play important regulatory roles. Other hormones, such as ethylene, abscisic acid, gibberellins, auxins, and cytokinins also have an effect on plant immunity, often via the modulation of the SA and JA signaling pathways (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012; Broekgaarden *et al.*, 2015). Although there are exceptions, SA-dependent defenses are generally considered to act against pathogens with a biotrophic lifestyle, whereas JA-dependent responses are often associated with defense against necrotrophic pathogens and herbivorous insects (Pieterse *et al.*, 2012). In response to different types or combinations of attackers, the plant produces specific blends of hormones that differ in composition, quantity and timing, which is instrumental in fine-tuning the induced defense response against the invading attacker (De Vos *et al.*, 2005).

Cross-communication between the SA and JA signaling pathways emerged as an important mechanism by which plants steer their induced defense responses and may reduce defense-associated fitness costs (Pieterse *et al.*, 2012; Vos *et al.*, 2013a; Vos *et al.*, 2015). Transcriptome profiling studies revealed extensive interplay between the two pathways, with antagonistic effects of SA on JA-responsive gene expression being most prominent (Glazebrook *et al.*, 2003; Van Verk *et al.*, 2011; Van der Does *et al.*, 2013). In *Arabidopsis thaliana* (Arabidopsis), activation of the SA pathway suppresses a large set of JA-responsive genes, including the JA marker genes *PLANT DEFENSIN1.2* (*PDF1.2*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Van Wees *et al.*, 1999; Van der Does *et al.*, 2013). Consequently, activation of the SA pathway diminishes JA-dependent defenses against necrotrophic pathogens and insect herbivores (reviewed in Pieterse *et al.*, 2012).

In order to study the mechanisms underlying the antagonistic effect of SA on JA-responsive gene expression (hereafter also referred to as SA/JA crosstalk), knowledge of the JA pathway that accumulated over the past recent years is highly instrumental. The F-box protein CORONATINE INSENSITIVE1 (COI1) was found to be an indispensable component of the JA signaling pathway (Devoto *et al.*, 2002). As part of the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI1}, COI1 interacts with JASMONATE ZIM-domain (JAZ) proteins to form a complex that functions as a receptor for JA-Ile, the most bioactive derivative of JA (Fonseca *et al.*, 2009). Binding of JA-Ile to the JAZ-SCF^{COI1}-receptor complex leads to degradation of JAZ via the proteasome, resulting in the onset of the JA response (Chini *et al.*, 2007; Thines *et al.*, 2007). In the absence of JAs, JAZ proteins act as transcriptional repressors

of JA-responsive genes by binding to positive transcriptional regulators, such as MYC2, 3 and 4 (Chini *et al.*, 2007; Fernández-Calvo *et al.*, 2011) and ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1) (Zhu *et al.*, 2011). To prevent activity of their bound transcription factors, JAZ proteins recruit the general co-repressor TOPLESS (TPL) and TOPLESS-Related (TPR) proteins either directly if they contain an EAR-motif or indirectly via the adaptor protein NINJA that contains an EAR-motif (Pauwels *et al.*, 2010; Shyu *et al.*, 2012). In JA-stimulated cells, degradation of JAZ proteins results in the release of transcription factors, leading to activation of a large set of JA-responsive genes, including JA marker gene *VSP2* and genes encoding APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors, such as ERF1 and OCTADECANOID-RESPONSIVE ARABIDOPSIS59 (ORA59) that lead to activation of the JA marker gene *PDF1.2* (Lorenzo *et al.*, 2003; Pré *et al.*, 2008). Besides transcription factors that act positively on transcription of JA-responsive genes there are also numerous repressive transcription factors that can inhibit transcription of JA-inducible genes such as *VSP2* and *PDF1.2* (McGrath *et al.*, 2005; Nakata *et al.*, 2013; Caarls *et al.*, 2015).

In Arabidopsis, significant progress has been made in the identification of targets in the JA pathway via which SA exerts its antagonistic effect (Pieterse *et al.*, 2012; Caarls *et al.*, 2015). SA has been shown to suppress the JA pathway downstream of JA biosynthesis and the JAZ-SCF^{COI1} complex, suggesting that SA antagonizes JA signaling at the level of transcriptional regulation (Leon-Reyes *et al.*, 2010b; Van der Does *et al.*, 2013). Whole-genome expression profiling revealed that the GCC-box motif (AGCCGCC) is an important promoter element in JA-responsive genes that are sensitive to suppression by SA (Van der Does *et al.*, 2013). Using a synthetic GCC-box-containing promoter fused to a reporter gene, the GCC-box was demonstrated to be sufficient for SA-mediated suppression of JA-induced expression (Van der Does *et al.*, 2013). The GCC-box is a binding site for members of the ERF family of AP2/ERF transcription factors (Hao *et al.*, 1998), which comprises 122 members in Arabidopsis (Nakano *et al.*, 2006) and has been grouped into the DREB and the ERF subfamilies based on homology in their DNA binding domains (Sakuma *et al.*, 2002). In the *PDF1.2* promoter, the GCC-box is essential for JA/ethylene-induced activation (Zarei *et al.*, 2011). The ERF transcription factor ORA59, which binds to the GCC-box and acts as an activator of *PDF1.2* (Pré *et al.*, 2008; Zarei *et al.*, 2011), was then shown to be a target of SA, as both *ORA59* transcription and ORA59 protein accumulation were affected by SA (Van der Does *et al.*, 2013; Zander *et al.*, 2014).

Besides transcriptional activators, the ERF family of transcription factors also harbors transcriptional repressors that act at the GCC-box cis-regulatory element (Fujimoto *et al.*, 2000; McGrath *et al.*, 2005; Yang *et al.*, 2005; Huang *et al.*, 2015). In

Arabidopsis, 14 of the 122 members of the ERF subfamily of AP2/ERF transcription factors contain an EAR motif (Ohta *et al.*, 2001; Nakano *et al.*, 2006). This motif interacts with general co-repressors such as TPL, which is involved in repression of genes that are responsive to JA and auxin (Szemenyei *et al.*, 2008; Pauwels *et al.*, 2010; Kagale and Rozwadowski, 2011). In addition, also ERFs that lack an EAR motif can have a role in transcriptional repression, for example through interaction with a repressor such as SILENCER ELEMENT BINDING FACTOR (SEBP), as was shown for the ERF Pti4 of potato (González-Lamothe *et al.*, 2008), or through activation of a negative regulator of the GCC-box (Caarls *et al.*, 2015). Several *ERF* genes are SA inducible (Krishnaswamy *et al.*, 2011). This led us to hypothesize that induction by SA of ERF repressors that act at the GCC-box could contribute to the antagonistic effect of SA on JA-responsive gene expression. To test this hypothesis, we selected and tested 17 loss-of-function *erf* mutants for their ability to display SA-mediated suppression of *PDF1.2* and *VSP2* expression. Moreover, we analyzed whether the *tpl-1* mutant is impaired in SA/JA crosstalk. By using the protein synthesis inhibitor cycloheximide (CHX), we also tested whether antagonism by SA requires *de novo* protein synthesis. Together, our results suggest that although *de novo* synthesis of a yet unknown protein is required for SA-mediated suppression of JA-responsive gene expression, a role for SA-induced ERF repressor proteins in the antagonism between SA and JA signaling is unlikely.

RESULTS

De novo protein synthesis is required for SA/JA crosstalk

If our hypothesis that JA-induced gene expression can be antagonized by SA-induced *ERF* transcriptional repressors is correct, then novel protein synthesis of these ERFs upon SA treatment is expected to be prerequisite for SA/JA crosstalk. To investigate this, the effect of the protein synthesis inhibitor CHX on the expression of JA-inducible *PDF1.2* was determined in Arabidopsis Col-0 wild-type plants after treatment with MeJA, SA, or a combination of both. *PDF1.2* was chosen as a read-out because its promoter region harbors two GCC-boxes that the ERF suppressors could potentially target. Plants were first treated with MeJA for 24 h, then with CHX, and 20 minutes later with SA (Fig. 1A). Six h after SA treatment, leaves were harvested for gene expression analysis. Figure 1B shows that in the absence of CHX, *PDF1.2* expression was induced by MeJA, and subsequent treatment with SA suppressed this induction by more than 2 fold. This confirms previous findings that SA antagonizes JA-induced *PDF1.2*, even when SA is applied after the induction of the JA response (Koornneef *et al.*, 2008a). Application of CHX 24 h after MeJA

treatment strongly reduced *PDF1.2* mRNA accumulation in comparison to plants that were not treated with CHX, showing that *de novo* protein synthesis is important for activation of *PDF1.2* expression by MeJA (Fig. 1B). However, a statistically significant 4-fold induction of *PDF1.2* expression was still detectable in MeJA/CHX-treated plants, which was likely mediated by the residual pool of transcriptional activators that was already present before the CHX treatment. Importantly, the level of MeJA-induced *PDF1.2* mRNAs that remained after the CHX treatment was no longer reduced by SA if CHX was present, and instead was even higher in the SA/JA combination treatment than in the MeJA treatment alone (Fig. 1B). In plants that received no CHX, SA activated the expression of the SA-responsive marker gene *PR1* both in the absence and the presence of MeJA (Fig. 1B). CHX treatment completely abolished the induction of *PR1* by SA, confirming previous findings (Uquillas *et al.*, 2004). Together, these results indicate that, similar to the SA-induced expression of *PR1, de novo* protein synthesis is required for the SA-mediated suppression of JA-induced *PDF1.2* expression.

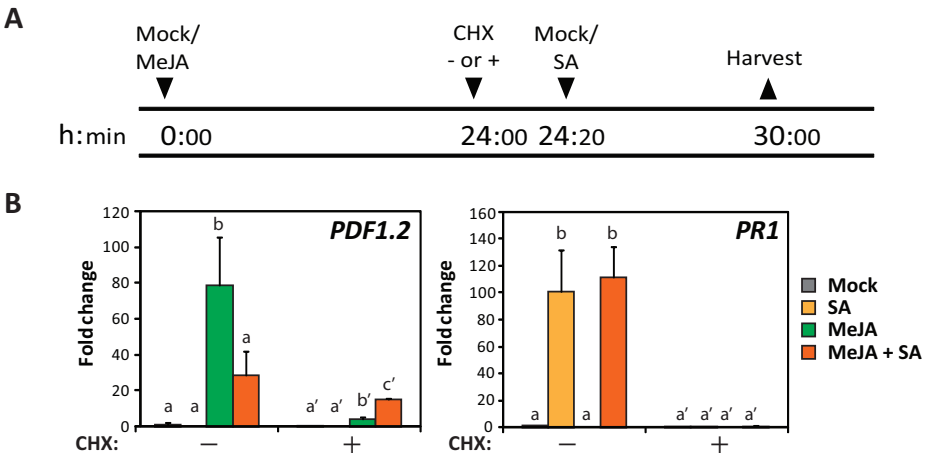


Figure 1: SA/JA crosstalk requires *de novo* protein synthesis. (A) Time line of different treatments. Five-week-old wild-type Col-0 plants were treated with 0.1 mM MeJA or a mock solution, CHX was applied 24 h later, and after a subsequent 20 min 0.5 mM SA was applied. Six h after CHX treatment, plant material was harvested for gene expression analysis. **(B)** qRT-PCR analysis of *PDF1.2* and *PR1* gene expression in Col-0 plants with MeJA, SA or MeJA followed by SA treatment. All treatments were combined with or without CHX. Fold change is relative to the expression in mock-treated plants without CHX, and normalized to the reference gene *At1g13320*. Shown are the averages of three independent biological replicas; error bars indicate standard deviations (SD). Different letters indicate statistically significant differences between hormone treatments of the –CHX or +CHX-treated plants (ANOVA, Holm-Sidak post-hoc test; $P \leq 0.05$). –, without CHX, +, with CHX.

SA signaling induces *ERF* transcription factor genes

Previously, we showed that the GCC-box promoter element is sufficient for SA-mediated suppression of JA-induced gene expression (Van der Does *et al.*, 2013). The GCC-box is a binding site for ERF-type transcription factors (Hao *et al.*, 1998), some of which can act as transcriptional repressors. Here, we tested if putative SA-inducible ERF transcriptional repressors could play a role in SA/JA crosstalk. First, we analyzed SA-responsiveness of the genes encoding the 122 members of the ERF family of AP2/ERF superfamily of transcription factors (Sakuma *et al.*, 2002; Nakano *et al.*, 2006). To this end, we mined publically available data on gene expression in Arabidopsis plants treated with SA, the SA analog benzo-(1,2,3)-thiadiazole-7-carbothioic acid (BTH), or the SA-inducing biotrophic pathogen *Hyaloperonospora arabidopsidis* (Supplemental Table S1; Atallah, 2005; Wang *et al.*, 2006; Krinke *et al.*, 2007; Goda *et al.*, 2008; Blanco *et al.*, 2009; Huibers *et al.*, 2009). Additionally, we analyzed the expression pattern of the 122 members of the ERF family by RNA sequencing of a high-density time series (14 time points) of SA-treated Arabidopsis plants (Supplemental Table S1). We selected putative SA-inducible ERF repressors in the following manner. First, the *ERFs* of which the expression was induced by one or more SA inducers (fold change $\geq$ 1.5 (public datasets) or $P \leq 0.05$ (RNA-seq data) in at least three different datasets were selected for further study (marked blue in Supplemental Table S1). This group comprised the following 10 ERF transcription factor genes: *AtERF-1*, *CEJ1/DEAR1, DREB2A, ERF1, ERF2, ERF11*, *ERF13*, *ERF112*, *RAP2.6* and *RAP2.6L*. Then, three additional *ERFs* were selected as interesting candidates, even though their expression was induced in only one or two of the datasets (indicated in orange in Supplemental Table S1): *ERF5* was reported to be induced by the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* in an SA-dependent and COI1-independent manner (Chen *et al.*, 2002), *ERF104* was described to play a role in the regulation of *PDF1.2* expression (Bethke *et al.*, 2009) and *HRE2* was relatively strongly induced by SA (fold change 9.0) in one of the selected studies.

Because of their putative function as transcriptional repressors (Ohta *et al.*, 2001), seven additional genes encoding EAR-motif-containing ERFs of the ERF subfamily (Nakano *et al.*, 2006), namely *ERF3*, *ERF4*, *ERF7*, *ERF8*, *ERF9*, *ERF10* and *ERF12*, were also considered as potential SA/JA crosstalk regulators (indicated in green in Supplemental Table S2). They all showed SA-induced expression in at least one of the datasets. *ERF11* also encodes an EAR-motif containing ERF, but was already selected because its expression was induced by SA in three different datasets. In total, out of the 122 members of the ERF family, we selected 20 genes for further study (Table 1).

Selection of ERFs with a potential role in SA/JA crosstalk

To determine the time-frame in which putative SA-activated transcriptional repressors need to act in order to suppress JA-induced gene expression, a time course experiment was performed in which *PDF1.2* and *VSP2* expression levels were determined in response to treatment with SA, MeJA, or a combination of SA and MeJA. Even though the JA marker gene *VSP2* is considered to be regulated by bHLH transcription factors that bind to the G-box, and not by ERF transcription factors, its expression is subject of this study because SA/JA crosstalk of this gene may be indirectly regulated by ERFs via (in)activation of other genes containing a GCC-box. *PR1* expression was taken along as a control for SA-inducibility. *PDF1.2* and *VSP2* transcripts accumulated at increasing levels between 1 to 6 h after treatment with MeJA (Fig. 2). Suppression of MeJA-induced expression of these genes by SA was most clearly detected at 4 and 6 h after treatment. Hence, if SA-induced transcriptional repressors play a role in SA/JA crosstalk, their action is expected to occur within 4 h after treatment. Moreover, they are expected to be induced in plants treated with both SA and MeJA. Therefore, we used qRT-PCR to analyze the level of expression of the selected *ERF* genes at 2 h after treatment with SA, MeJA or a combination of SA and MeJA.

Table 1 shows that of these 20 *ERF* genes, 14 were induced (fold change ≥ 1.5) within 2 h after application of a combination of SA and MeJA (i.e. *AtERF-1, CEJ1/DEAR1, DREB2A, ERF1, ERF3, ERF10, ERF11, ERF12, ERF13, ERF104, ERF112, HRE2, RAP2.6,* and *RAP2.6L*). Except for two genes, these *ERF* genes were also induced 2 h after application of SA alone. For the remaining selected *ERF* genes, transcription was not induced by any of the treatments (Table 2; fold change ≤ 1.5) in this experiment. This group comprises of the genes encoding for EAR-motif-ERFs *ERF4*, *ERF7*, *ERF8*, and *ERF9,* which we decided to leave in our selection because of their great potential to act as repressors. Furthermore, *ERF2* and *ERF5* were not induced by SA in this experiment. We removed *ERF2* from our selection, but *ERF5* was retained as an interesting candidate. Son *et al.* (2012) reported a negative effect of ERF5 on defense against the necrotrophic fungus *Alternaria brassicicola*, and a positive effect on SA-dependent gene expression and defense against *P. syringae*, suggesting a role for ERF5 in SA and JA signaling. Finally, we continued with 19 ERFs for further study: 14 *ERF*s that are induced by treatment with SA or a combination of SA and MeJA, four additional EAR-motif-containing ERFs, and ERF5 (Table 2).

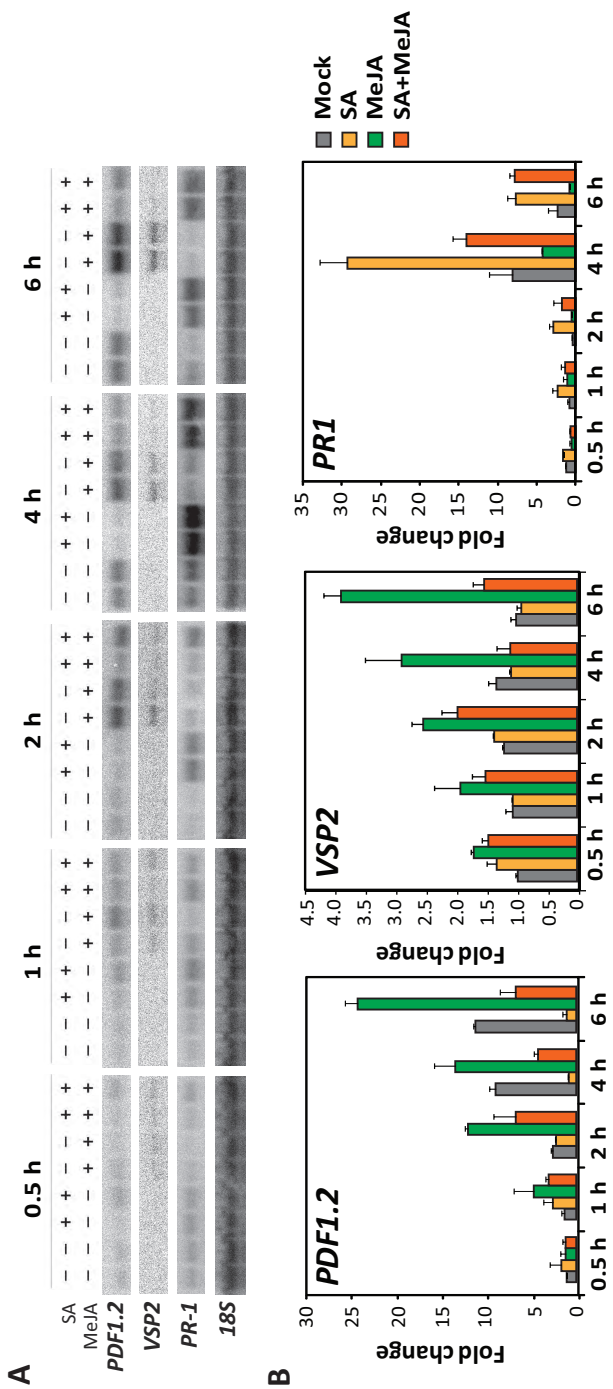


Figure 2: SA- and JA-responsive gene expression in a time course experiment. (A) RNA gel blot analysis of *PDF1.2*, *VSP2* and *PR1* expression in 5-week-old wild-type Col-0 plants treated with water (Mock), 1 mM SA and/or 0.1 mM MeJA and harvested at indicated times post-treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. For each sample two biological replicas are shown. (B) Quantification of *PDF1.2*, *VSP2* and *PR1* expression as shown in (A). Fold change is relative to the expression in plants 0.5 h after mock treatment and normalized to *18S* rRNA expression values. Shown are the means of two independent biological replicas; error bars indicate SD.

Table 1: Expression of selected *ERF* genes in response to SA and MeJA treatment. qRT-PCR analysis of expression of a selection of *ERF* genes in 5-week-old Col-0 plants 2 h post-treatment with 1 mM SA and/or 0.1 mM MeJA. Depicted is the fold change, which is expression in plants after treatment relative to expression in mock-treated plants. Green cells indicate that expression of the gene was induced (fold change ≥ 1.5), while orange cells indicate reduction of gene expression (fold change ≤ 0.67). The experiment was repeated with similar results. The twenty *ERFs* are divided into groups based on hierarchal clustering done by Nakano *et al.* (2006) and by Sakuma *et al.* (2002). CEJ1/DEAR1 and all proteins of group VIIIa/B-1 contain an EAR domain. The mutants lines that are selected are indicated is the most right column.

	AGI code	ERF# [1]	Alternative name	Group [2]	Group [3]	EAR Motif [4]	Fold change Expression after treatment (2h)			Mutant line [5]
							SA	MeJA	SA + MeJA	
1	AT4G17500	AtERF#100	**AtERF-1**	IXa	B-3		2.11	1.24	3.77	Rioja *et al.*(2013)
2	AT5G05410	AtERF#045	**DREB2A**	IVa	A-2		2.26	0.72	5.30	SAIL_365_F10
3	AT3G50260	AtERF#011	**CEJ1/DEAR1**	IIa	A-5	CMII-2 EAR	1.84	0.76	2.23	FLAG_293H04
4	AT3G23240	AtERF#092	**ERF1**	IXc	B-3		2.60	0.71	2.38	see M&M
5	AT1G50640	AtERF#082	**ERF3**	VIIIa	B-1	CMVIII-1 EAR	2.34	0.98	2.02	Not available
6	AT1G03800	AtERF#077	**ERF10**	VIIIa	B-1	CMVIII-1 EAR	1.71	1.76	2.07	SAIL_95_A08
7	AT1G28370	AtERF#076	**ERF11**	VIIIa	B-1	CMVIII-1 EAR	2.31	0.71	3.31	SALK_116053
8	AT1G28360	AtERF#081	**ERF12**	VIIIa	B-1	CMVIII-1 EAR	1.07	0.71	1.54	SAIL_873_D11
9	AT2G44840	AtERF#099	**ERF13**	IXa	B-3		2.69	4.47	11.50	GK_121A12
10	AT5G61600	AtERF#104	**ERF104**	IXb	B-3		1.63	0.57	2.05	Bethke *et al.* (2009)
11	AT2G33710	AtERF#112	**ERF112**	Xc	B-4		2.84	1.00	3.40	GK_604D02
12	AT2G47520	AtERF#071	**HRE2**	VIIa	B-2		1.90	1.32	2.63	Licausi *et al.* (2010)
13	AT1G43160	AtERF#108	**RAP2.6**	Xa	B-4		0.71	13.03	25.44	SAIL_1225_G09
14	AT5G13330	AtERF#113	**RAP2.6L**	Xa	B-4		3.15	3.10	9.88	SALK_051006
15	AT5g47220	AtERF#101	**ERF2**	IXa	B-3		1.30	0.65	1.13	Not selected
16	AT3G15210	AtERF#078	**ERF4**	VIIIa	B-1	CMVIII-1 EAR	0.87	1.17	1.29	McGrath *et al.*(2005)
17	AT5G47230	AtERF#102	**ERF5**	IXb	B-3		1.10	0.52	1.02	Son *et al.*(2012)
18	AT3G20310	AtERF#083	**ERF7**	VIIIa	B-1	CMVIII-1 EAR	1.33	0.88	1.43	Not available
19	AT1G53170	AtERF#079	**ERF8**	VIIIa	B-1	CMVIII-1 EAR	0.99	0.64	0.47	FLAG_157D10
20	AT5G44210	AtERF#080	**ERF9**	VIIIa	B-1	CMVIII-1 EAR	1.49	0.34	0.42	SALK_043407C

1. Numbering as introduced by the phylogenetic analysis of Nakano *et al.* (2006).
2. Group classification by Nakano *et al.*(2006).
3. Group classification based on Sakuma *et al.*(2002).
4. The presence of an EAR domain as described by Nakano *et al.* (2006). CMVIII-1: (L/F)DLN(L/F)xP. CMII-2: DLNxxP.
5. Mutants were obtained from (i) the authors of the papers, (ii) the Nottingham Arabidopsis Stock Centre (NASC) for SALK, SAIL and GABI-KAT lines or (iii) the French National Institute for Agricultural Research (INRA) for FLAG lines.

Responsiveness to MeJA, SA and SA/JA crosstalk of loss-of-function *erf* mutants

To investigate whether the selected 19 ERFs are involved in SA/JA crosstalk, their respective *erf* knockout mutants were obtained (Table 1). No suitable mutants were available for *ERF3* and *ERF7*. The remaining 17 *erf* mutants were analyzed for their ability to display SA/JA crosstalk in comparison to wild-type Col-0 or Ws-0 (in case of *erf8* and *cej1/dear1*). The *erf* mutant *ora59* that is impaired in *PDF1.2* expression was included as a negative control (Zander *et al.*, 2014). Expression of the JA marker genes *PDF1.2* and *VSP2* and the SA marker gene *PR1* was determined 5 and 24 h after treatment with MeJA, SA or a combination of MeJA with SA (Fig. S1).

Four *erf* mutants (*hre2*, *rap2.6*, *erf5* and *erf104*) displayed a reduced activation of *PDF1.2* expression upon MeJA treatment compared to wild-type plants, although the level of *PDF1.2* reduction was not completely compromised, as it was in the *ora59* mutant (Fig. 3 and Fig. S1). This suggests that the corresponding wild-type ERF proteins have a positive effect on MeJA-induced *PDF1.2* transcription. In contrast, an enhanced expression level of *PDF1.2* after MeJA treatment in comparison to wild-type plants was observed in six of the selected *erf* mutants. Among these is *erf4*, representing an EAR-motif ERF that was previously reported to negatively regulate *PDF1.2* expression, corroborating our findings (McGrath *et al.*, 2005). A similar expression behavior was found for *erf8*, *aterf-1*, *erf13, erf112*, and *dreb2a* (Fig. 3 and Fig. S1), suggesting that the corresponding ERFs are also potential negative regulators of JA-responsive gene expression.

Induction of *VSP2* expression after MeJA treatment was reduced in the mutants *rap2.6L*, *rap2.6*, *cej1/dear1*, *erf8* and *dreb2a* (Fig. 3 and Fig. S1), suggesting that the corresponding ERF proteins indirectly contribute to activation of the *VSP2* gene. Enhanced levels of *VSP2* induction upon MeJA treatment were observed in mutants *erf5*, *aterf-1* and *erf112* (Fig. 3 and Fig. S1). Mutant *ora59* also expressed enhanced *VSP2* levels, which is in line with an integrative role for ORA59 in the mutually antagonistic interaction between the so-called ERF-branch and MYC-branch of the JA signaling pathway, controlling expression of the marker genes *PDF1.2* and *VSP2*, respectively (Lorenzo *et al.*, 2004; Verhage *et al.*, 2011). Likewise, mutant *erf5* showed reduced *PDF1.2* and enhanced *VSP2* levels. However, the high *VSP2* levels as observed in MeJA-induced mutants *aterf-1* and *erf112* were not accompanied by low *PDF1.2* levels, but rather by high *PDF1.2* levels. This indicates that AtERF-1 and ERF112 repress *VSP2* expression, independent of the classic ERF-branch that is controlled by ORA59. Interestingly, mutants *dreb2a* and *erf8* displayed a behavior that is opposite to that of *ora59*, showing enhanced *PDF1.2* and reduced *VSP2* expression, suggesting that DREB2A and ERF8 may also be involved in the mutual antagonistic interaction between the classic ERF- and MYC-branch. Induction of the

PR1 gene upon SA treatment was unaffected in all but two of the *erf* mutants (Fig. 3 and Fig. S1), suggesting that the corresponding ERFs do not play a major role in SA signaling. In *rap2.6*, SA treatment led to a lower level of *PR1* expression than in wild-type Col-0 plants. It was suggested before that RAP2.6 has a role in SA-induced signaling (Ali *et al.*, 2013). In *erf8, PR1* expression was higher compared to wild-type Ws-0 plants, suggesting a role for ERF8 in suppression of SA-dependent defense besides its role in JA-dependent defenses.

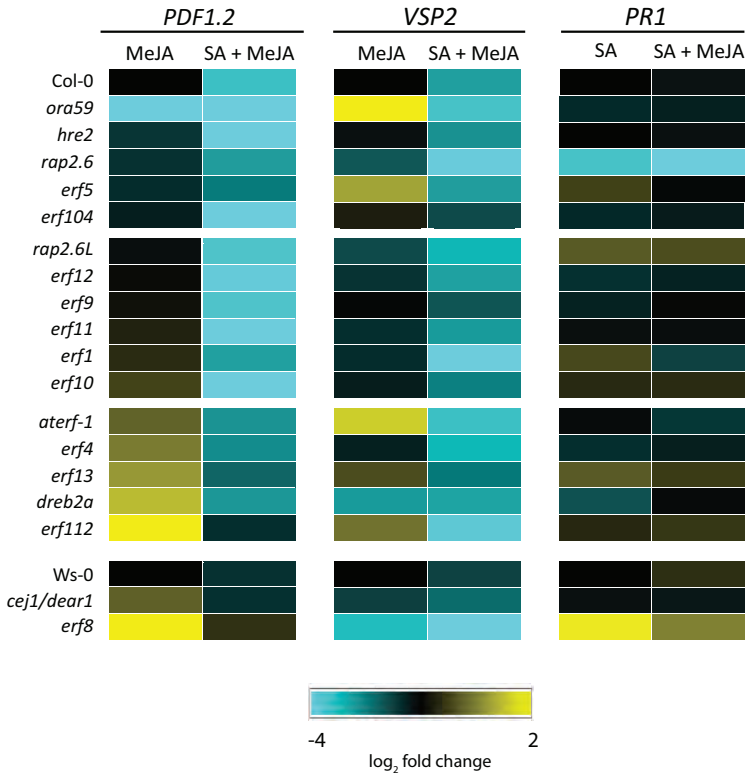


Figure 3: Expression of *PDF1.2*, *VSP2* and *PR1* after MeJA SA, or SA + MeJA treatment in all tested *erf* mutants. Expression levels of *PDF1.2, VSP2* and *PR1* in wild-type and 17 selected *erf* mutant lines after application of 0.1 mM MeJA (for *PDF1.2* and *VSP2*) or 1 mM SA (for *PR1*) and a combination of SA and MeJA. Shown is a heat map representation of $\log_2$ fold expression changes relative to wild-type plants in the same experiment that were MeJA-treated (for *PDF1.2* and *VSP2*) or SA-treated (for *PR1*). For wild-type plants, the average is shown of the different experiments. Cyan and yellow represent reduced and elevated expression, respectively, as indicated by the color bar. Fold change was calculated by expression quantification of RNA gel blots (for genotypes *hre2*, *erf4*, *erf9*, *erf10*, *erf11*, *erf12* and *erf104*) or qRT-PCR (for other genotypes). See Supplemental Figure S1 for untransformed data depicted in RNA gel blot pictures and diagrams (qRT-PCR), including statistical analysis.

Importantly, in the presence of SA, MeJA-induced *PDF1.2* and *VSP2* expression was strongly suppressed in both wild-types and all the tested *erf* mutants (Fig. 3 and Fig.

S1). Even when MeJA alone induced enhanced levels of *PDF1.2* or *VSP2*, as was the case in several mutants, as described above, the combination with SA evidently reduced the expression to similar levels as in combination-treated wild-type plants. Only in mutants *ora59* and *dreb2a* that already exhibited extremely low levels of *PDF1.2* (*ora59*) or *VSP2* (*dreb2a*) upon single MeJA treatment, the combination with SA did not lead to further reduction.

SA-induced *PR1* expression was not significantly affected by the combination with MeJA, neither in wild-type nor in the *erf* mutant plants (Fig. 3 and Fig. S1), confirming previous studies in which various wild-type and mutant Arabidopsis plants were subjected to exogenous application of both hormones (Koornneef *et al.*, 2008a; Leon-Reyes *et al.*, 2010a; Van der Does *et al.*, 2013). Because all the tested *erf* mutants are still highly sensitive to SA-mediated suppression of MeJA-induced *PDF1.2* and *VSP2* expression, we must conclude that none of the corresponding ERFs, that had been selected as putative SA-mediated transcriptional repressors of JA signaling, are essential for SA/JA crosstalk of the markers *PDF1.2* and *VSP2*.

SA/JA crosstalk functions independently of TOPLESS

TPL is a general co-repressor that is recruited by numerous repressors and transcription factors that contain an EAR-domain, including NINJA or JAZ to repress JA-responsive genes in the absence of a JA stimulus. The *tpl-1* mutant exhibits enhanced sensitivity to JA in a root growth inhibition assay (Pauwels *et al.*, 2010). Several EAR-motif AP2/ERFs can also interact with TPL (Causier *et al.*, 2012). Hence, we hypothesized that TPL, by interaction with different EAR-motif-ERFs or with NINJA/JAZs, could play a central role in repression of JA-responsive gene expression by SA. To investigate whether TPL is involved in SA/JA crosstalk, we monitored the expression of *PDF1.2* and *VSP2* in *tpl-1* in response to SA, MeJA, or a combination of SA and MeJA.

In the *tpl-1* mutant, basal *PDF1.2* and *VSP2* expression levels after mock treatment were respectively 40 and 60 times higher than in wild-type L*er*-0 adult plants (Fig. 4). Likewise, treatment with MeJA induced *PDF1.2* and *VSP2* expression to a higher extent in *tpl-1* than in L*er*-0 (Fig. 4). These effects were less prominent in seedlings, but also here MeJA induced *PDF1.2* expression to a higher level (Fig. S2). These results reassert the important role of TPL in repression of JA signaling, both in basal and MeJA-induced conditions. Induction of *PR1* by SA treatment was equally effective in *tpl-1* and L*er*-0 (Fig. 4 and Fig. S2). Importantly, SA strongly repressed the MeJA-induced expression level of both *PDF1.2* and *VSP2* in *tpl-1* adult plants and seedlings (Fig. 4 and Fig. S2). These results indicate that despite its role in repression of JA signaling, TPL is not essential for SA/JA crosstalk of *PDF1.2* and *VSP2*.

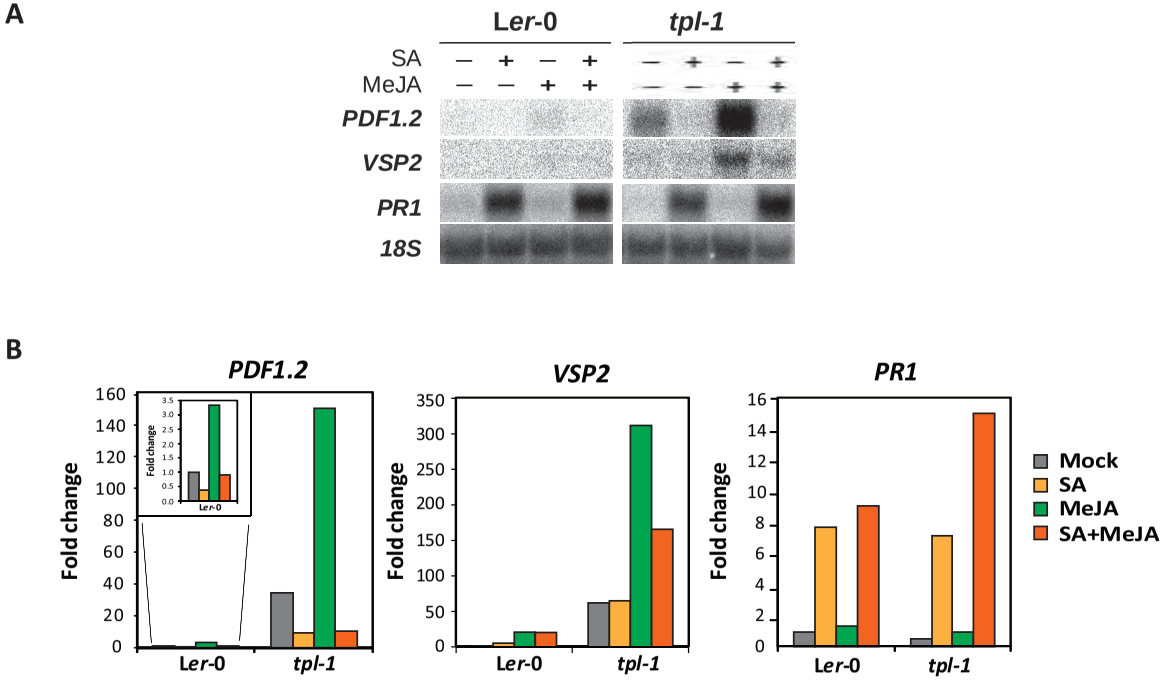


Figure 4: Co-repressor of JA signaling TPL is not required for SA/JA crosstalk. (A) RNA gel blot analysis of *PDF1.2, VSP2* and *PR1* expression in 5-week-old L*er*-0 wild-type and *tpl-1* mutant plants treated with water (Mock), 1 mM SA and/or 0.1 mM MeJA, and harvested at 24 h post-treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. (B) Quantification of *PDF1.2*, *VSP2* and *PR1* expression as shown in (A). Fold change is relative to the expression in L*er*-0 plants after mock treatment and normalized to *18S* rRNA expression values.

DISCUSSION

No evidence for a role of ERF repressors in SA/JA crosstalk

The antagonistic effects of SA on the JA signaling pathway have been well documented (Pieterse *et al.*, 2012), but the mechanisms underlying this phenomenon are complex and need further exploration. Here, we show that *de novo* synthesis of proteins is required for suppression of JA-induced *PDF1.2* expression by SA signaling. In the presence of CHX the induction of *PR1* by SA was completely inhibited, and while also the induction of *PDF1.2* by MeJA was strongly reduced, there was still a statistically significant 4-fold induction compared to the mock (Fig. 1). Only if CHX was present, the *PDF1.2* expression level could not be further antagonized by SA. This indicates that novel protein synthesis is required for SA to exert both its positive action on *PR1* expression and its repressive action on JA-induced *PDF1.2* expression. These SA-induced proteins could potentially interact with JA-induced transcriptional activators, and thereby reduce JA-dependent transcription. Alternatively, the SA-induced proteins could act as transcriptional repressors by occupying cis-regulatory elements in the promoters of JA-responsive genes and hence compete with JA-regulated transcriptional activators, leading to repression of transcription of JA-inducible genes (Caarls *et al.*, 2015).

In this study, we investigated the potential role of SA-inducible ERF transcriptional repressors in the SA-mediated attenuation of JA-responsive gene expression. We focused on the ERF family of the AP2/ERF superfamily of transcription factors, because the 122 members of this family share a common DNA binding domain with affinity for the GCC-box promoter element, which was previously shown to be a central target site of SA/JA antagonism (Nakano *et al.*, 2006; Van der Does *et al.*, 2013). Moreover, the ERF transcriptional activator ORA59 has been reported to be targeted by SA to suppress JA/ethylene signaling, indicating that ERFs can have a significant role in SA/JA crosstalk (Van der Does *et al.*, 2013; Zander *et al.*, 2014). Finally, several ERFs were described before as repressors of *PDF1.2* expression (Huang *et al.*, 2015). Based on the SA-inducible expression pattern of *ERFs* and/or the presence of an EAR-domain in their protein sequence, we selected and tested 17 ERF transcription factors as potential SA-induced repressors of JA signaling. To our knowledge, this is the first study in which a large set of ERF repressors is systematically screened for their potential role in SA/JA antagonism and in SA- and JA-responsiveness.

Induction of *PDF1.2* and *VSP2* by single MeJA treatment was significantly affected in the majority of the *erf* mutants (discussed below), while *PR1* induction by SA was affected only in two *erf*s, *rap2.6* (reduced) and *erf8* (enhanced). Strikingly, all

tested *erf* mutants displayed wild-type levels of SA-mediated suppression of JA-induced *PDF1.2* and *VSP2* expression, suggesting that the corresponding ERFs do not play an essential role in SA/JA crosstalk (Fig. 3 and Fig. S1). There may be functional redundancy among different ERF proteins affecting SA/JA crosstalk, in which case mutation of single genes does not result in a significant effect on SA-mediated suppression of JA-induced gene expression. However, several *erf* mutants did display an effect on *PDF1.2* or *VSP2* expression induced by only MeJA treatment, showing that even mutations in single *ERF* genes can result in measurable effects on transcription of these two JA-responsive genes. Moreover, a loss-of-function mutation in TPL, which is a general repressor of JA signaling and interacts with different EAR-motif-AP2/ERFs (Pauwels *et al.*, 2010; Causier *et al.*, 2012), also did not affect SA-mediated suppression of JA-induced *PDF1.2* and *VSP2* expression (Fig. 4 and Fig. S2). Together, these results make it unlikely that the tested ERF transcription factors or TPL play a major role as repressor in the antagonistic effect of SA on JA-responsive gene expression.

However, a role for the tested ERFs in SA/JA crosstalk cannot be fully excluded. Firstly, we were not able to assess the role of two of the selected ERFs, ERF3 and ERF7, as we did not obtain homozygous loss-of-function mutants after screening several T-DNA insertion lines. Overexpression of *ERF7* has been shown to result in suppression of *PDF1.2* expression in stable transgenic lines, indicating that it functions as a repressor (Song *et al.*, 2005). Secondly, we selected only those ERFs of which the gene expression was induced after treatment with SA or BTH or infection by *H. arabidopsidis* or that contain an EAR-domain. SA might however activate other repressive ERFs at the protein level, as post-translational regulation has previously been demonstrated for several ERFs (Koyama *et al.*, 2013; Van der Does *et al.*, 2013). Therefore, we cannot exclude a role in SA/JA crosstalk for other ERF transcription factors.

ERF transcriptional repressors of JA-inducible genes

It has been demonstrated that all the transcription factors of the ERF subfamily that contain an EAR-motif are capable of suppressing gene transcription in protoplast transactivation assays (Fujimoto *et al.*, 2000; Song *et al.*, 2005; Yang *et al.*, 2005; Wehner *et al.*, 2011). In accordance, we found a role for two EAR-motif-ERFs, ERF4 and ERF8, as negative regulators of *PDF1.2* expression, evidenced by increased *PDF1.2* expression after MeJA treatment in the *erf4* and *erf8* mutants (Fig. 3 and Fig. S1). For ERF4, this confirmed previous results by McGrath *et al.* (2005), who showed that overexpression of *ERF4* resulted in suppression of MeJA-induced *PDF1.2* expression and reduced resistance against *Fusarium oxysporum*. ERF8 was previously described to be able to suppress induced luciferase activity in transgenic

GCC:LUC lines (Wehner *et al.*, 2011); here, its relevance as a suppressor of the GCC-box containing *PDF1.2* gene is confirmed.

In addition, loss-of-function mutants of four ERF transcription factors without an EAR-domain also displayed enhanced *PDF1.2* expression levels after MeJA treatment, namely *aterf-1*, *erf13*, *erf112*, and *dreb2a* (Fig. 3 and Fig. S1). This indicates that the corresponding ERFs can repress MeJA-induced *PDF1.2* expression through other means than through interaction with EAR-binding co-repressors.

Opposite to the increased *PDF1.2* levels in the *dreb2a* and the *erf8* mutants, the MeJA-induced *VSP2* levels were extremely low (Fig. 3 and Fig. S1), suggesting that DREB2A and ERF8 can activate parts of the MYC-branch of JA signaling. In accordance, overexpression of DREB2A leads to increased expression of *VSP1* (Sakuma *et al.*, 2006). The mutual antagonism between the classical ERF- and MYC-branch of JA signaling (Lorenzo *et al.*, 2004; Verhage *et al.*, 2011) could possibly explain the decrease in MeJA-induced *VSP2* levels by an increase of the antagonistic classical ERF-branch. In contrast, mutants *erf5, erf112* and *aterf-1* displayed a significantly enhanced expression of *VSP2* upon MeJA treatment (Fig. S1). Similar to *ora59*, the increased *VSP2* level in *erf5* is associated with a reduced *PDF1.2* level, which is in line with the mutually antagonistic interaction between the ERF- and MYC-branch of JA signaling. Mutants *erf112* and *aterf-1* displayed enhanced activation of both *PDF1.2* and *VSP2*, indicating that the corresponding ERFs antagonize both branches of JA signaling.

ERF transcriptional activators of JA-induced genes

Previously, the ERF transcription factor ORA59 was shown to be a dominant positive regulator of JA-induced *PDF1.2* (Pré *et al.*, 2008). We find that *PDF1.2* expression in the *ora59* mutant was virtually absent, independent of the treatment applied (Fig. 3 and Fig. S1), confirming previous findings (Zander *et al.*, 2014). In addition to *ora59,* several other *erf* mutants that we assayed show reduced *PDF1.2* expression after induction by MeJA, namely *erf104*, *rap2.6*, *hre2,* and *erf5* (Fig. 3, Fig. S1). In line with our results, overexpression of *ERF104* was shown to lead to increased *PDF1.2* expression (Bethke *et al.*, 2009). *RAP2.6* overexpression was reported to enhance the expression of several JA-related genes (Ali *et al.*, 2013), and accordingly, we find here that both *PDF1.2* and *VSP2* expression are reduced upon MeJA treatment of the *rap2.6* mutant. Two contrasting reports on ERF5 have been published. Son *et al.* (2012) demonstrated a negative effect of ERF5 on defense against the necrotrophic fungus *Alternaria brassicicola*, and a positive effect on SA-dependent gene expression and defense against *P. syringae*. On the other hand, a study by Moffat *et al.* (2012) showed that ERF5 increased resistance against the necrotroph *Botrytis cinerea*, while

it negatively regulated UV-C-induced expression of *PR1*. Our *erf5* mutant analysis shows no significant effect on SA-induced *PR1* expression, while it confirms the positive role of ERF5 in *PDF1.2* induction, suggesting a contribution towards resistance against necrotrophs. Interestingly, although overexpression of the ERF gene *ERF1* was previously shown to strongly activate *PDF1.2* (Lorenzo *et al.*, 2003), we did not observe an effect on the level of *PDF1.2* transcription in the *erf1* mutant, and also *VSP2* and *PR1* expression were not affected (Fig. S1).

MeJA treatment led to a reduced *VSP2* induction by MeJA in five mutants. In *erf8*, *dreb2a* and *rap2.6*, reduced *VSP2* expression correlated with reduced *(rap2.6*) versus enhanced (*erf8* and *dreb2a*) *PDF1.2* expression, as discussed above. In contrast, *rap2.6L* and *cej1/dear1* displayed reduced *VSP2* expression without a significant effect on *PDF1.2* expression, which suggests a role for the corresponding ERFs specifically in activation of the MYC-branch. Together these results indicate that different ERFs can have redundant roles in the activation of JA-responsive genes. However, the fact that effects on *PDF1.2* and *VSP2* expression are detectable in the respective single *erf* mutants suggests that different ERFs might act additively or be active in different tissues, developmental stages, or at different times following induced signaling.

General co-repressor TOPLESS

Co-repressor TPL is recruited to repress gene expression in several hormonal signaling pathways. It interacts with EAR-domain containing suppressors NINJA or JAZ in the JA signaling pathway, but also with the auxin repressors AUX/IAA via their EAR-motifs (Szemenyei *et al.*, 2008; Pauwels *et al.*, 2010; Shyu *et al.*, 2012). AP2/ERF transcription factor APETALA2 (AP2) was shown to interact with TPL, and the recruitment of TPL and its interactor HDA19 represses downstream targets (Krogan *et al.*, 2012). Several EAR-motif-ERF transcriptional repressors, including ERF4 and ERF11, were shown to interact with TPL (Causier *et al.*, 2012). We investigated if suppression via TPL could play a role in SA/JA crosstalk. *PDF1.2* and *VSP2* expression in basal and MeJA-induced conditions were significantly higher in the *tpl-1* mutant compared to wild-type L*er*-0 (Fig. 4), thus demonstrating convincingly the repressive effect of TPL on JA-inducible gene expression. SA-inducible *PR1* expression was unaffected in the *tpl-1* mutant and also suppression of MeJA-induced *PDF1.2* and *VSP2* expression by SA was still intact in *tpl-1* (Fig. 4). Hence, TPL is not required for SA/JA crosstalk of *PDF1.2* and *VSP2* expression. Indirectly, this also suggests that EAR-motif-ERF transcriptional repressors are unlikely to play a role in SA/JA crosstalk.

Potential SA-inducible actors in suppression of JA signaling

In summary, we demonstrate that SA-mediated suppression of JA-dependent *PDF1.2* gene expression requires *de novo* SA-induced protein synthesis and that it is not likely that ERF repressive transcription factors or the transcriptional co-repressor TPL play a major role in this process. So, which SA signaling components are *de novo* synthesized upon induction by SA and may act in the suppression of the JA pathway? Previously, the transcriptional co-activator NPR1, the TGA transcription factors TGA2, TGA5 and TGA6, glutaredoxin GRX480, and the WRKY transcription factors WRKY50, WRKY51 and WRKY70 were reported to play important roles in SA/JA crosstalk (reviewed by Caarls *et al.*, 2015). Regulation of *ORA59* expression by TGA transcription factors, possibly in an interaction with SA-induced GRX480, is likely essential in suppression of the GCC-box (Leon-Reyes *et al.*, 2010a; Zander *et al.*, 2014). A future challenge for research on SA/JA crosstalk will be to identify if these, or yet unknown, SA-induced proteins can interact with JA signaling components to suppress JA-dependent gene transcription.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana seeds were sown on river sand. Two weeks after germination, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture (5:12 v/v) that had been autoclaved twice for 20 min with a 24-h interval. For plate assays, seeds were sown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) including vitamins, at pH 6.0, supplemented with 1% (w/v) sucrose and 0.85% (w/v) plant agar. Plants were cultivated in a growth chamber with a 10-h day (75 μmol m^{-2} s^{-1}) and 14-h night cycle at 70% relative humidity and 21°C.

For several of the studied genes, knockout mutants were previously published and available, namely: *aterf-1* (At4g17500) (Rioja *et al.*, 2013), *erf4* (At3g15210) (McGrath *et al.*, 2005), *erf5* (At5g47230) (Son *et al.*, 2012), *erf104* (At5g61600) (Bethke *et al.*, 2009), *hre2* (At2g47520) (Licausi *et al.*, 2010), *ora59* (At1g06160) (Zander *et al.*, 2014) (all in Col-0 background) and *tpl-1 (*At1g15750) (L*er*-0 background) (Long *et al.*, 2002).

Knock-outs of other genes were obtained by acquiring T-DNA insertion lines from the Nottingham Arabidopsis Stock Centre (NASC): *dreb2a* (SAIL_365_F10; At5g05410), *erf9* (SALK_043407C; At5g44210), *erf10* (SAIL_95_A08; At1g03800), *erf11* (SALK_166053; At1g28370), *erf12* (SAIL_873_D11; At1g28360), *erf13* (GK_121A12; At2g44840), *erf112* (GK_604D02; At2g33710), *rap2.6* (SAIL_1225_G09; At1g43160) and *rap2.6L*

(SALK_051006;At5g13330), all in Col-0 background (Sessions *et al.*, 2002; Alonso *et al.*, 2003; Kleinboelting *et al.*, 2011). Other knockout lines were obtained from the French National Institute for Agricultural Research (INRA): *cej1/dear1* (FLAG_293H04; At3g50260) and *erf8* (FLAG_157D10; At1g53170), both in Ws-0 background (Samson *et al.*, 2002). Lines that were homozygous for the T-DNA insert were selected with PCR using the primers listed in Supplemental Table S2. Mutants were used to analyze SA/JA crosstalk only when the T-DNA insertion was located in an exon, or when the expression of the mutated gene was proven absent by qRT-PCR in mutants carrying the T-DNA up- or downstream of the coding sequence. The primers used in the qRT-PCR for analysis of expression of the *ERF* target gene in the mutants were as described by Czechowski *et al.* (2004). For details on the qRT-PCR analysis see section "RNA extraction, RNA gel blot and qRT-PCR analysis".

For *ERF1* (At3g23240), a T-DNA insertion line was obtained through screening of 80,000 lines of the SALK collection (Alonso *et al.*, 2003), which was based on a four-dimensional pooling strategy. In order to identify a T-DNA insertion in the *ERF1* gene, a PCR-based approach was taken, using two T-DNA specific primers for the LB (JMLB1) and RB (JMRB) of the pROK2 vector and the corresponding *ERF1* Forward and Reverse primer (Supplemental Table S2). In each screening at least 4 primer combinations were tested (JMLB1 and *ERF1* Forward; JMLB1 and *ERF1* Reverse; JMRB and *ERF1* Forward; JMRB and *ERF1* Reverse). A PCR product was considered valid when it hybridized with an *ERF1* specific probe and was present in each of the 4 DNA pools. PCR products were run in a 1% agarose gel (1x TBE) containing ethidium bromide and transferred to a Hybond-N^+ membrane using 0.4 N NaOH. For pre-hybridization and hybridization Church and Gilbert solution was used (7% SDS, 0.3M NaPi pH 7.0 and 1 mM EDTA). In the *erf1* T-DNA insertion line, specific amplification with the primer combination JMLB1 and *ERF1* Reverse was obtained.

Chemical treatments

Five-week-old 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. For mock treatments, plants were dipped into a solution containing 0.015% (v/v) Silwet L77. MeJA was added to the solutions from a 1000-fold concentrated stock in 96% ethanol. To the solutions without MeJA, a similar volume of 96% ethanol was added.

For the CHX experiments, five-week-old plants were dipped in mock or MeJA solution. Twenty four hours later, leaf discs (19.6 mm^2) were cut from the 4^{th} to 6^{th} leaf and placed in 6-well plates containing 3 mL of MES buffer (5 mM MES, 1 mM KCl, pH 5.7)

per well. Fifteen leaf discs were used per sample; 3 independent biological replicas were included per treatment. CHX (Sigma-Aldrich, St. Louis, MO, USA) was added from a 100-fold concentrated stock in 10% ethanol/MES buffer, resulting in a final concentration of 0.1 mM CHX, after which leaf discs were kept under vacuum for 20 min. Subsequently, SA was added from a 4-fold concentrated stock in MES buffer, resulting in an end concentration of 0.5 mM SA. Similar volumes of ethanol or MES buffer were added to solutions without CHX or SA. After 6 h, leaf discs were snap frozen in liquid nitrogen.

For treatment of L*er*-0 and *tpl-1* seedlings, 14-day-old plate-grown plants were transferred to fresh MS agar plates with or without 0.5 mM SA, 20 μM MeJA, or a combination of both. As described above, MeJA was added from a 1000-fold concentrated stock in 96% ethanol, media without MeJA received a similar volume of 96% ethanol. Seedlings were harvested 48 h after transfer.

RNA extraction, RNA gel blot and qRT-PCR analysis

RNA was extracted from leaf material from 5 plants per treatment per sample. For gene expression analysis with RNA gel blotting, the protocol as described by Van Wees (1999) was followed for RNA extraction, RNA blotting and blot hybridization with gene-specific probes for *PR1* (At2g14610), *PDF1.2* (At5g44420), *VSP2* (At5g24770) and *18S* rRNA. Probes were synthesized by PCR amplification on cDNA using previously described primers (Van der Does *et al.*, 2013). After hybridization with α-^{32}P-dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of probes were quantified using a Bio-Rad Molecular Imager FX with Quantity One software (Bio-Rad, Veenendaal, the Netherlands).

For gene expression analysis with a two-step qRT-PCR, RNA was extracted as described for vegetative tissues by Oñate-Sánchez and Vicente-Carbajosa (2008). RNA that was used for qRT-PCR was pretreated with DNAse I (Fermentas, St. Leon-Rot, Germany) to remove genomic DNA. RevertAid H minus Reverse Transcriptase (Fermentas) was used to convert DNA-free total RNA into cDNA using oligo(dT) primers. PCR reactions were performed in optical 384-well plates with a ViiA 7 realtime PCR system (Applied Biosystems, Carlsbad, CA, USA), using SYBR® Green to monitor the synthesis of double-stranded DNA. A standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60 to 95°C with a ramp speed of 1.0°C min^{-1}. Fold change was calculated relative to the reference gene *At1g13320* (Czechowski *et al.*, 2005) using the $2^{-\Delta\Delta}$Ct method described previously (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The primers used to analyze gene expression with qRT-PCR were as described by Czechowski *et al.* (2004), except the primers for expression of *ERF5*, *PDF1.2*, *VSP2*, *PR1* and *At1g13320* (Supplemental Table S2; Van der Does *et al.*, 2013).

RNA sequencing of SA-treated plants

For RNA sequencing, leaf 6 was harvested from 4 individual SA- or mock-treated wild-type Col-0 plants at each of the following time points post treatment: 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 10 h, 12 h, and 16 h. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), including a DNase treatment step in accordance with manufacturer's instructions. RNA-seq library preparation and sequencing was performed by UCLA Neuroscience Genomics Core (Los Angeles, CA, USA). Sequencing libraries were prepared using the Illumina TruSeq RNA Sample Prep Kit, and sequenced on the Illumina HiSeq 2000 platform with single read lengths of 50 bases. Basecalling was performed using the Casava v1.8.2. pipeline with default settings except for the additional argument '--use-bases-mask y50,y6n', to provide an additional fastq file containing the barcodes for each read in each sample. Sample demultiplexing was performed by uniquely assigning each barcode to sample references, allowing for a maximum of 2 mismatches and only considering barcode nucleotides with a quality score of 28 or greater. Reads were mapped to the Arabidopsis genome (TAIR version 10) using TopHat version 2.0.4 (Trapnell *et al.*, 2009) with parameter settings: 'max-intron-length 2000', 'transcriptome-mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genome-read-mismatches 3', ' p 6', 'read-mismatches 3', 'G', 'min-intron-length 40'. Aligned reads were summarized over annotated gene models (TAIR version 10) using HTseq-count version 0.5.3p9 (http://www-huber.embl.de/users/anders/HTSeq/) with parameters: 'stranded no', '-i gene_id'. Sample counts were depth-adjusted using the median-count-ratio method available in the DESeq package (Anders and Huber, 2010). Genes that were significantly altered over time in response to SA when compared to the mock treatment were identified using a generalized linear model (GLM) with a log link function and negative binomial distribution. Within this model we considered both the time after treatment (time) and the treatment itself (treat) as a factor. To assess the effect of SA application on the total read count for each gene, a saturated model (total counts ~ treatment + time + treatment:time) was compared to a reduced model considering time alone (total counts ~ time) using an ANOVA Chi-square test. The obtained p-values for all genes were corrected for multiple testing using a Bonferroni correction. All statistics associated with testing for differential gene expression were performed with R (www.r-project.org).

ACKNOWLEDGMENTS

This work was supported by the Dutch Technology Foundation STW (VIDI grant no. 11281 to S.C.M.W.), the Netherlands Organization of Scientific Research (VICI grant no. 865.04.002 to C.M.J.P.), and the European Research Council (ERC Advanced Investigator Grant no. 269072 to C.M.J.P.).

Supplemental Table S1 . Expression of *ERF* genes in response to SA/BTH treatment or *H. arabidopsidis* infection. Overview of gene expression levels of all 122 *ERF* genes in Arabidopsis seedlings, mature plants or cell suspension cultures treated with SA or BTH, or infected with the biotrophic pathogen *H. arabidopsidis*. Numbers indicate fold change (FC) of expression level in the induced treatments relative to the level in mock treatments, or indicate the h after SA treatment that induction of expression was observed for the first time. In the most right column, a 'yes' indicates that the *ERF* was significantly induced in plants treated with SA compared to mock-treated plants in a 16 h-timeseries ($P \leq 0.05$). Data were included in the table only if FC > 1.5. Empty cells indicate that expression was either not significantly affected, or not detected in the selected dataset. Colored cells mark the genes that were selected for further SA/JA crosstalk research. Blue cells show the genes that were upregulated in three or more datasets, green cells indicate ERFs with a CMVIII-1 EAR domain, and orange cells show the genes that were upregulated in one or two datasets, but selected in further research for another reason (see main text).

						Seedlings [4]	Seedlings [5]	Cell suspension [6]	4-wk-old plants [7]	Seedlings [8]	Seedlings [9]	5-wk-old plants [10]
						CATMAv2 array	Affymatrix ATH1 array	CATMA array	Affymetrix ATH1 Array	Northern blot	Northern blot	RNA sequencing
						0.5 mM SA	0.01 mM SA	0.25 mM SA	0.06 mM BTH	1 mM SA	*H.a.* avirulent	1 mM SA
						2.5 h	3 h	4 h	8 h		3 dpi	0-16 h
AGI code	**ERF nr** [1]	**Group** [2]	**Group** [2]	**Other name**	**EAR domain** [3]	Fold Change (SA vs Mock)	Fold Change (SA vs Mock)	Fold Change (SA vs Mock)	Fold Change (BTH vs Mock)	Start time of induction after SA treatment	Fold Change (SA vs Mock)	*P* value ≤ 0.05?
At1g15360	AtERF#001	Va	B-6	WIN1/SHN1								
At5g19790	AtERF#002	Vb	B-6	RAP2.11								
At5g25190	AtERF#003	Va	B-6	ESE3								yes
At5g11190	AtERF#004	Va	B-6	SHN2								yes
At5g25390	AtERF#005	Va	B-6	SHN3								yes
At1g46768	AtERF#006	IIa	A-5	RAP2.1	CMII-2 EAR		1.50			8 h		
At4g06746	AtERF#007	IIa	A-5	RAP2.9/DEAR5	CMII-2 EAR							yes
At2g23340	AtERF#008	IIa	A-5	DEAR3	CMII-2 EAR		1.60					
At4g36900	AtERF#009	IIa	A-5	RAP2.10/DEAR	CMII-2 EAR		1.60					yes
At5g67190	AtERF#010	IIa	A-5	DEAR2	CMII-2 EAR							yes
At3g50260	AtERF#011	IIa	A-5	CEJ1/DEAR1	CMII-2 EAR	2.57		1.72	5.53		2.66	yes

At1g21910	AtERF#012	IIb	A-5	DREB26			yes
At1g77640	AtERF#013	IIb	A-5			2 h	
At1g44830	AtERF#014	IIb	A-5				
At4g31060	AtERF#015	IIb	A-5				yes
At5g21960	AtERF#016	IIb	A-5				
At1g19210	AtERF#017	IIb	A-5		3.10		yes
At1g74930	AtERF#018	IIb	A-5	ORA47			yes
At1g22810	AtERF#019	IIc	A-5				
At1g71520	AtERF#020	IIc	A-5			1 h	yes
At1g71450	AtERF#021	IIIa	A-4				
At1g33760	AtERF#022	IIIa	A-5				yes
At1g01250	AtERF#023	IIIa	A-4				yes
At2g36450	AtERF#024	IIIb	A-4	HARDY			
At5g52020	AtERF#025	IIIb	A-4			8 h	
At1g63040	AtERF#026	IIIb	A-4				
At1g12630	AtERF#027	IIIb	A-4		1.50		yes
At5g51990	AtERF#028	IIIc	A-1	CBF4/DREB1D			
At4g25490	AtERF#029	IIIc	A-1	CBF1/DREB1B			yes
At4g25470	AtERF#030	IIIc	A-1	CBF2/DREB1C			yes
At4g25480	AtERF#031	IIIc	A-1	CBF3/DREB1A			yes
At1g63030	AtERF#032	IIIc	A-1	DDF2	1.60		
At1g12610	AtERF#033	IIIc	A-1	DDF1	3.50		
At2g44940	AtERF#034	IIId	A-4				
At3g60490	AtERF#035	IIId	A-4				
At3g16280	AtERF#036	IIIe	A-4				yes
At1g77200	AtERF#037	IIIe	A-4				
At2g35700	AtERF#038	IIId	A-4	ERF38			yes
At4g16750	AtERF#039	IIId	A-4				
At5g25810	AtERF#040	IIIe	A-4	TINY			yes
At5g11590	AtERF#041	IIIe	A-4	TINY2			yes

At2g25820	AtERF#042	IIIe	A-4	ESE2					8 h	yes
At4g32800	AtERF#043	IIIe	A-4							yes
At3g11020	AtERF#044	IVa	A-2	DREB2B					16 h	
At5g05410	AtERF#045	IVa	A-2	DREB2A	2.00	1.90			1 h	yes
At2g38340	AtERF#046	IVa	A-2	DREB19						
At2g40350	AtERF#047	IVa	A-2						4 h	
At2g40340	AtERF#048	IVa	A-2	DREB2C					2 h	yes
At1g75490	AtERF#049	IVb	A-2							yes
At5g18450	AtERF#050	IVb	A-2							
At3g57600	AtERF#051	IVb	A-2							yes
At2g40220	AtERF#052	IVb	A-3	ABI4						
At2g20880	AtERF#053	Ia	A-6						2 h	yes
At4g28140	AtERF#054	Ia	A-6							yes
At1g36060	AtERF#055	Ib	A-6							
At2g22200	AtERF#056	Ib	A-6							yes
At5g65130	AtERF#057	Ib	A-6							
At1g22190	AtERF#058	Ib	A-6	RAP2.4/OSH1						
At1g78080	AtERF#059	Ib	A-6	RAP2.4/WIND1						yes
At4g39780	AtERF#060	Ib	A-6				1.76			yes
At1g64380	AtERF#061	Ib	A-6							
At4g13620	AtERF#062	Ib	A-6							
At4g11140	AtERF#063	VI	B-5	CRF1						yes
At4g23750	AtERF#064	VI	B-5	CRF2/TMO3						yes
At5g53290	AtERF#065	VI	B-5	CRF3						
At4g27950	AtERF#066	VI	B-5	CRF4						yes
At3g61630	AtERF#067	VI	B-5	CRF6				3.00		yes
At2g46310	AtERF#068	VI	B-5	CRF5					4 h	
At1g22985	AtERF#069	VI	B-5	CRF7						
At1g71130	AtERF#070	VI	B-5	CRF8						yes
At2g47520	AtERF#071	VIIa	B-2	HRE2		9.00				

At3g16770	AtERF#072	VIIa	B-2	EBP/RAP2.3						yes
At1g72360	AtERF#073	VIIa	B-2	HRE1						yes
At1g53910	AtERF#074	VIIa	B-2	RAP2.12						yes
At3g14230	AtERF#075	VIIa	B-2	RAP2.2		1.78				
At1g28370	AtERF#076	VIIIa	B-1	ERF11	CMVIII-1 EAR		4.44		2.41	yes
At1g03800	AtERF#077	VIIIa	B-1	ERF10	CMVIII-1 EAR					yes
At3g15210	AtERF#078	VIIIa	B-1	ERF4/RAP2.5	CMVIII-1 EAR		1.59			
At1g53170	AtERF#079	VIIIa	B-1	ERF8	CMVIII-1 EAR					yes
At5g44210	AtERF#080	VIIIa	B-1	ERF9	CMVIII-1 EAR					yes
At1g28360	AtERF#081	VIIIa	B-1	ERF12	CMVIII-1 EAR					yes
At1g50640	AtERF#082	VIIIa	B-1	ERF3	CMVIII-1 EAR			2 h		yes
At3g20310	AtERF#083	VIIIa	B-1	ERF7	CMVIII-1 EAR					yes
At1g80580	AtERF#084	VIIIb	B-1			1.71				yes
At5g13910	AtERF#085	VIIIb	B-1	LEP						
At5g18560	AtERF#086	VIIIb	B-1	PUCHI						
At1g28160	AtERF#087	VIIIb	B-1							
At1g12890	AtERF#088	VIIIb	B-1							yes
At1g12980	AtERF#089	VIIIb	B-1	ESR1/DRN						
At1g24590	AtERF#090	VIIIb	B-1	ESR2/DRNL						
At4g18450	AtERF#091	IXc	B-3							
At3g23240	AtERF#092	IXc	B-3	ERF1		2.07	2			yes
At2g31230	AtERF#093	IXc	B-3	ERF15						yes
At1g06160	AtERF#094	IXc	B-3	ORA59						yes
At3g23220	AtERF#095	IXc	B-3	ESE1						yes
At5g43410	AtERF#096	IXc	B-3							
At1g04370	AtERF#097	IXc	B-3	ERF14						yes
At3g23230	AtERF#098	IXc	B-3	TDR1						yes
At2g44840	AtERF#099	IXa	B-3	ERF13		2.40			2.55	yes
At4g17500	AtERF#100	IXa	B-3	AtERF-1		1.50		4 h	5.86	yes
At5g47220	AtERF#101	IXa	B-3	ERF2		1.90	3.84	4 h	6.82	yes

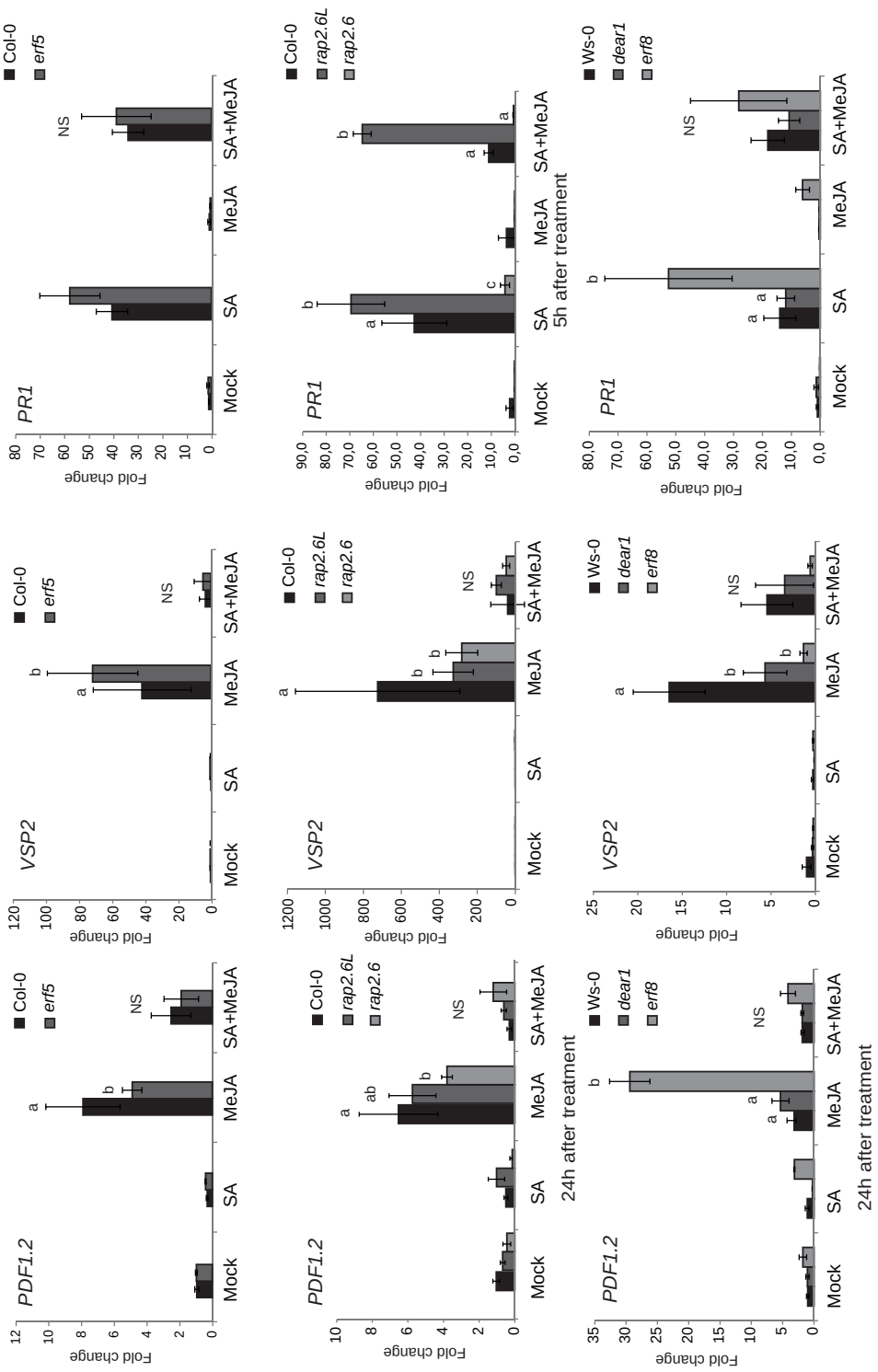
At5g47230	AtERF#102	IXb	B-3	ERF5						2.41	
At4g17490	AtERF#103	IXb	B-3	ERF6							
At5g61600	AtERF#104	IXb	B-3	ERF104				4.26		3.23	
At5g51190	AtERF#105	IXb	B-3	ERF105				2.57			
At5g07580	AtERF#106	IXb	B-3								yes
At5g61590	AtERF#107	IXb	B-3	DEWAX			3.10				yes
At1g43160	AtERF#108	Xa	B-4	RAP2.6					8 h	6.06	yes
At4g34410	AtERF#109	Xb	B-3	RRTF1							
At5g50080	AtERF#110	Xa	B-4								
At5g64750	AtERF#111	Xa	B-4	ABR1							
At2g33710	AtERF#112	Xc	B-4	ERF112					2 h	16	yes
At5g13330	AtERF#113	Xa	B-4	RAP2.6L	2.19	3.30	1.83		4 h	2.83	yes
At5g61890	AtERF#114	Xa	B-4								
At5g07310	AtERF#115	Xa	B-4	ERF115							
At1g25470	AtERF#116	VI-L	B-6	CRF12							
At1g49120	AtERF#117	VI-L	B-6	CRF9							
At1g68550	AtERF#118	VI-L	B-6	CRF10			0.54				
At3g25890	AtERF#119	VI-L	B-6	CRF11							yes
At2g20350	AtERF#120	Xb-L	B-6						2 h		
At5g67010	AtERF#121	Xb-L	B-6								
At5g67000	AtERF#122	Xb-L	B-6								

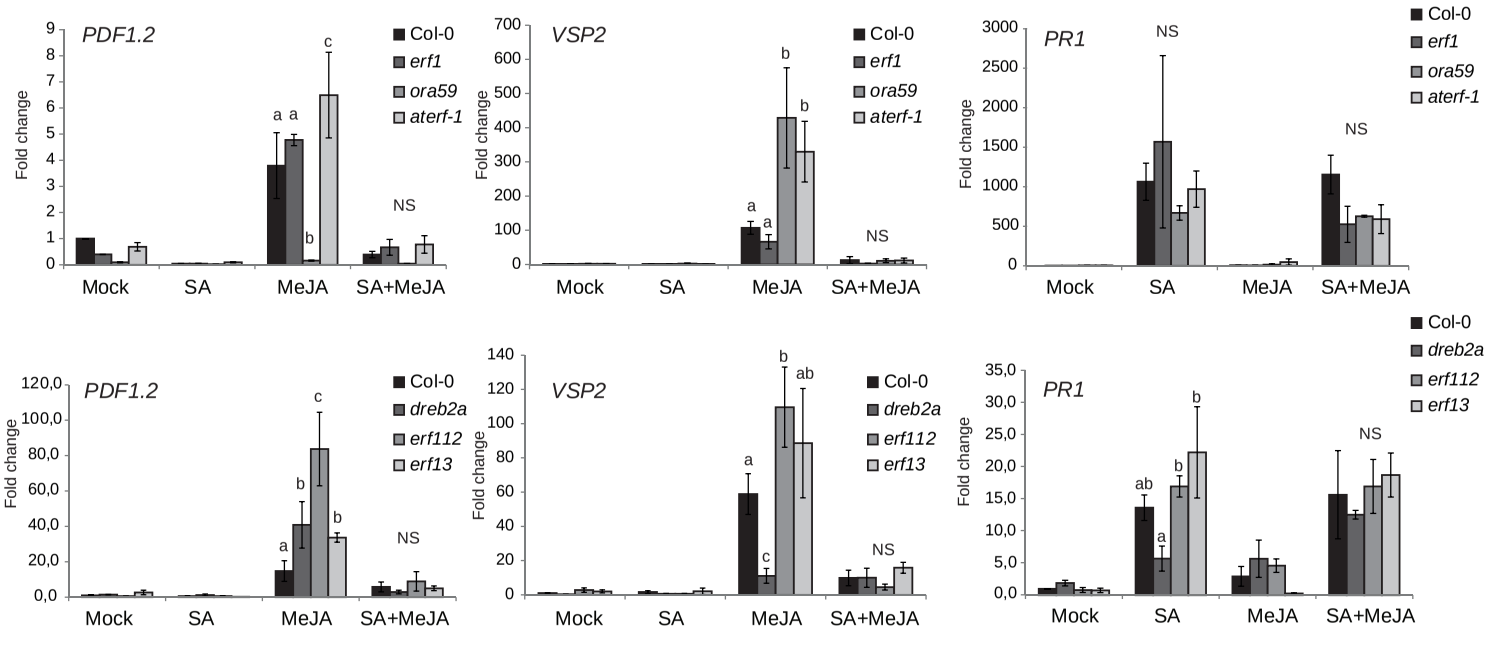
1. ERF number (#) and group based on the phylogenetic analysis done by Nakano *et al.* (2006) and the numbering system introduced there.
2. Group classification based on Sakuma *et al.* (2002).
3. The presence of an EAR domain as described by Nakano *et al.* (2006). CMVIII-1: (L/F)DLN(L/F)xP. CMII-2: DLNxxP.
4. Blanco *et al.* (2009). Table S1.;
5. BAR.utoronto.ca; Goda *et al.* (2008) NASC array 192.;
6. Krinke *et al.* (2007) Table S1.;
7. Wang *et al.* (2006);
8. Atallah *et al.* (2005);
9. Huibers *et al.* (2009). Table S1.
10. SA-treated plants compared to mock-treated plants by RNA sequencing. Yes means gene is significantly induced in SA-induced plants vs mock-treated plants ($P \leq 0.05$).

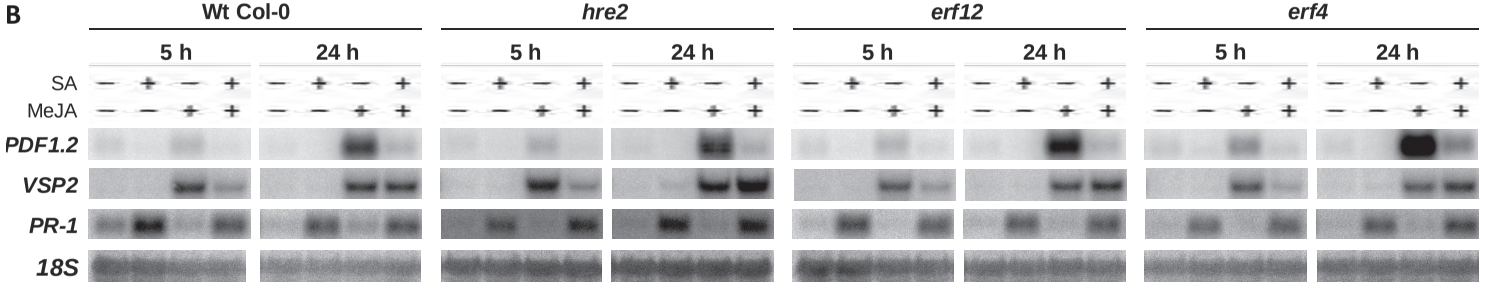
Supplemental Table S2. List of all primers designed for genotyping and qRT-PCR in this study.

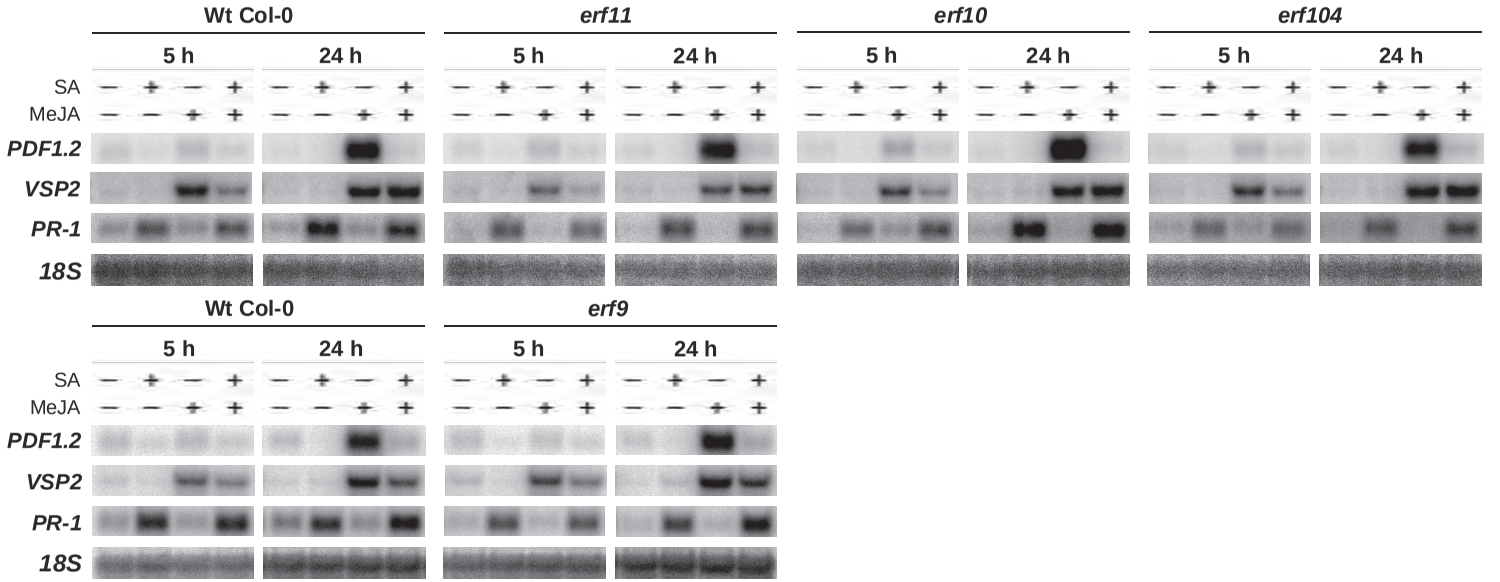
Name	Sequence (5'→ 3')
Primers used for genotyping:	
erf8 FLAG_157D10 Forward	ACGATGATGCATTTCCTTGG
erf8 FLAG_157D10 Reverse	GGATAAATGCAGCAAAAACCA
erf9 SALK_043407C Forward	CACCATGGCTCCAAGACAGGCG
erf9 SALK_043407C Reverse	CTAAACGTCCACCACCGGT
erf10 SAIL_95_A08 Forward	TGGATCAGTTTGTTACTGGGG
erf10 SAIL_95_A08 Reverse	GAATGTAACTAAGGCCCTGGC
erf11 SALK_116053 Forward	CCACACGTCGTCCTTCATATC
erf11 SALK_116053 Reverse	TGCAAAGCCTAAAATTAAAAACG
erf12 SAIL_873_D11 Forward	ACACGCTTGCTCAGCATTTAC
erf12 SAIL_873_D11 Reverse	AGTCTAACAATGGCGTCAACG
erf13 GK_121A12 Forward	GGCCACAAAAAGTGAATTTAAC
erf13 GK_121A12 Reverse	CGAAGTAAAACTGATCCACCG
erf112 GK_604D02 Forward	CCGGAGGTCAATGCGTTT
erf112 GK_604D02 Reverse	TGGCCACAATGCATTAGTT
rap2.6 SAIL_1225_G09 Forward	TCAATCAACGTGTCATGAAGG
rap2.6 SAIL_1225_G09 Reverse	TCAGACTGAAGTTGTATTGGGAG
rap2.6l SALK_051006 Forward	TTCGGTTCGTGTGTTTTTCA
rap2.6l SALK_051006 Reverse	TATGCTGATCGGTGGTTCAA
dear1 FLAG_293H04 Forward	TAAGTAACGTCCTGCCAAACC
dear1 FLAG_293H04 Reverse	AAGCATTCCGCGTACGATAG
dreb2a SAIL_365_F10 Forward	GGAGTGGAGCCGATGTATTGT
dreb2a SAIL_365_F10 Reverse	TGCCACCAACAAGCATTCCT
SALK LBb1.3 (for *erf9 and rap2.6l*)	ATTTTGCCGATTTCGGAAC
SALK LBA1 (for *erf11*)	TGGTTCACGTAGTGGGCCATCG
SAIL LB (for *erf10* and *erf12)*	TTTCATAACCAATCTCGATACACG
SAIL LB mod.(for *rap2.6* and *dreb2a*)	GCATCTGAATTTCATAACCAATCTC
FLAG_RB4(for *erf8* and *dear1*)	TCACGGGTTGGGGTTTCTACAGGAC
GABI_08409(for *aterf13,* a*terf112*)	ATATTGACCATCATACTCATTGC
ERF1 Forward	TCAAGACCTTCCGATCAAATCCGTAAGCTC
ERF1 Reverse	ACCCCAAAAGCTCCTCAAGGTACTGTTCTC
JMLB1(for *erf*1)	GGCAATCAGCTGTTGCCCGTCTCACTGGTG
JMRB (for *erf1)*	TGATAGTGACCTTAGGCGACTTTTGAACG
Primers used for qRT-PCR[1]:	
VSP2 Forward	ACGGAACAGAGAAGACCGAC
VSP2 Reverse	TCTTCCACAACTTCCAACGG
[2]*PDF1.2* Forward	TTTGCTGCTTTCGACGCAC
[2]*PDF1.2* Reverse	CGCAAACCCCTGACCATG
ERF5 Forward	ACGTTAACGGTGGAGAGA
ERF5 Reverse	GAGATAACGGCGACAGAAGC

1. All other qRT-PCR primers used in this study are described elsewhere. Aside from *ERF5*, all other *ERF* gene expression analyses (e.g. Table 1) were performed with primers from Czechowski *et al.* (2004). Primers for *PR1*, *PDF1.2* (see footnote 2) and *Atg13320* are described by Van der Does *et al.* (2013).
2. Besides this primer set for *PDF1.2* another *PDF1.2* primer set was also used (for qRT-PCR analysis of the experiment depicted in Fig. 1). This second set is described by Van der Does *et al.* (2013), where they were named *PDF1.2* FW3 and RV3. Both primer sets give the same fold change results.

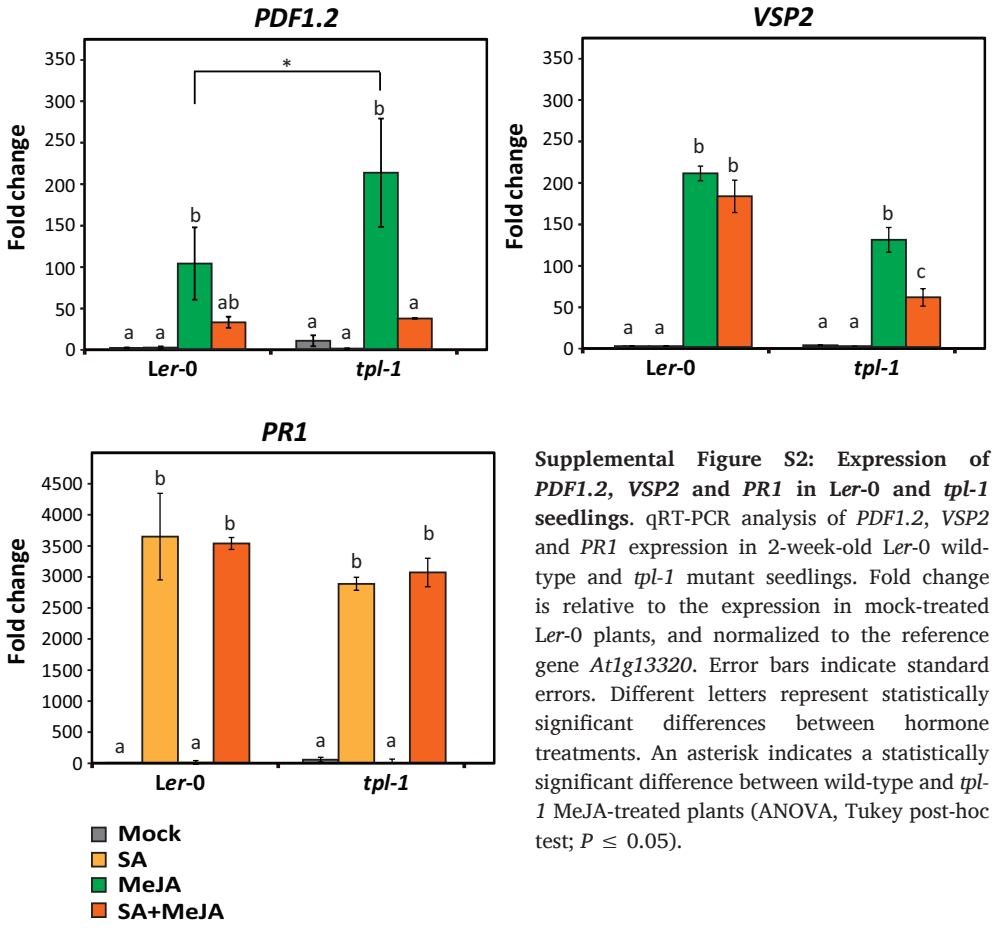

A
PDF1.2
VSP2
PR1
Fold change
Col-0
erf5
rap2.6L
rap2.6
Ws-0
dear1
erf8
Mock
SA
MeJA
SA+MeJA
NS
24h after treatment
5h after treatment


PDF1.2
VSP2
PR1
Fold change
Col-0
erf1
ora59
aterf-1
dreb2a
erf112
erf13
Mock
SA
MeJA
SA+MeJA
NS


B
Wt Col-0
hre2
erf12
erf4
5 h
24 h
SA
MeJA
PDF1.2
VSP2
PR-1
18S



Supplemental Figure S1: Expression of *PDF1.2*, *VSP2* and *PR1* in all tested *erf* mutants determined by qRT-PCR and RNA blots. (A) qRT-PCR analysis of *PDF1.2*, *VSP2* and *PR1* expression in 5-week-old plants treated with water (Mock), 1 mM SA and/or 0.1 mM MeJA, and harvested at 5 h (*VSP2*) and 24 h (*PDF1.2*, *PR1*) post-treatment. Gene expression in *erf* mutants was compared to that in wild-type Col-0 or Ws-0 plants of the same experiment (each graph represents one experiment). Different letters indicate statistically significant differences between the genotypes per treatment, NS indicates no statistically significant difference (ANOVA, Tukey post-hoc test; $P \leq 0.05$). (B) RNA gel blot analysis of *PDF1.2*, *VSP2* and *PR1* expression in 5-week-old plants treated with water (Mock), 1 mM SA and/or 0.1 mM MeJA, and harvested at 5 h and 24 h post-treatment. Equal loading of RNA samples was checked using a probe for *18S* rRNA. Gene expression in *erf* mutants was compared to that in Col-0 plants of the same experiment (each row represents one experiment).



Supplemental Figure S2: Expression of *PDF1.2*, *VSP2* and *PR1* in L*er*-0 and *tpl-1* seedlings. qRT-PCR analysis of *PDF1.2*, *VSP2* and *PR1* expression in 2-week-old L*er*-0 wild-type and *tpl-1* mutant seedlings. Fold change is relative to the expression in mock-treated L*er*-0 plants, and normalized to the reference gene *At1g13320*. Error bars indicate standard errors. Different letters represent statistically significant differences between hormone treatments. An asterisk indicates a statistically significant difference between wild-type and *tpl-1* MeJA-treated plants (ANOVA, Tukey post-hoc test; $P \leq 0.05$).

CHAPTER 3

Dissecting the role of nuclear NPR1 in SA/JA crosstalk

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ABSTRACT

The plant immune signaling network exists of different signaling pathways that exhibit extensive interplay. The plant hormones salicylic acid (SA) and jasmonic acid (JA) play an essential role in immune regulation and have been shown to antagonize each other's activity. NPR1, the master transcriptional regulator of the SA pathway, is known to be required for the antagonistic action of SA on JA signaling, but the mechanism of NPR1-mediated suppression of JA responses by SA is unclear. Here, we studied natural variation in the NPR1 protein in Arabidopsis and try to correlate this to the level of SA/JA crosstalk in Arabidopsis accessions. We further analyzed several mutants that alter the localization, conformation or stability of NPR1. This revealed evidence for a role of nuclear NPR1 in the suppression of JA-responsive gene expression. Moreover, it demonstrated that redox regulation of the NPR1 protein and its degradation by interaction with NPR3 and NPR4 are not important in this process. Furthermore, mutation of two cysteine residues in NPR1 (Cys^{82} or Cys^{216}), which changes the conformation of NPR1, disrupted suppression of JA marker genes *PDF1.2* and *VSP2* by SA, while SA-induced *PR1* expression was not reduced. This allowed us to dissect the function of NPR1 in activation of *PR1* expression from its function in SA/JA crosstalk. We hypothesized that the cysteine-mutated NPR1 impaired part of the NPR1-regulated SA-induced transcriptome that is important for SA/JA crosstalk. By performing RNAseq, 32 SA-responsive genes were identified that are lower expressed in plants expressing the Cys^{82}-mutated NPR1 than in Col-0, and thus may be involved in the SA-mediated suppression of JA-responsive genes. This included four *WRKY* transcription factors genes that are known targets of NPR1 and have been suggested to regulate suppression of JA-responsive gene expression. Furthermore, we showed that the GCC-box was overrepresented in the promoters of JA-inducible genes that are suppressed by SA in an NPR1-dependent manner, suggesting that NPR1 targets ERF transcription factors. Our data thus suggest that after SA accumulation, NPR1 translocates to the nucleus where it activates *WRKYs*, and directly or indirectly targets ERF transcription factors, resulting in antagonism of JA-inducible genes.

INTRODUCTION

The plant immune system relies on the recognition of pathogen- or insect-derived molecules and altered self-molecules by host receptors (Dodds and Rathjen, 2010). Recognition leads to the activation of defenses that, when successful, stop infection and signal systemic tissue to become primed for enhanced defense against future attack. Downstream of recognition, plant hormones play vital roles in triggering the plant immune signaling network (Pieterse *et al.*, 2012). Salicylic acid (SA) and jasmonic acid (JA) are the major defense hormones and are important for inducing both the local defense response to the attacker at hand and in establishing systemic resistance. In general, SA is essential in the defense response against biotrophic pathogens, which feed on living host cells. Conversely, the JA pathway is generally effective against necrotrophic pathogens, which kill host tissue and feed on the contents, and against herbivorous insects. JA-dependent defense against necrotrophic pathogens is co-regulated by ethylene, while for defense against chewing herbivores, JA works in concerted action with abscisic acid (ABA) (Pieterse *et al.*, 2012; Vos *et al.*, 2013b).

Activation of the SA or JA pathway triggers massive transcriptional reprogramming, which includes activation of a distinct set of pathogenesis-related (*PR*) genes by both hormones. Signaling downstream of SA is largely regulated by the transcriptional regulator NON-EXPRESSOR OF PR GENES1 (NPR1), which is required for the activation of many SA-responsive genes including the SA marker gene *PR1*, and for SA-dependent disease resistance (Cao *et al.*, 1994; Delaney *et al.*, 1995; Vlot *et al.*, 2009). NPR1 interacts with TGA transcription factors to activate the expression of *PR1* and activates expression of genes encoding WRKY transcription factors, which fine-tune and amplify downstream responses (Wang *et al.*, 2006; Blanco *et al.*, 2009). Activation of JA-responsive genes relies on the degradation of JASMONATE ZIM-domain (JAZ) repressor proteins that suppress the activity of JA-responsive transcription factors. CORONATINE INSENSITIVE1 (COI1) is the F-box protein in the SCF^{COI1} complex that targets JAZ for degradation when binding JA-Ile, the biologically active form of JA (Pauwels and Goossens, 2011). Mutants in the JA-receptor COI1 are more susceptible to herbivorous insects and necrotrophic pathogens (Thomma *et al.*, 1998; Stotz *et al.*, 2002). Two branches are distinguished in JA-dependent defense signaling, the first is co-regulated by JA and ethylene and controlled by ERF transcription factors such as OCTADECANOID-RESPONSIVE ARABIDOPSIS59 (ORA59), which activates the expression of marker gene *PLANT DEFENSIN1.2 (PDF1.2)* (Zarei *et al.*, 2011). The other branch is co-regulated by JA and ABA and controlled by MYC transcription factors, which activate JA marker gene *VEGETATIVE STORAGE PROTEIN2 (VSP2)* (Fernández-Calvo *et al.*, 2011).

Increasing knowledge shows that the hormonal signaling pathways involved in plant defense are interconnected in an intricate signaling network (Tsuda *et al.*, 2009; Moore *et al.*, 2011; Kim *et al.*, 2014). This complex network enables antagonistic and synergistic interactions between the pathways, a phenomenon that is referred to as hormonal crosstalk (Robert-Seilaniantz *et al.*, 2011; Pieterse *et al.*, 2012). Several studies have tried to elucidate the molecular mechanism underlying SA-mediated suppression of the JA response (from hereon: SA/JA crosstalk). In the model plant *Arabidopsis thaliana* (Arabidopsis), SA targets the JA pathway downstream of JA biosynthesis and of COI1, and likely acts by directly targeting JA-dependent gene expression, partly through degradation of positive regulator ORA59 (Leon-Reyes *et al.*, 2010b; Van der Does *et al.*, 2013). Several SA-controlled transcriptional (co) regulators that can suppress JA-dependent gene expression have been identified (Caarls *et al.*, 2015). The transcription factors TGA2, TGA5 and TGA6 (Leon-Reyes *et al.*, 2010a; Zander *et al.*, 2014) and WRKY41, WRKY46, WRKY53, WRKY62 and WRKY70 (Li *et al.*, 2004; Mao *et al.*, 2007; Higashi *et al.*, 2008; Hu *et al.*, 2012) have all been shown to be involved in suppression of JA-responsive genes. Transcriptional co-regulator NPR1 is also required for SA-mediated suppression of JA marker genes *VSP2*, *PDF1.2* and *LOX2* (Spoel *et al.*, 2003). Moreover, NPR1 controls the SA-mediated suppression of JA-induced resistance against insects and necrotrophic pathogens (Spoel *et al.*, 2007; Leon-Reyes *et al.*, 2009). Also in tomato, rice and *Nicotiana attenuata,* NPR1 plays an important role in the interaction between SA and JA signaling pathways (Rayapuram and Baldwin, 2007; Yuan *et al.*, 2007; El Oirdi *et al.*, 2011), indicating that NPR1 has evolved as an important SA-induced modulator of the JA pathway.

SA-mediated regulation of the NPR1 protein and NPR1-induced activation of SA-responsive gene expression have been extensively studied (reviewed by Pajerowska-Mukhtar *et al.*, 2013). The NPR1 protein contains a number of conserved domains, such as a C-terminal transactivation domain, a nuclear localization signal (NLS) and four domains involved in protein-protein interaction, that enable it to act as a transcriptional co-activator (Kuai *et al.*, 2015). The ankyrin repeat domain mediates the interaction of the NPR1 protein with TGA transcription factors that are required for *PR* gene activation (Zhang *et al.*, 1999; Zhou *et al.*, 2000; Després *et al.*, 2003). Secondly, NPR1 contains a BTB/POZ domain, that interacts with TGA2 to negate its N-terminal repression domain (Boyle *et al.*, 2009). Moreover, under non-inducing conditions it acts as an auto-inhibitory domain that masks the C-terminal transactivation domain under non-inducing conditions. Binding of SA to NPR1 at the C-terminal cysteine residues Cys^{521} and Cys^{529} disrupts the interaction between the BTB/POZ domain and the transactivation domain, converting NPR1 into an activated transcriptional co-activator (Rochon *et al.*, 2006; Wu *et al.*, 2012; Manohar

et al., 2015). Finally, NPR1 interacts with NIMIN proteins, which are negative regulators of NPR1, via two distinct domains (Hermann *et al.*, 2013).

As a transcriptional co-activator, NPR1 targets the expression of *PR* genes and several *WRKY*s (Wang *et al.*, 2006; Blanco *et al.*, 2009). Activation of these genes by NPR1 is controlled by the subcellular localization of NPR1 and several SA-controlled modifications of the NPR1 protein. Under non-inducing conditions, intramolecular disulfide bonds are formed between cysteine residues of NPR1 monomers, resulting in the formation of oligomers, which are sequestered in the cytosol due to their large size. Changing either one of these cysteines in NPR1, Cys^{82} or Cys^{216}, to an alanine, results in increased monomerization and nuclear localization of NPR1 (Mou *et al.*, 2003). S-nitrosylation of NPR1 cysteine residues facilitates oligomerization (Tada *et al.*, 2008). Accumulation of SA causes a redox change in the cell after which thioredoxins (TRX) TRX-h3 and TRX-h5 reduce the cysteine residues, breaking the intramolecular disulfide bonds, and thus releasing NPR1 monomers that can move to the nucleus via nuclear pore proteins (Mou *et al.*, 2003; Tada *et al.*, 2008; Cheng *et al.*, 2009). In the nucleus, NPR1 is further modified by sumoylation of serine residues, which enhances the interaction of NPR1 with TGA3 and promotes expression of *PR1* (Saleh *et al.*, 2015). The stability of NPR1 is regulated by phosphorylation of serine residues (Spoel *et al.*, 2009) and by an interaction with NPR3 and NPR4. These NPR1-homologs act as CUL3 ligase adapter proteins in proteasome-mediated degradation of NPR1. NPR3 and NPR4 differ in both their binding affinity for SA and binding capacity to NPR1, allowing an interplay between SA levels and NPR1 degradation (Fu *et al.*, 2012).

Many questions still remain on the mechanism of NPR1-mediated suppression of JA responses. In Arabidopsis, a fusion protein of NPR1 and the hormone binding domain (HBD) that was retained in the cytosol was shown to suppress *PDF1.2* expression in seedlings treated with a combination of SA and methyl JA (MeJA). This suggested that the function of NPR1 in suppression of JA-induced gene expression takes place in the cytosol (Spoel *et al.*, 2003). Moreover, in rice, overexpression of OsNPR1 resulted in suppression of JA-responsive gene expression and reduced defense against an insect, but overexpression of a form of NPR1 that was constitutively localized in the nucleus due to mutation of two cysteines (Cys^{76} and Cys^{216}) impaired suppression of JA-responsive genes defense against insects (Yuan *et al.*, 2007). These results suggested that the suppression of JA responses by NPR1 occurs in the cytosol. However, it has not been elucidated how a cytosol-located NPR1 can repress JA-inducible gene expression.

Redox-based protein modifications regulate NPR1 activity and are likely also important for the establishment of SA/JA crosstalk. SA treatment increases the

total glutathione levels and results in a higher ratio of reduced (GSH) to oxidized glutathione (GSSG). Interestingly, JA treatment decreases the total amount of glutathione, and increases the amount of GSSG relative to GSH (Spoel and Loake, 2011). The timeframe in which SA was able to suppress *PDF1.2* coincided with the cellular redox change. In addition, treatment with glutathione biosynthesis inhibitor BSO prevented SA-mediated suppression of *PDF1.2* (Koornneef *et al.*, 2008a). These results suggested a role for redox regulation in prioritization of the SA pathway over the JA pathway. Glutaredoxins (GRXs) are small ubiquitous enzymes that use glutathione to reduce disulfides and have also been implicated in suppression of JA-responsive gene expression. Several members of the group III class of GRXs in Arabidopsis interact with TGA transcription factors and suppress expression of *ORA59* and *PDF1.2* (Ndamukong *et al.*, 2007; Zander *et al.*, 2012). Redox-induced activation of NPR1 might also be important for suppression of JA responses, but this has not been studied yet.

In this study, we investigated the role of NPR1 in SA-mediated suppression of JA-induced gene expression. Suppression by SA of a subset of SA-antagonized JA-inducible genes was dependent on NPR1, and the GCC-box was enriched in the promoters of these genes. Next, naturally occurring polymorphisms in NPR1 were compared between Arabidopsis accessions that differed in their ability to display SA/JA crosstalk. We further analyzed several mutants in redox transmission and mutants that change the location, activity or stability of NPR1. In this way, we show that redox regulation of NPR1 and degradation of NPR1 by NPR3 and NPR4 are not important for SA/JA crosstalk. In addition, we showed that, in contrast to previous findings in seedlings (Spoel *et al.*, 2003), nuclear localization of NPR1 is required in mature plants. We further demonstrate that two lines overexpressing cysteine mutated versions of the NPR1 protein, 35S:*npr1*C82A-GFP (C82A), 35S:*npr1*C216A-GFP (C216A) in the *npr1-1* background, were impaired in suppression of JA marker genes *PDF1.2* and *VSP2*, but were not affected in SA-induced *PR1* expression. The C82A mutant was subjected to high-throughput RNA-sequencing (RNA-seq) after SA treatment, as it could aid in identifying SA-regulated genes that are specifically involved in SA/JA crosstalk and not in activation of SA-inducible responses. We found that expression of several SA-induced NPR1-dependent genes, including four *WRKY*s, is reduced in this mutant. Our results point to a model for SA/JA crosstalk in mature plants in which NPR1 acts in the nucleus by activating downstream targets, including *WRKY* transcription factors genes, of which the corresponding proteins could suppress JA-responsive genes.

RESULTS

NPR1-dependency of a subset of SA-antagonized JA-inducible genes

Previously, expression of the JA marker genes *VSP2, LOX2* and *PDF1.2* was shown to be antagonized by SA in an NPR1-dependent manner. Here, we performed transcript profiling to determine the NPR1-dependency of additional JA-responsive genes that are sensitive to SA-mediated suppression. Three separate experiments were performed with five-week-old wild-type Col-0 and mutant *npr1-1* plants that were mock-treated or treated with SA, MeJA, or a combination of both hormones. Leaf tissue was harvested 28 h after treatment and the transcript profile of each independent experiment was analyzed with Affymetrix ATH1 GeneChips. The transcriptome data of Col-0 plants have been described by Van der Does *et al.* (2013) and details on the procedure and results can be found therein. Here, the SA and MeJA-responsiveness of genes in *npr1-1* were compared to that of genes in Col-0. In Col-0, MeJA treatment resulted in significant upregulation of 175 genes compared to mock treatment (Van der Does *et al.*, 2013). Most of these genes, 128 of the 175 (73%), were also significantly upregulated by MeJA in the *npr1-1* mutant, confirming that although NPR1 seems to regulate a subset of JA-inducible genes, it is not necessary for the induction of the majority of JA-responsive genes (Supplemental Table S1).

In contrast, only 10 of the 50 genes (20%) that were significantly upregulated in response to SA treatment in Col-0 plants, were also upregulated in *npr1-1* plants, indicating that the majority of SA-induced genes (80%) in this setup depended on NPR1. This confirms the importance of NPR1 for SA-induced gene expression (Supplemental Table S1). The proportion of SA-induced genes that are NPR1-dependent is comparable to that found by Blanco *et al.* (2009), although the total number of genes we found to be significantly upregulated is lower, possibly because of the late time point of sampling. The genes that we identified as SA-upregulated and NPR1-dependent include known NPR1-targets, such as *PR1*, *WRKY18*, *WRKY38*, *NIMIN1* and *UGT76B1* (Wang *et al.*, 2006; Blanco *et al.*, 2009). GRXS13 was previously described as SA-induced and NPR1-independent (Blanco *et al.*, 2009), and also this was confirmed in our dataset. These results indicate that we were able to determine NPR1-dependency of induced gene expression, and confirm that NPR1 plays a greater role in SA- than in JA-upregulated gene expression.

In Col-0 plants, 34% of the MeJA-induced genes was affected by SA/JA crosstalk: 59 of the 175 MeJA-induced genes were at least 1.5-fold repressed ($\log_2$ fold change SA + MeJA/MeJA $\leq$ -0.6) by the combined treatment with SA and MeJA, compared to MeJA alone (Supplemental Table S1; Van der Does *et al.*, 2013). Of the 59 analyzed

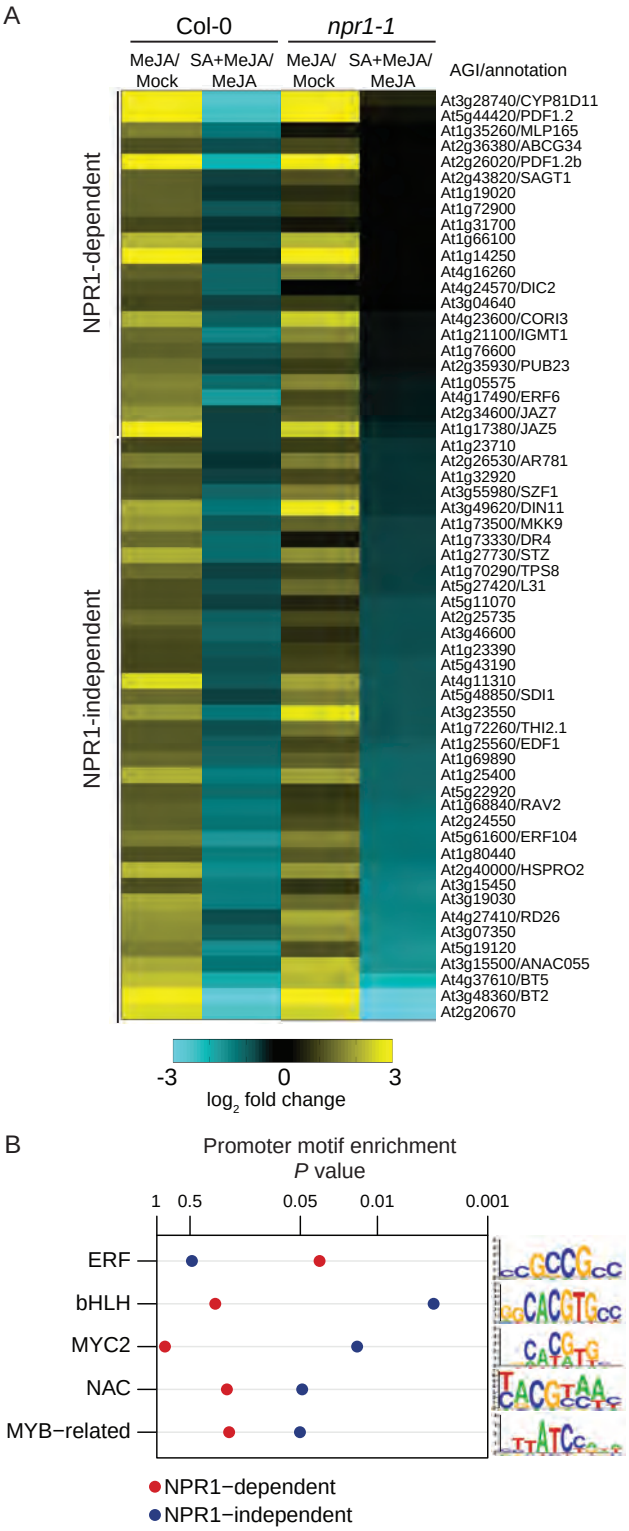


Figure 1: NPR1-(in)dependency of MeJA-induced genes that are antagonized by SA.
(A) Expression of 59 genes in MeJA-treated Col-0 and *npr1-1* plants relative to mock-treated plants, or SA + MeJA-treated Col-0 and *npr1-1* plants relative to MeJA-treated plants (log_2 fold change). Genes are ranked on difference in log_2 fold change in SA + MeJA/MeJA in *npr1-1*. For genes that showed a log_2 fold change SA + MeJA/MeJA ≥ -0.5, suppression was considered NPR1-dependent (upper 22 genes). If the difference between SA + MeJA/MeJA was ≤ -0.6 in *npr1-1*, then suppression was considered NPR1-independent (lower 37 genes). See Supplemental Table S1 for expression values of all genes. **(B)** A promoter motif analysis was performed on the promoters of SA/JA-suppressed genes of the NPR1-regulated gene set (22 genes) and the NPR1-independent gene set (37 genes), as identified in Figure 1A. Five selected motifs are depicted with the associated *P* values for the NPR1-dependent (red dots) or NPR1–independent group (blue dots). For each motif, the sequence logo is shown, while the associated type of transcription factor is indicated on the left of the graph. For the complete list of motifs, see Supplemental Table S2.

MeJA-induced, SA-suppressed genes, 22 were not suppressed by SA in the *npr1-1* mutant ($\log_2$ fold change SA + MeJA/MeJA $\geq$ -0.5), indicating that the suppression of these genes by SA depended on a functional NPR1 protein (Fig. 1A; Supplemental Table S1). This set of genes included JA marker gene *PDF1.2*, of which SA-mediated suppression has previously been shown to be NPR1-dependent (Spoel *et al.*, 2003). Here, we demonstrated that suppression of 21 other genes including *ERF6*, *CORI3*, *JAZ5* and *JAZ7*, was also dependent on NPR1. Surprisingly, 37 of the 59 genes were still suppressed by SA in the *npr1-1* mutant, suggesting that although NPR1 was essential for SA-mediated suppression of a significant group of JA-responsive genes, there was an even greater proportion of JA-responsive genes that could be suppressed by SA in an NPR1-independent manner in this experiment.

To learn more about the transcriptional regulators that are involved in SA/JA crosstalk in an NPR1-dependent or -independent manner, we analyzed the core promoters of SA/JA-suppressed genes of the NPR1-regulated gene set (22 genes) and the NPR1-independent gene set (37 genes). For this, we scanned the 500-bp upstream of the annotated 5'-UTR of all genes. Interestingly, different promoter motifs were enriched in the NPR1-dependent set compared to the NPR1-independent set. The complete list of transcription factor binding motifs and their significance values can be found in Supplemental Table S2. In the NPR1-dependent set, a promoter motif resembling a GCC-box motif was enriched (Fig. 1B), which suggests an effect of NPR1 on ERF transcription factors, which are known to bind the GCC-box (Franco-Zorrilla *et al.*, 2014). In contrast, in the NPR1-independent set of SA/JA crosstalk genes, enrichment of motifs recognized by bHLH, NAC or MYB-related transcription factors was found (Fig. 1B). The GCC-motif has previously been shown to be sufficient for SA-mediated suppression of JA-responsive gene expression (Van der Does *et al.*, 2013). The specific enrichment for the GCC-box in the NPR1-dependent group suggests that NPR1 is required for SA-induced targeting of ERF transcription factors that bind to this motif. The ERF transcription factor ORA59 is a central target in SA/JA crosstalk, as both its transcription and protein levels are negatively affected by SA (Van der Does *et al.*, 2013; Zander *et al.*, 2014). Therefore it is likely that NPR1 targets this specific transcription factor. The JA marker genes *VSP1* and *LOX2*, which are known to be suppressed by SA in an NPR1-dependent manner, were not included in this subset of genes, probably because of the late time point of harvesting. Suppression of these type of genes by SA is usually measured at 5-6 h after treatment (Leon-Reyes *et al.*, 2009; Chapter 2). In conclusion, we showed that suppression of a subset of SA/JA crosstalk-sensitive genes was repressed by SA in an NPR1-dependent manner and found evidence that NPR1 may target ERF transcription factors in this process.

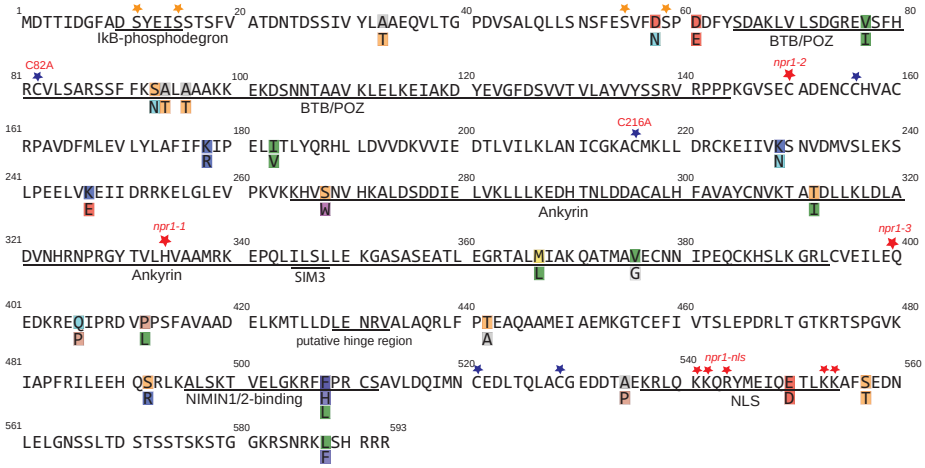
Natural variation in the NPR1 protein in Arabidopsis accessions defective in crosstalk

We next aimed to understand more about the domains of the NPR1 protein that are important for SA/JA crosstalk. Many *npr1* mutants have been identified that disrupt NPR1-modulated SA-induced gene expression. Three of these mutants, *npr1-1*, *npr1-2* and *npr1-3*, have previously been tested for suppression of JA-induced genes by SA. The *npr1-1* and *npr1-2* mutants are affected in both SA-induced *PR1* expression and SA-suppressed JA-inducible *PDF1.2* expression (Cao *et al.*, 1994; Cao *et al.*, 1997; Spoel *et al.*, 2003; Leon-Reyes *et al.*, 2010a). The *npr1-1* mutation alters His^{334} to a tyrosine and the *npr1-2* mutation Cys^{150} to a tyrosine (Fig. 2A; Cao *et al.*, 1997), which disrupt the interaction between NPR1 and TGA transcription factors (Zhang *et al.*, 1999; Zhou *et al.*, 2000), suggesting that this interaction is important for SA-mediated induction of *PR1* as well as suppression of *PDF1.2*. The *npr1-3* mutation causes a premature stop codon, resulting in a truncated protein that lacks among others the C-terminal nuclear localization signal and therefore localizes to the cytosol (Fig. 2A; Cao *et al.*, 1997). The observation that *npr1-3* is less affected in SA-antagonism of JA-induced *PDF1.2* expression than *npr1-1,* has been interpreted as a function of NPR1 in the cytosol in SA/JA crosstalk (Leon-Reyes *et al.*, 2009).

Here, we explored the natural variation in NPR1 in Arabidopsis accessions and tried to correlate naturally occurring polymorphisms in NPR1 to a disrupted SA/JA crosstalk. To test SA/JA crosstalk in 349 Arabidopsis accessions, expression of *PDF1.2* after treatment with MeJA alone or a combination or SA and MeJA was determined. Results of this natural variation study will be described in detail elsewhere. We found that in 280 of the 349 tested accessions, *PDF1.2* was suppressed by the combination treatment of SA and MeJA compared to MeJA treatment alone. This indicates that SA/JA crosstalk is conserved in Arabidopsis, as was suggested earlier based on an investigation of 16 Arabidopsis accessions (Koornneef *et al.*, 2008a). Sequences for *NPR1* were available for 152 of the tested accessions (http://signal.salk.edu/atg1001). We aligned all NPR1 protein sequences and identified 25 unique amino acid variations between Col-0 and one or more of the 151 other accessions (Fig. 2A). Interestingly, the cysteine and serine residues that are modified or phosphorylated to regulate NPR1's activity and stability, namely Cys^{82}, Cys^{216}, Cys^{156}, Cys^{521} and Cys^{529} and Ser^{11}, Ser^{15}, Ser^{55} and Ser^{59} are conserved in all studied sequences (Fig. 2A).

In the set of 152 accessions, we identified nine accessions in which *PDF1.2* expression was induced by MeJA (log_2 fold change MeJA/Mock ≥ 0.6) but not suppressed after the SA and MeJA combination treatment (log_2 fold change (SA + MeJA)/MeJA ≥ -0.5). These nine accessions were HR-5, Si-0, L*er*-1, T1080, Mz-0, Jl-3, Ha-0, Se-0 and Tsu-0 (Fig. 2B). In the NPR1 sequence of these accessions, nine unique amino

A



B

Accessions	log$_2$ fold change *PDF1.2*		Amino acid changes in NPR1 vs Col-0
	MeJA/ Mock	SA+MeJA/ MeJA	
HR-5	11.25	0.08	K178R I183V L588F
Si-0	4.91	0.31	
L*er*-1	2.52	0.33	K178R I183V S268W Q406P F507H E550D
T1080	3.26	1.03	K178R I183V S268W M367L Q406P
Mz-0	0.84	1.16	K178R I183V Q406P L588F
Jl-3	5.07	1.33	K178R I183V S268W M367L Q406P
Ha-0	1.53	1.60	K178R I183V L588F
Se-0	3.78	1.76	K178R I183V S268W M367L Q406P A535P
Tsu-0	3.10	1.91	
Col-0	4.12	-2.50	
Edi-0	4.70	-4.83	K178R I183V Q406P L588F
LL-0	1.71	-2.98	K178R I183V S268W M367L Q406P A535P
Ct-1	2.41	-2.81	K178R I183V S268W M367L Q406P F507H

Figure 2: Natural variation in the NPR1 protein and SA/JA crosstalk in Arabidopsis accessions. **(A)** Representation of the amino acid sequence of the NPR1 protein in Col-0. Known domains are underlined and named: N-terminal IκB-like phosphodegron (D10-S15), of which Ser[11] and Ser[15] can be sumoylated and phosphorylated, leading to degradation of NPR1. Phosphorylation of Ser[55] and Ser[59] inhibits sumoylation and keeps NPR1 stable (Spoel *et al.*, 2009; Saleh *et al.*, 2015). The BTB/POZ domain (S65-P144) suppresses the C-terminal activation domain when no SA is present (Rochon *et al.*, 2006; Wu *et al.*, 2012). The ankyrin repeat (K265-L393) interacts with TGA transcription factors (Zhang *et al.*, 1999; Zhou *et al.*, 2000; Després *et al.*, 2003). The SIM3 domain (I345-L349) is required for sumoylation of NPR1 (Saleh *et al.*, 2015). Further C-terminal, there is a putative hinge region (L428-V432) (Maier *et al.*, 2011) and a binding site for NIMIN1 and 2 (A495-S512) (Hermann *et al.*, 2013). The nuclear localization signal (NLS; K537-K554) contains five basic amino acids that are critical for nuclear translocation of NPR1 (Kinkema *et al.*, 2000). Conserved cysteine residues (indicated with blue stars) and serine residues (indicated with orange stars) can be modified to regulate function of NPR1. The locations of the mutations in *npr1-1*, *npr1-2*, *npr1-3*, and *npr1-nls* are indicated with red stars. Amino acids in the NPR1 protein sequence that differ between Col-0 and one or more of 152 analyzed accessions are shaded in the Col-0 sequence (top). The amino acid that is found in another accession(s) is indicated with shading

underneath. RasMol colors are used for the shading according to traditional amino acid properties. **(B)** Nine accessions were identified in which *PDF1.2* was induced by MeJA (log_2 fold change MeJA/Mock ≥ 0.6), and not suppressed by the combination treatment of SA and MeJA compared to MeJA treatment alone (log_2 fold change (SA + MeJA)/MeJA ≥ -0.5). For comparison, four accessions in which *PDF1.2* is suppressed by SA are included. All amino acid changes in NPR1 in these accessions relative to Col-0 are shown. One of the amino acid changes (E550D, underlined) is only present in L*er*-1, and not present in any of the other analyzed accessions in which *PDF1.2* is suppressed by SA treatment.

acids were different compared to the Col-0 NPR1 sequence (Fig. 2B). We found no amino acid differences that were shared between all nine accessions, but a number of accessions had amino acids polymorphisms in common. Three of these changes, K178R, I183V and E550D, are conservative amino acid differences, meaning that they are not expected to change NPR1 protein function (similar colors in Fig. 2A). Six differences are non-conservative; two of them, S268W and M367L, are located in the ankyrin repeat and four others (Q406P, F507H, A535P and L588F) locate to regions without a known function (Fig. 2A). It is possible that these six non-conservative differences alter the function of NPR1, however, these six polymorphisms are all also present in accessions that still displayed SA-antagonized expression of *PDF1.2,* as shown in Figure 2B for the accessions Edi-0, LL-0, and Ct-1. This suggests that these differences in NPR1 by themselves do not result in a disruption of SA/JA crosstalk. One amino acid difference detected was only present in an accession that did not display SA/JA crosstalk, i.e. E550D in L*er*-1 (underlined in Fig. 2B), however this results in a similar amino acid in the nuclear localization signal. In conclusion, we do not find a polymorphism in the NPR1 amino acid sequence that can be linked to the ability of accessions to display SA/JA crosstalk.

Screening mutants in redox transmitters, in NPR3 and NPR4, and mutants that change NPR1 localization or conformation for SA/JA crosstalk

Next, we assessed whether mutants that disrupt the regulation or activity of NPR1 are affected in SA/JA crosstalk. Figure 3A is a schematic representation of the regulation of NPR1 before and after SA accumulation, in which the mutant and transgenic lines that were tested are indicated in red. To study SA/JA crosstalk in these lines, five-week-old plants were mock-treated or treated with SA, MeJA, or a combination of SA and MeJA and the expression of *PR1*, *VSP2* and *PDF1.2* was measured relative to mock-treated Col-0 plants. For each treatment and genotype, three individual plants were harvested separately as three biological replicates. In Col-0, MeJA-induced expression of both *PDF1.2* (measured 24 h after treatment) and *VSP2* (measured 5 h after treatment) was suppressed by the addition of SA (Fig. 3B). The *npr1-1* mutant was affected in *PR1* induction by SA treatment and in the antagonism of MeJA-induced *PDF1.2* and *VSP2* expression by SA, confirming previous results (Fig. 3B).

SA/JA crosstalk in trx, ntra and gsnor1 mutants

Under non-inducing conditions, NPR1 is present in the cytosol as an oligomer, formed by intramolecular disulfide bonds between cysteine residues. Following a SA-mediated redox change, TRX-h3 and TRX-h5 reduce these disulfide bonds, generating NPR1 monomers that translocate to the nucleus. Thioredoxins are reduced themselves, and thus regenerated, by NADPH-thioredoxin reductase (NTR) (Reichheld *et al.*, 2007; Fig. 3A). The TRX-NTR system is required for full induction of *PR* genes by SA and for systemic acquired resistance (SAR; Tada *et al.*, 2008). We were interested if these redox transmitters are also required for suppression of JA-responsive genes. Here, we tested the *trx-h3* and *trx-h5* mutants and thioredoxin reductase mutant *ntra* for suppression of *PDF1.2* and *VSP2* by SA. We found that that SA-induced *PR1* expression was reduced in *trx-h3* and *trx-h5* mutants (Fig. 3B; Supplemental Figure S1), which is in line with results from Tada *et al.* (2008). In the *ntra* mutant, induction of *PR1* by SA was not lower than in Col-0 at 5 h after treatment, but at 24 h a significantly reduced *PR1* expression was detected ($P \leq 0.05$; Supplemental Figure S1). In both *trx* mutants and in *ntra*, the upregulation of *PDF1.2* and *VSP2* after MeJA treatment was comparable to that in Col-0. Expression of both genes was suppressed by the combination treatment in all three mutants. This indicates that the individual components of the NTR-TRX system are by themselves not required for the SA-mediated suppression of *PDF1.2* and *VSP2*.

While thioredoxin-mediated reduction of NPR1 leads to its monomerization, its oligomerization is facilitated by S-nitrosylation of cysteine residues of NPR1 by S-nitrosoglutathione (GSNO; Fig. 3A). The enzyme GSNOR regulates GSNO levels. In the *gsnor1* mutant, which accumulates high levels of GSNO, SA-induced monomerization and nuclear localization are inhibited (Tada *et al.*, 2008). We tested whether SA-mediated suppression of *PDF1.2* and *VSP2* expression was affected in this mutant. We found that *PR1* expression was induced by SA in *gsnor1* (Fig. 3B). However, the level of induction was lower than that in Col-0, as was reported previously (Supplemental Figure S1; Tada *et al.*, 2008). MeJA-induced activation of *PDF1.2* was comparable to Col-0, while MeJA-induced activation of *VSP2* was low. However, induction of *VSP2* was below average in Col-0 in this experiment as well (Supplemental Figure S1), and in Figure 3B, the average fold change for expression in Col-0 (from different experiments) is depicted. Most importantly, suppression of *PDF1.2* and *VSP2* by SA was not affected (Fig. 3B). In conclusion, we found that knock-out lines of the four genes, which encode redox transmitters, did not affect SA-mediated suppression of JA-dependent genes, while SA-mediated *PR1* activation was affected. This suggests that the redox-mediated switch from NPR1 oligomers to monomers is not important for suppression of JA-responsive genes by SA.

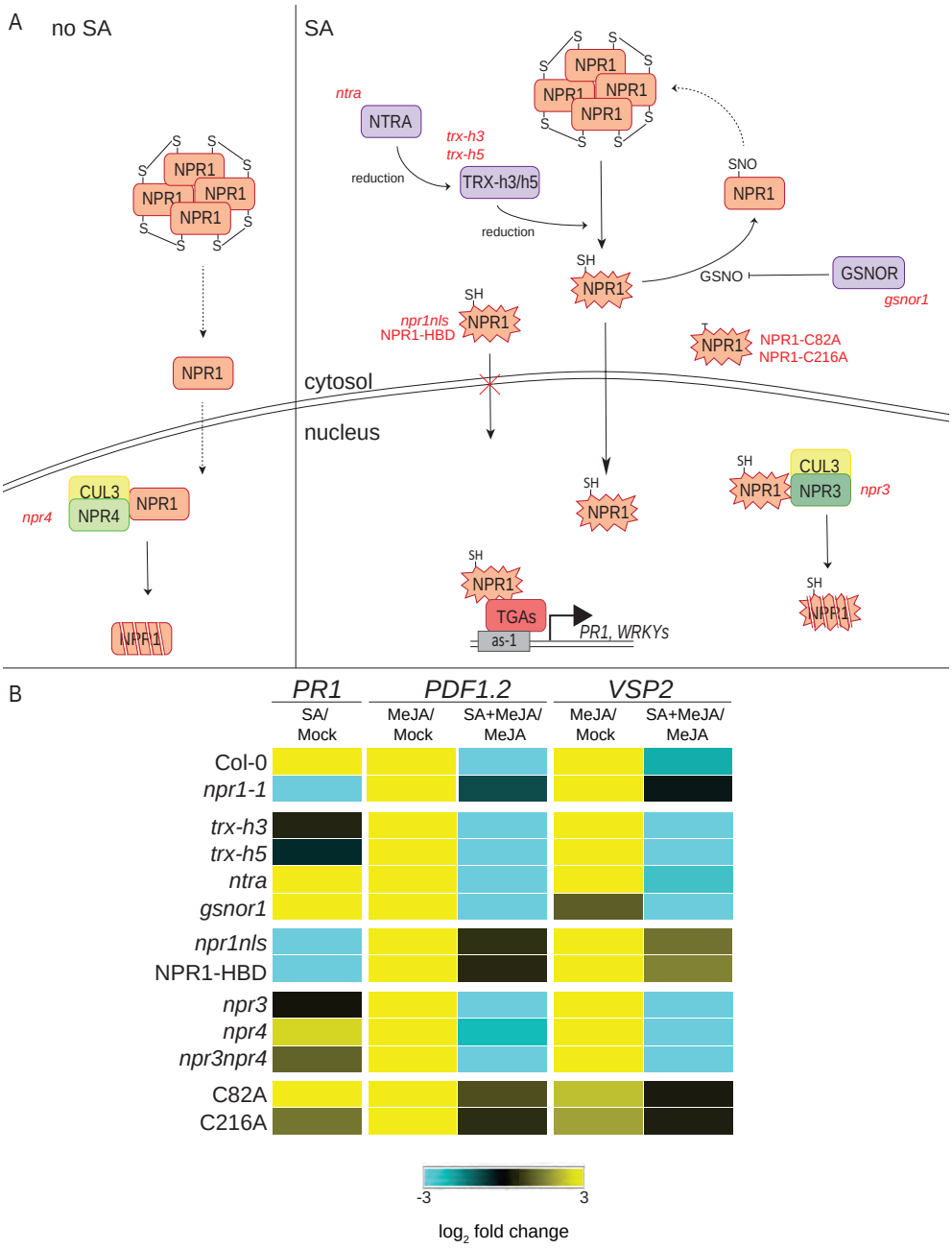


Figure 3: Effect of NPR1-related mutations on SA/JA crosstalk (A) Schematic representation of the regulation of NPR1 in response to SA. Analyzed mutants and transgenic lines are placed next to the processes or proteins that they affect. In uninduced conditions (left), NPR1 forms an oligomer by disulfide bridges between cysteines. Any NPR1 that moves to the nucleus is degraded by interaction with the CUL3-adapter protein NPR4 (Fu *et al.*, 2012). SA accumulation (right) causes a change in cellular redox that is perceived by NPR1. The disulfide bridges are reduced by TRX-h3 and TRX-h5, and subsequently

NPR1 translocates to the nucleus (Tada *et al.*, 2008). Thioredoxins are reduced and thus regenerated by NTRA (Reichheld *et al.*, 2007). Oligomerization is facilitated by S-nitrosylation of cysteine residues by S-nitrosoglutathione (GSNO). High GSNO levels in the *gsnor1* mutant inhibit NPR1 monomerization and nuclear localization (Tada *et al.*, 2008). In the nucleus, NPR1 interacts with TGAs resulting in activation of *PR1*. When SA is present, NPR1 is degraded via interaction with NPR3 (Fu *et al.*, 2012). In the *npr1nls* mutant and the NPR-HBD line, NPR1 is hypothesized to not translocate to the nucleus (Kinkema *et al.*, 2000). In the C82A and C216A mutants, cysteine bridges are not formed and more monomers of NPR1 are present (Mou *et al.*, 2003). **(B)** Relative expression of *PR1*, *PDF1.2* and *VSP2* in Col-0, *npr1-1,* and all tested mutants. Shown is a heat map representation of the $\log_2$ fold change in plants treated with SA compared to mock-treated Col-0 plants in the same experiment (for *PR1*), MeJA-treated plants vs mock-treated Col-0 plants in the same experiment (for *PDF1.2* and *VSP2*) or combined SA and MeJA-treated plants compared to MeJA-treated Col-0 plants in the same experiment (for *PDF1.2* and *VSP2*). The mutants were tested in experiments performed at different times, in which Col-0 and *npr1-1* were always taken along as a control. For Col-0 and *npr1-1*, the average $\log_2$ fold change of several experiments is therefore shown. Expression of *PR1* and *VSP2* shown here was measured at 5 h after treatment. *PDF1.2* expression was measured at 24 h after treatment. See Supplemental Figure S1 for graphs showing fold change expression of *PR1*, *PDF1.2* and *VSP2* in all mutants.

SA/JA crosstalk in NPR1-HBD and npr1nls mutants

After SA induction, translocation of NPR1 into the nucleus is required for expression of NPR1 targets (Fig. 3A). In Arabidopsis seedlings that overexpress a fusion protein of NPR1 (NPR1-HBD) that was retained in the cytosol, *PR1* expression was not induced by SA. However, SA treatment did result in suppression of MeJA-induced *PDF1.2* (Spoel *et al.*, 2003). Moreover, in the *npr1-3* mutant, which lacks the nuclear localization signal, SA-mediated *PDF1.2* suppression was not as severely affected as in *npr1-1* (Leon-Reyes *et al.*, 2009). Therefore, NPR1's function in SA-mediated suppression of JA responses has been suggested to be in the cytosol. To confirm the function of cytosolic NPR1, here we tested adult plants of the NPR1-HBD construct, instead of seedlings that had been tested previously (Spoel *et al.*, 2003). In addition, we used the *npr1nls* mutant, in which five amino acids in the nuclear localization signal are mutated, resulting in exclusively cytosolic localized NPR1 (Kinkema *et al.*, 2000; Fig. 2A). Similar to the *npr1-1* mutant, there was no induction of *PR1* expression following SA treatment in both NPR1-HBD and *npr1nls* (Fig. 3B). MeJA-induced expression levels of *PDF1.2* and *VSP2* were similar to that in Col-0 (Supplemental Figure S1). We found that *PDF1.2* and *VSP2* expression were not suppressed after combined SA and JA treatment in both lines. These results suggest that, in contrast to what was reported earlier for seedlings (Spoel *et al.*, 2003), in mature plants cytosolic NPR1 does not contribute to the suppression of JA-responsive gene expression upon SA treatment.

SA/JA crosstalk in npr3, npr4 and npr3npr4 mutants

NPR3 and NPR4 contain similar domains as NPR1 such as the BTB/POZ domain, ankyrin repeat and NLS, and can also interact with TGA transcription factors (Liu *et al.*, 2005; Zhang *et al.*, 2006). NPR3 and NPR4 are CUL3 adapter proteins that target NPR1

for degradation depending on SA levels: when SA levels are low, NPR4 is suggested to bind NPR1 and to target it for degradation, and when SA levels are high, NPR3 binds NPR1 leading to its degradation (Fig. 3A; Fu *et al.*, 2012). In this way, NPR1 levels are tightly controlled to ensure timely activation of SA-dependent defenses. The *npr3* and *npr4* mutants accumulate increased levels of NPR1 protein. Interestingly, in response to MeJA, the *npr4* mutant was shown to have reduced *VSP2* levels and delayed *PDF1.2* expression (Liu *et al.*, 2005). We tested expression of *PR1*, *PDF1.2* and *VSP2* in the single mutants *npr3*, *npr4* and the double mutant *npr3npr4*. Compared to Col-0, SA-induced *PR1* expression was reduced in *npr3* and in the double mutant, but not in the *npr4* mutant (Fig. 3B). Expression of *VSP2* was induced after MeJA treatment in all mutants (Fig. 3B), although the level of expression was lower in *npr4* and *npr3npr4* (Supplemental Figure S1). This is in line with what was reported by Liu *et al.* (2005). Induction of *PDF1.2* was however not reduced in these mutants. Importantly, suppression of *PDF1.2* and *VSP2* after the combination treatment of SA and JA was as effective as in Col-0. This indicates that NPR3 and NPR4 do not affect the SA-mediated suppression of these genes and suggests that the NPR3/NPR4-mediated degradation of NPR1 is not important for SA/JA crosstalk.

SA/JA crosstalk in C82A and C216A mutants

Cysteine-based modifications of the NPR1 protein are important for its regulation (Spoel and Loake, 2011). When either one of two cysteine residues in NPR1 (Cys^{82} or Cys^{216}) are mutated to alanine in the transgenic lines 35S:*npr1*C82A-GFP (from hereon: C82A), 35S:*npr1*C216A-GFP (from hereon: C216A), and overexpressed in the *npr1-1* background the protein displays increased monomerization, nuclear localization, and constitutive elevated *PR1* expression (Mou *et al.*, 2003). We hypothesized that increased monomerization of NPR1 in these lines would also result in a greater repression of JA-responsive transcription by NPR1. We found that *PR1* induction was enhanced in C82A and reduced in C216A 5 h after SA treatment, but higher in both C82A and in C216A at 24 h after SA treatment (Supplemental Figure S1). Both *PDF1.2* and *VSP2* expression were induced by MeJA in C82A and C216A, but surprisingly, in both mutants, treatment with SA and MeJA did not suppress this expression (Fig. 3B). This shows that while expression of NPR1-C82A and NPR1-C216A complemented the *PR1* activation of the *npr1-1* mutant background, it did not restore suppression of JA-responsive genes by SA. This suggests that the mutation of Cys^{82} and Cys^{216} disrupts the function of NPR1 in SA/JA crosstalk while leaving the function of NPR1 on SA-responsive gene expression intact. Three explanations for the affected crosstalk in the C82A and C216A mutants are considered: (i) increased monomerization and nuclear localization of NPR1 in these mutants disrupts crosstalk, (ii) loss of interaction with a crosstalk-regulatory protein, or (iii) disruption of part of the transcriptional regulation

by NPR1. However, the first explanation is unlikely as we found that in mature plants, nuclear localization is in fact required for SA-mediated suppression of JA-responsive genes.

In conclusion, this mutant analysis with mature plants provides evidence for a specific role of NPR1 in the nucleus in SA-mediated suppression of JA-responsive gene expression that function independently of the role of NPR1 in SA-responsive *PR1* expression. It also shows that redox-mediated modification of the NPR1 protein and degradation of NPR1 via NPR3 and NPR4 are not required for SA/JA crosstalk.

Resistance of mutants C82A and C216A against *Mamestra brassicae*

To study the biological impact of the defect in SA-mediated antagonism of JA-inducible defense genes, resistance of the C82A and C216A mutants to caterpillars of the moth *Mamestra brassicae*, which is controlled by JA-dependent defense signaling, was tested. To this end, wild-type Col-0, *npr1-1*, C82A and C216A plants were infested with *M. brassicae* caterpillars. One first instar larva per plant was allowed to feed for 14 days. When feeding on Col-0 plants, the average weight of the caterpillars was 23 mg after 14 days. When *M. brassicae* fed on the *npr1-1* mutant, this was only 7 mg, indicating a significant reduction of weight ($P \leq 0.05$). On the mutant C82A, caterpillars also gained significantly less weight, i.e. they reached an average weight of 13 mg ($P \leq 0.05$). On C216A, caterpillars weighed on average 18 mg, which was not statistically significant less than on Col-0 (Fig. 4A). The *npr1-1* mutant has been shown to be more resistant against other chewing herbivores as well, which was correlated with increased amounts of glucosinolates (Cui *et al.*, 2002; Stotz *et al.*, 2002; Mewis *et al.*, 2006). Our results suggests that, similar to *npr1-1,* C82A has enhanced defenses to *M. brassicae* feeding, and a similar trend was observed in the C216A mutant. This supports the idea that in these mutants suppression of JA-dependent defense is reduced.

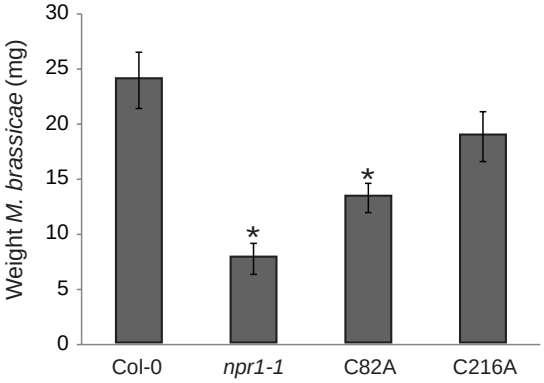


Figure 4: Resistance of C82A and C216A against *M. brassicae*. Growth of *M. brassicae* caterpillars on Col-0, *npr1-1*, C82A and C216A. One first-instar caterpillar was allowed to feed on one plant for 14 days, after which it was weighed. An asterisk indicates a statistically significant lower weight in each genotype compared to Col-0 (one-way ANOVA; Dunnett post-hoc test; $N = 22$, $P \leq 0.05$). Error bars represent standard error.

RNA-sequencing of *npr1-1*, C82A and C216A

In order to gain more knowledge on the mechanism of NPR1-mediated suppression of JA responses, we next investigated which processes are impaired in the C82A and C216A mutants causing a defect in SA-mediated suppression of JA-induced *PDF1.2* and *VSP2*. Since NPR1 is required in the nucleus for SA-mediated suppression of JA-inducible gene expression (Fig. 3B), we hypothesized that NPR1 either acts directly by targeting JA-inducible transcription, or indirectly by activating SA-responsive genes that in turn suppress JA-responsive gene expression. To investigate the last option, we studied the transcriptome of the mutants *npr1-1*, C82A and C216A after induction of SA signaling. In the *npr1-1* mutant, expression of many SA-inducible genes is affected (Glazebrook *et al.*, 2003; Blanco *et al.*, 2009), of which some may have a role in suppression of JA responses. However, in the C82A and C216A mutants, at least part of the SA-induced gene expression is still intact, as shown by the induction of *PR1* expression by application of SA. SA-responsive genes whose expression is affected in the C82A and C216A mutants are thus potentially involved in SA/JA crosstalk. To identify SA-induced genes that encode potential suppressors of *PDF1.2* and *VSP2* transcription, we studied the transcriptome of C82A and C216A in comparison to that of Col-0 and *npr1-1*.

An RNA sequencing experiment was performed on leaf material of Col-0, *npr1-1*, C82A and C216A plants treated with 1 mM SA. For Col-0, a water (mock) treatment was taken along. Three biological replicates per line and treatment were analyzed. For each biological replicate, developmental leaf number 8 of one plant was harvested at 5 h after treatment, because at this time SA-responsive genes such as *PR1* are induced by SA, and early JA-responsive genes such as *VSP2* are suppressed by SA (Fig. 3B). Leaf material was processed and isolated RNA was subjected to RNA-Illumina sequencing.

First, we analyzed the expression level of the *NPR1* gene. As shown in Figure 5A, the expression of *NPR1* was 2.5 times upregulated in Col-0 after SA treatment, which is in accordance with findings by Ryals *et al.* (1997). In all biological replicates of the C82A and C216A lines *NPR1* was higher expressed compared to Col-0. However, in three replicates (i.e. individual plants), namely C82A replicate 2 (C82A-2), C82A replicate 3 (C82A-3) and C216A replicate 3 (C216A-3), the *NPR1* transcript levels were around 20-fold induced, whereas in the other three replicates the induction was around 5-fold (Fig. 5A). The RNA-seq data enabled us to verify the presence of the expected mutations in *NPR1* in all tested lines. In the *npr1-1* mutant, the *npr1-1* mutant allele was lowly expressed, which is indicated by a dark grey bar in Figure 5A. The C82A and C216A mutants overexpress NPR1 that is mutated in Cys^{82} and Cys^{216} in the *npr1-1* background, and these lines contain both an *npr1-1* allele (dark grey) and a Cys-mutated version of the *NPR1* allele (light gray).

We then determined if the different expression levels of *NPR1* in the three replicates of C82A and C216A influenced NPR1-mediated transcriptional activity. Two distinct transcriptome patterns after SA treatment could be distinguished in the replicates, one was comparable to that of expression in SA-treated *npr1-1* plants, and the other to that of SA-treated Col-0 plants. As an example, expression of SA marker gene *PR1* is shown in Figure 5B. In the three plants that expressed relatively low levels of the Cys^{82}- and Cys^{216}-mutated *NPR1* allele, SA treatment did not result in expression of *PR1*. In contrast, *PR1* was induced in the three plants that have relatively high expression of the Cys-mutated *NPR1* allele (Fig. 5A). We speculate that expression of Cys-mutated *NPR1* in C82A-1, C216A-1 and C216-2 was too low to result in complementation of the *npr1-1* mutant.

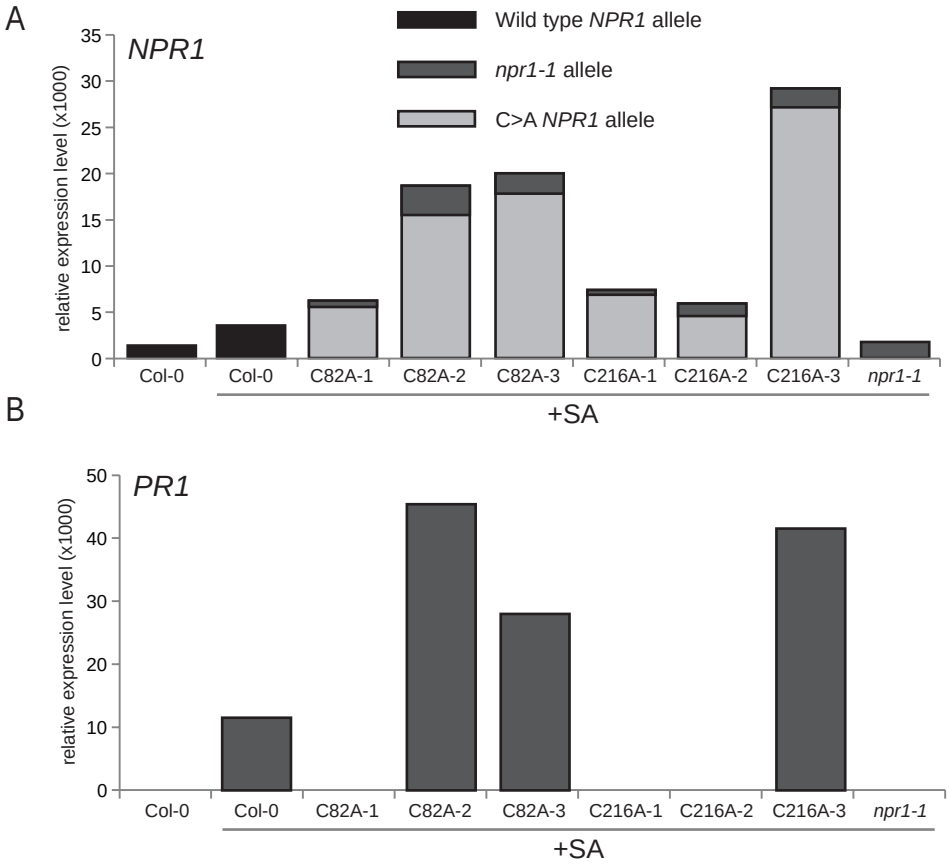


Figure 5: Expression levels of *NPR1* and *PR1* as revealed by RNA-seq. (A,B) Expression levels of *NPR1* (A) and *PR1* (B) in mock-treated Col-0 plants or SA-treated Col-0, *npr1-1* (average of three replicates), and C82A and C216A mutants (three separate replicates) as measured with RNA-seq.

Reduced expression of SA-responsive genes in C82A that may regulate SA/JA crosstalk

We are interested in the conditions where *PR1* is induced by SA, but *PDF1*.2 and *VSP2* are not suppressed by SA (Fig. 3B). Consequently, we choose to analyze in closer detail the two replicates of the C82A line that showed enhanced *PR1* expression after SA treatment. Differentially expressed genes in SA-treated Col-0 plants relative to SA-treated *npr1-1* or SA-treated C82A-2 and C82A-3 mutants (C82A-2/3) were compared. In SA-treated *npr1-1*, 5499 genes were significantly differentially expressed compared to SA-treated Col-0 ($P \leq 0.05$; Supplemental Table S3). Of these genes, 2480 were higher expressed in Col-0 than in *npr1-1*. This group included many known genes that are inducible by SA in an NPR1-dependent manner. The other 3019 genes were significantly lower expressed in Col-0 than in *npr1-1*. In contrast, in SA-treated C82A-2/3 plants, only 97 genes were significantly differentially expressed compared to SA-treated Col-0 ($P \leq 0.05$; Supplemental Table S3). This is in line with the hypothesis that only a part of the SA-responsive transcriptome is affected in C82A. Of these genes, 46 were lower expressed in C82A-2/3 than in Col-0, and 51 were higher expressed in C82A-2/3 compared to Col-0. The majority of the genes that were lower expressed in C82A-2/3, namely 40 of the 46 genes, were also lower expressed in SA-treated *npr1-1* compared to SA-treated Col-0, suggesting that the Cys^{82} mutation results in a reduced expression of a subset of genes whose expression is dependent on NPR1.

Genes that are induced by SA in an NPR1-dependent manner and are also lower expressed in C82A-2/3, are potential candidates for a role in NPR1-regulated SA-induced suppression of JA-responsive genes. In Col-0, SA induced the expression of 33 of the 46 genes that were lower expressed in SA-treated C82A-2/3 than in SA-treated Col-0 ($\log_2$ fold change SA/Mock (Col-0) ≥ 0.6). The expression of 32 of these genes (all genes except *At5g36925*) was significantly lower in the *npr1-1* mutant compared to Col-0 as well. Figure 6A shows a heat map of the $\log_2$ fold changes of the 33 genes in Col-0, C82A-2/3 or *npr1-1* treated with SA, relative to mock-treated Col-0. For many of these genes, the function is still unknown and further investigation is needed to study their role in SA-mediated suppression of JA-inducible genes. Interestingly, four genes affected in C82A-2/3 have been described to be direct targets of NPR1 (Wang *et al.*, 2006). These are *WRKY18*, *WRKY38*, *WRKY53*, and *WRKY70*, indicated with a red dot in Figure 6A. This suggests that the Cys^{82} mutation in NPR1 leads to reduced activation of these genes by NPR1. Expression of NPR1 targets *WRKY18* and *WRKY38* was shown to be reduced in plants treated with proteasome inhibitor MG115 (Spoel *et al.*, 2009). Possibly, the Cys^{82} mutation prevents degradation of NPR1, resulting in reduced expression of *WRKY18* and *WRKY38* and other NPR1 targets.

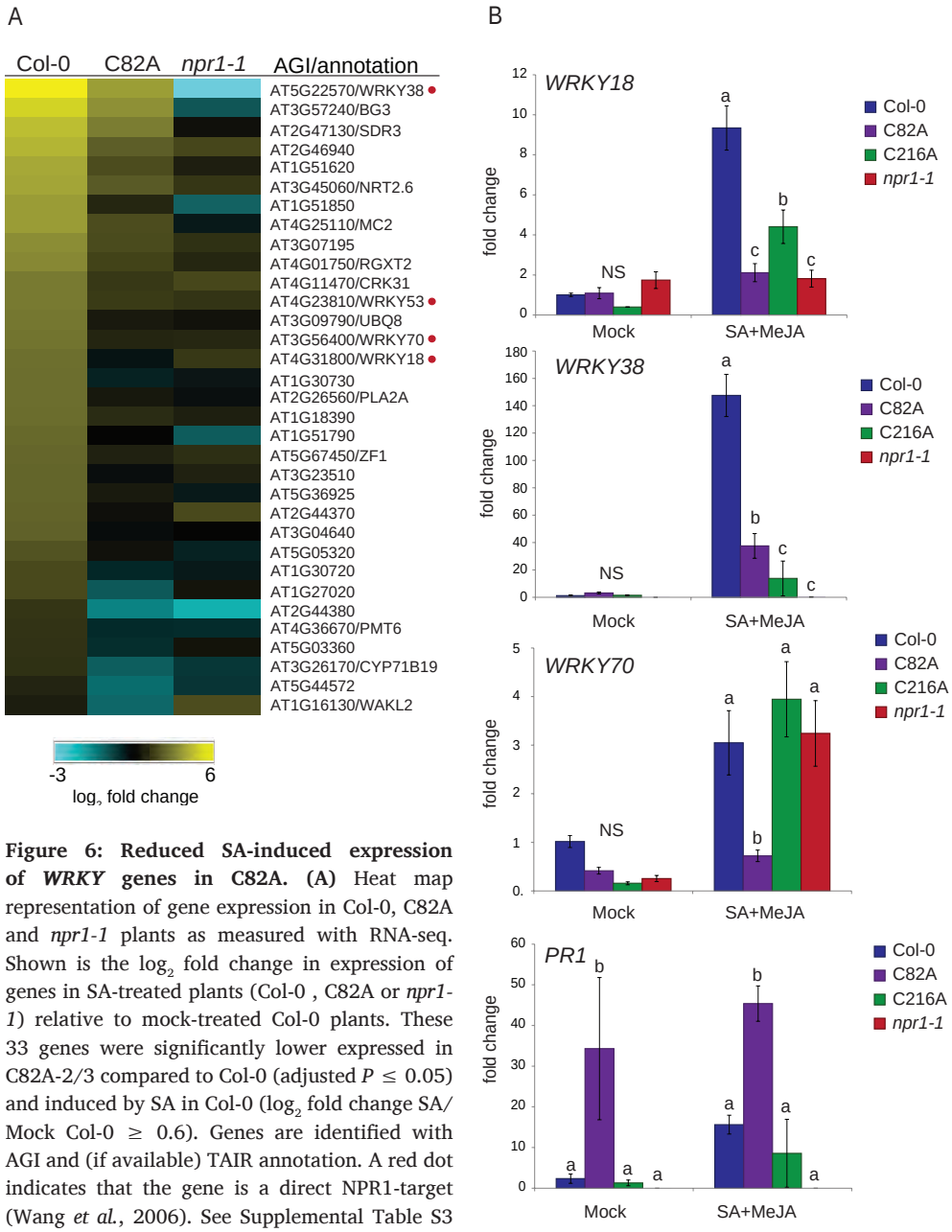


Figure 6: Reduced SA-induced expression of *WRKY* genes in C82A. (A) Heat map representation of gene expression in Col-0, C82A and *npr1-1* plants as measured with RNA-seq. Shown is the $\log_2$ fold change in expression of genes in SA-treated plants (Col-0 , C82A or *npr1-1*) relative to mock-treated Col-0 plants. These 33 genes were significantly lower expressed in C82A-2/3 compared to Col-0 (adjusted $P \leq 0.05$) and induced by SA in Col-0 ($\log_2$ fold change SA/Mock Col-0 ≥ 0.6). Genes are identified with AGI and (if available) TAIR annotation. A red dot indicates that the gene is a direct NPR1-target (Wang *et al.*, 2006). See Supplemental Table S3 for all differentially expressed genes between Col-0 and *npr1-1* and between Col-0 and C82A-2/3. **(B)** Expression of *WRKY18*, *WRKY38*, *WRKY70* and *PR1* in Col-0, C82A, C216A and *npr1-1* after mock or combined SA and MeJA treatment, as measured with qRT-PCR. Gene expression was normalized to expression of reference gene *At1g13320* and calculated relative to mock-treated Col-0 plants using the $2^{-\Delta\Delta}$Ct method. Different letters indicate a significant difference between genotypes per treatment (two-way ANOVA, Tukey post-hoc test; N = 3; $P \leq 0.05$). Error bars represent standard error.

Reduced expression of these *WRKY* genes could provide an explanation for the loss of SA/JA crosstalk in C82A: overexpression of *WRKY70* was shown to lead to reduced levels of MeJA-induced *PDF1.2* and *VSP2* (Li *et al.*, 2006), while conversely, the triple knock-out mutant of *wrky70wrky53wrky46* showed increased MeJA-induced *PDF1.2* expression (Hu *et al.*, 2012). We next investigated the expression behavior of the selected *WRKY* genes in the SA/JA crosstalk-defective mutants C82A, C216A and *npr1-1* at 5 h after treatment with a combination of SA and MeJA. As a control, expression of *PR1* was verified in this experiment and also found to be induced by the combination treatment in Col-0, C82A and C216A, but not in *npr1-1*, as expected (Fig. 6B). In Col-0, expression of *WRKY18, WRKY38* and *WRKY70* was induced by the combination treatment of SA and MeJA (Fig. 6B), but *WRKY53* was not (data not shown). Induction of both *WRKY18* and *WRKY38* was significantly reduced in C82A, C216A and *npr1-1*. Expression of *WRKY70* was reduced in C82A only, but not C216A and *npr1-1*. The reduction in expression of NPR1 targets *WKRY18* and *WRKY38* was thus confirmed to occur in mutants impaired in SA/JA crosstalk under conditions where a combination of SA and MeJA were supplied. Therefore, their induction by SA may be important for suppression of JA-induced *VSP2* and *PDF1.2* in Col-0.

DISCUSSION

NPR1 is an indispensable regulator of SA-induced gene expression and defense, and is essential for SA-modulated suppression of JA-induced marker genes and defense. While regulation of the NPR1 protein in response to SA has been studied extensively, the mechanism of NPR1-mediated suppression of JA-dependent gene expression by SA is less clear. This study investigated the function of NPR1 in SA/JA crosstalk. To this end, several mutants and transgenic lines that affect NPR1 localization, conformation or stability were screened for SA-mediated suppression of JA marker genes *PDF1.2* and *VSP2*. We showed that, in contrast to what was reported for seedlings (Spoel *et al.*, 2003), in mature plants, nuclear NPR1 does play a role in suppression of JA marker genes by SA. We further tested two lines overexpressing cysteine mutated versions of the NPR1 protein, C82A and C216A, which were impaired in suppression of JA marker genes *PDF1.2* and *VSP2*, but were not affected in SA-induced *PR1* expression. This allowed us to dissect the function of NPR1 in SA-responsive *PR1* gene activation and *PDF1.2* and *VSP2* repressions. Reduced expression of several NPR1 target genes in the C82A mutant might be responsible for its impaired SA/JA crosstalk, suggesting that NPR1 could act in the nucleus in SA/JA crosstalk by activating SA-responsive genes whose corresponding proteins then play a role in the SA-mediated suppression of JA-responsive genes.

Cytosolic NPR1 does not suppress JA marker genes in adult plants

In seedlings that express an NPR1 protein that is constitutively localized to the cytosol (NPR1-HBD), MeJA-induced *PDF1.2* expression was reported to be suppressed after SA treatment (Spoel *et al.*, 2003). In addition, the *npr1-3* mutant, which lacks the C-terminal part of the NPR1 protein that contains the nuclear localization signal (Fig. 2), was demonstrated to still exhibit SA-mediated suppression of *PDF1.2*, albeit to a lower extent as wild-type plants (Leon-Reyes *et al.*, 2009). This suggested a cytoplasmic function of NPR1 in SA/JA crosstalk. However, here it was shown, using adult plants, that *PDF1.2* and *VSP2* were not suppressed after the SA and MeJA combination treatment in two different mutants that express a cytosolic version of NPR1, i.e. NPR1-HBD and *npr1nls* (Fig. 3B). This indicates that, at least in adult Arabidopsis plants, NPR1 needs to translocate to the nucleus in order to suppress JA-dependent genes. The difference between our results with NPR1-HBD and those reported by Spoel *et al.* (2003), could be caused by the differences in age or in experimental conditions of the agar-grown seedlings and soil-grown adult plants. We are currently investigating if SA can suppress *PDF1.2* in seedlings of the *npr1nls* mutant. In this study, we provided evidence that nuclear localized NPR1 can function in SA/JA crosstalk in mature Arabidopsis plants.

Redox-mediated modification of NPR1 in SA/JA crosstalk

Redox-mediated post-translational modifications regulate the conformation of the NPR1 protein and consequently its nuclear localization and activity in SA-responsive gene expression. The TRX-NTR system reduces disulfide bonds in NPR1 and can also contribute to denitrosylation of NPR1, both resulting in monomerization and nuclear localization of the NPR1 protein. Especially TRX-h5 is important for the establishment of plant immunity (Tada *et al.*, 2008; Kneeshaw *et al.*, 2014). S-nitrosylation of cysteine residues of NPR1 by GSNO facilitates oligomerization of NPR1. In the *gsnor1* mutant, GSNO levels are increased resulting in high levels of protein-SNO. Possibly through a decrease in NPR1 monomerization and nuclear localization, *gsnor1* mutants express reduced levels of *PR1* and *WRKY*s and are increased susceptible to *Pst* DC3000 (Kneeshaw *et al.*, 2014). Because redox changes are also associated with SA/JA crosstalk (Koornneef *et al.*, 2008a), we investigated the role of TRX, NTRA and GSNOR in the SA-induced, NPR1-mediated suppression of JA-responsive *PDF1.2* and *VSP2*. While SA-induced *PR1* expression was reduced in all four mutants at either 5 or 24 h after SA treatment (Supplemental Figure S1), they were not impaired in suppression of JA-inducible *PDF1.2* and *VSP2* by SA (Fig. 3B). This indicates that that redox regulation of the protein NPR1, as carried out by the tested enzymes, is not important for its role in SA-mediated suppression of JA-dependent genes.

As the time-frame in which *PDF1.2* can be suppressed by SA coincides with the cellular redox change, it is likely that redox regulation of other factors than NPR1 is important for SA/JA crosstalk. Overexpression of oxidoreductase GRX480 was reported to impair the induction of *PDF1.2* by JA (Ndamukong *et al.*, 2007). In addition, ten other GRXs were shown to suppress the promoter of *ORA59* (Zander *et al.*, 2012). GRX480 likely functions downstream of NPR1, as its expression is reduced in the *npr1-1* mutant and overexpression of *GRX480* in the *npr1-1* background still results in suppression of *PDF1.2*. Expression of *GRX480* was also found to be reduced in *npr1-1* in our RNA-seq experiment (Supplemental Table S3). In suppression of *PDF1.2*, GRXs likely act together with TGA transcription factors, as GRXs were shown to interact with TGA2, and the suppression of *PDF1.2* by overexpression of GRX480 was lost in the triple mutant *tga2tga3tga6* (Ndamukong *et al.*, 2007; Zander *et al.*, 2012). Besides playing an important role in *PR1* expression, which is mediated by interacting with NPR1, the TGAs are essential for SA-induced suppression of *PDF1.2* as well as for ethylene-induced activation of *PDF1.2* (Leon-Reyes *et al.*, 2010a; Zander *et al.*, 2010). GRXs and TGAs are both interesting candidates that could explain the redox requirement in SA/JA crosstalk. TGAs were further shown to bind to the *as-1* element in the promoter of *ORA59,* which encodes an ERF transcription factor, and regulate both induction of *ORA59* by ACC and suppression by SA (Zander *et al.*, 2014). We found that the GCC-box, which is the binding site for ERF transcription factors, is enriched in the promoters of JA-induced genes that were suppressed in an NPR1-dependent manner, suggesting that NPR1 can target these transcription factors (Fig. 1B). As our research now places NPR1 in the nucleus to carry out suppression of *PDF1.2,* it would be interesting to test its role as a TGA-interactor in this suppression.

No role for NPR3- or NPR4-mediated degradation of NPR1 in SA/JA crosstalk

NPR3 and NPR4 are CUL3-adapter proteins that target NPR1 for degradation (Fu *et al.*, 2012). We found that SA-induced *PR1* levels were reduced in the *npr3* and *npr3npr4* mutants, while basal *PR1* expression was not affected (Supplemental Figure S1), which is in contrast with previous reports showing an increase in basal *PR1* expression and a faster induction of *PR1* in the *npr3npr4* mutant (Zhang *et al.*, 2006; Fu *et al.*, 2012). We further found that single and double mutants of NPR3 and NPR4 were not impaired in SA-mediated suppression of *PDF1.2* and *VSP2*, which suggests that NPR3 or NPR4-mediated degradation of NPR1 is not necessary in this process (Fig. 3B). MeJA-induced expression of *VSP2* was also lower in the *npr4* and *npr3npr4* mutants, suggesting a role for NPR4 in activation of this gene after MeJA treatment (Supplemental Figure S1), which is in line with findings by Liu *et al.* (2005).

Cysteine mutations in NPR1 disrupt suppression of JA marker genes by SA

The mutation of two cysteines in NPR1, (Cys^{82} and Cys^{216}) into alanines, causes the transgenic C82A and C216A lines that overexpress these mutated versions of the NPR1 protein in the *npr1-1* background, to lose suppression of JA-induced *PDF1.2* and *VSP2* by SA (Fig. 3B). Interestingly, similar mutations in rice NPR1 (Cys^{76} and Cys^{216} changed to alanine) were previously tested and also shown to result in a loss of suppression of JA-dependent genes and defense (Yuan *et al.*, 2007). As the Cys-mutated NPR1 in rice was constitutively localized to the nucleus, these results were interpreted as evidence for a cytosolic role of NPR1 in suppression of JA responses. Mutation of Cys^{82} or Cys^{216} in the Arabidopsis C82A and C216A lines caused the Cys-mutated NPR1 to localize in both the nucleus and the cytosol (Mou *et al.*, 2003). Therefore, the impairment in SA/JA crosstalk in both Cys-mutated NPR1-overexpressing Arabidopsis and rice is unlikely due to exclusion of NPR1 from the cytosol, but rather caused by other effects of the mutations in the Cys residues, for example loss of interaction with other proteins or reduced activation of NPR1 target genes.

We considered the increased monomerization and nuclear localization Cys-mutated NPR1 in C82A and C216A as one of three hypotheses to explain why in the C82A and C216A mutants SA/JA crosstalk was disrupted. However, as we showed that nuclear localization is required for SA/JA crosstalk, this is an unlikely explanation (Fig. 3B). As a matter of fact, NPR1-C82A and NPR1-C216A have high levels of NPR1 monomerization and are localized in the nucleus, but fail to show SA/JA crosstalk while SA-inducible gene expression is largely unaffected. We further hypothesized that the cysteine mutations could disrupt binding with an NPR1 interactor that has a role in SA/JA crosstalk. Investigations of the interaction partners of NPR1, NPR1-C82A and NPR1-C216A are underway. Finally, we hypothesized that the mutations resulted in disruption of transcriptional regulation by NPR1, and specifically reduced SA-activated NPR1-dependent genes that function in SA/JA crosstalk.

Identification of *WRKY* genes that may have a role in SA/JA crosstalk

To test the hypothesis that the cysteine mutations affected part of the SA-responsive, NPR-dependent transcriptome, the C82A mutant was subjected to RNA-seq after SA treatment. In the SA-treated C82A mutant, 46 genes were lower expressed compared to SA-treated Col-0. Of these genes, we considered the 32 genes that were induced by SA in Col-0 in an NPR1-dependent manner as interesting candidates for a role in SA/JA crosstalk. Many of the identified genes have no known function in defense (Fig. 6A; Supplemental Table S3) and are interesting candidates for crosstalk regulation that require further investigation. Interestingly, this set of genes also

contained four direct targets of NPR1, namely *WRKY18*, *WRKY38*, *WRKY53* and *WRKY70*. Overexpression of *WRKY70* has been shown to result in upregulation of SA-induced genes and suppression of JA-regulated genes (Li *et al.*, 2004). WRKY70 likely works together with WRKY53 and WRKY46, as the double and triple mutants of the encoding genes have increased MeJA-induced *PDF1.2* expression (Hu *et al.*, 2012). A similar role has been described for OsWRKY13 in rice (Qiu *et al.*, 2007). However, *WRKY53* was not induced by combination treatment of SA and MeJA in Col-0. In addition, while expression of *WRKY70* was reduced in C82A compared to Col-0 upon the combination treatment, *WRKY70* was not reduced in C216A and *npr1-1* (Fig. 6B), suggesting that loss of suppression of *PDF1.2* and *VSP2* in these two mutants is likely caused by reduced activation of other genes.

The two other *WRKY* genes, *WRKY18* and *WRKY38*, were reduced expressed in all three mutants *npr1-1*, C82A and C216A (Fig. 6B). WRKY18 was shown before to be involved in the activation of a subset of NPR1-dependent genes and is required for SAR (Wang *et al.*, 2006; Spoel *et al.*, 2009). WRKY18 also play complex roles in both SA- and JA-dependent pathways together with WRKY40 and WRKY60. Overexpression of *WRKY18* led to increased resistance to *Pst*, but co-expression with *WRKY40* and *WRKY60* abolished this. Conversely, a triple *wrky18wrky40wrky60* mutant is more susceptible to *B. cinerea*, and has low *PDF1.2* expression in response to infection with this pathogen (Xu *et al.*, 2006). WRKY38 is likely a negative regulator of defense against *Pst*, as the mutant shows reduced growth of this pathogen (Kim *et al.*, 2008). Further research into the function of these WRKYs is necessary to elucidate their role and function in the suppression of JA-dependent gene expression, for example by studying SA/JA crosstalk in *wrky* mutants.

It is not clear how the cysteine mutations lead to a changed in transcriptional regulation by NPR1. However, proteasome-mediated degradation of NPR1 has been shown to be important for complete induction of its targets *WRKY18*, *WRKY38* and *WRKY60* (Spoel *et al.*, 2009). Possibly, the cysteine mutations result in reduced degradation of NPR1 and thus reduced expression of the *WRKY* genes. This would mean that degradation of NPR1 is important for expression of SA-induced targets that indirectly suppress JA-inducible *PDF1.2* and *VSP2*.

Cysteine mutations in NPR1 disrupt defense against *M. brassicae*

A defect in SA/JA crosstalk could impact JA- or SA-controlled resistance against different attackers. The C82A mutant was found to be more resistant to feeding by *M. brassicae* caterpillars, which are sensitive to JA-dependent defenses (Fig. 4). The increased resistance to the insect could be the result of a loss of suppression of JA signaling, which would result in enhanced JA-dependent defense gene expression

or accumulation of glucosinolates (De Geyter *et al.*, 2012). However, we have also found using RNA-seq that the complementation of *npr1-1* by overexpression of the Cys-mutated NPR1 can differ per individual plant. In the bioassay, this complementation has not been not tested, whereas determination of complementation for *PR1* expression in each plant used is necessary in order to conclude whether the phenotype is caused by the expression of the Cys-mutated NPR1 in that plant. In our qRT-PCR experiments, *PR1* expression was always induced by SA in C82A (Fig. 3B).

NPR1 suppresses at least part of the ERF-branch of the JA pathway

Using microarray studies, NPR1 was shown to be essential for suppression of 22 of the 59 genes that are MeJA-induced and suppressed by SA in Col-0 at 28 h after treatment. These genes included *ERF6*, *CORI3*, *JAZ5* and *JAZ7*. We found that the GCC-box, which is a binding site for ERF transcription factors, is enriched in the promoters of the 22 genes that are suppressed in a NPR1-dependent manner. This suggests that NPR1 targets ERF transcription factors, for example ORA59, to suppress gene expression. However, the targeting of ERF transcription factors by NPR1 is unlikely to be the complete picture, as suppression of *VSP2*, a gene regulated by the MYC-branch, also depends on NPR1 (Fig. 3B). The tissue used for the microarray experiment was harvested at 28 h after treatment, while the suppression of *VSP2* is evident at earlier times after SA and MeJA treatment (Fig. 3B; Supplemental Figure S1). Studying expression of JA targets at additional times will likely result in the identification of more genes that are suppressed by SA in a NPR1-dependent manner.

Interestingly, 37 out of the 59 genes that were sensitive to SA/JA crosstalk in wild-type plants, were still suppressed by SA in the *npr1-1* mutant, showing that the majority of MeJA-induced genes in this experiment was suppressed by SA in a NPR1-independent manner. This set also contained ERF transcription factors, suggesting that some members of this family can be targeted by SA in an NPR1-independent manner. Promoter analysis of the NPR1-independent set of genes showed an enrichment of motifs recognized by bHLH, NAC or MYB-related transcription factors. Further investigation of an NPR1-independent mechanism for SA/JA crosstalk could improve our understanding of interactions between the SA- and JA-signaling pathways.

The important role of NPR1 in SA/JA crosstalk in Arabidopsis

SA/JA crosstalk has been suggested before to have evolved at least at the base of angiosperms and was shown to be conserved in Arabidopsis accessions (Koornneef *et al.*, 2008a; Thaler *et al.*, 2012). Here, we found that suppression of *PDF1.2* was

maintained in the majority of the 349 tested Arabidopsis accessions, which confirms the importance of the SA/JA crosstalk phenomenon for Arabidopsis. In the nine accessions that did not display SA/JA crosstalk and for which NPR1 sequences were available, no overall shared amino acid variations in the NPR1 protein could be detected. Moreover, most of the differences in amino acids were also present in accessions that displayed SA/JA crosstalk effectively. Only one variation was only present in one accession that was defective in SA/JA crosstalk, namely E550D in L*er*-1. However, this is an conservative amino acid difference, and thus unlikely to be responsible for the loss of SA/JA crosstalk. The role of polymorphisms in other proteins in SA/JA crosstalk is currently under investigation in another study by our group. The *NPR1* gene has previously been suggested to show evidence of balancing selection, meaning that different polymorphism are kept throughout the population of different accessions (Caldwell and Michelmore, 2009). We identified several polymorphisms in the NPR1 protein of 152 accessions, some of which might potentially alter its function (Fig. 2), which could be assessed by complementation assays with the various NPR1 protein versions in the *npr1-1* mutant of Col-0 background.

In this study, we investigated the function and regulation of NPR1 in SA/JA crosstalk. We showed that nuclear localization of NPR1 is required to lead to suppression of JA-inducible genes *PDF1.2* and *VSP2* by SA. In contrast, redox-modulated modifications of NPR1 seem not important for SA/JA crosstalk. Further, and importantly, mutation of either one of two cysteines in NPR1, Cys^{82} or Cys^{216}, resulted in a loss of suppression of JA-inducible *PDF1.2* and *VSP2*, whereas SA-induced *PR1* expression was not affected. The C82A and C216A mutants are thus unique, because they separated the role of NPR1 in activation of *PR1* and in SA/JA crosstalk. Here, transcriptome analysis of C82A identified *WRKY* targets that are reduced expressed in C82A and may have an important role in SA/JA crosstalk. In the future, protein interaction studies and targeted gene expression analysis of different types of *npr1* mutants will help to further elucidate the function of NPR1 in SA/JA crosstalk. In conclusion, this study suggests that modification of NPR1 by redox transmitters, NPR3 or NPR4 is not required for its regulatory function in SA/JA crosstalk. However, in mature plants, translocation of NPR1 to the nucleus upon SA treatment is required, where it activates *WRKY*s and downregulates ERF transcription factors, leading to suppression of JA-responsive gene expression.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis seeds were sown on river sand. Two weeks after germination, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture (5:12 v/v) that had been autoclaved twice for 45 min with a 24-h interval. Plants were cultivated in a growth chamber with a 10-h day and 14-h night cycle at 70% relative humidity and 21˚C. In all experiments, Col-0 was used as wild type. The mutants and overexpression lines used were generated by previous studies and are: *35S*:NPR1-HBD, 35S:*npr1nls-GFP* (Kinkema *et al.*, 2000), *npr1-1* (Cao *et al.*, 1994), 35S:*npr1*C82A-GFP (C82A), 35S:*npr1*C216A-GFP (C216A) (in *npr1-1* background) (Mou *et al.*, 2003), *npr3*, *npr4* and *npr3npr4* (Fu *et al.*, 2012), *trx-h3* and *trx-h5* (Tada *et al.*, 2008) and *atgsnor1* (*par2-1* allele) (Kneeshaw *et al.*, 2014). For the *ntra* mutant, T-DNA insertion line SALK_539152 was obtained from the Nottingham Arabidopsis Stock Centre, and plants homozygous for the insert were selected by genotyping.

Chemical treatments and herbivore assays

Five-week-old 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. For mock treatments, plants were dipped into a solution containing 0.015% (v/v) Silwet L77. MeJA was added to the solutions from a 1000-fold concentrated stock in 96% ethanol. To the solutions without MeJA, a similar volume of 96% ethanol was added. Leaves were harvested for RNA isolation 5 and 24 h after treatment.

M. brassicae eggs were provided by the Entomology department of Wageningen University and Research Centre (the Netherlands) where they were reared as described previously (Pangesti *et al.*, 2015). For the performance assay of *M. brassicae* caterpillars, one freshly hatched first-instar (L1) larva was placed on one plant inside a plastic cup covered with an insect-proof mesh to contain the caterpillars. Caterpillars were allowed to feed on the plants for 14 days, after which they were weighed.

RNA extraction and qRT-PCR analysis

For qRT-PCR analysis, RNA was extracted as described for vegetative tissues by Oñate-Sánchez and Vicente-Carbajosa (2008). RNA was pretreated with DNAse I (Fermentas, St. Leon-Rot, Germany) to remove genomic DNA. RevertAid H minus Reverse Transcriptase (Fermentas) was used to convert DNA-free total RNA into cDNA. PCR reactions were performed in optical 384-well plates with a ViiA 7 realtime PCR system (Applied Biosystems, Carlsbad, CA, USA), using SYBR® Green to monitor the synthesis of double-stranded DNA. The primers used to analyze expression by qRT-PCR are found in Table 1. A standard thermal profile was used: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon dissociation curves were recorded after cycle 40 by heating from 60 to 95° C with a ramp speed of 1.0° C min^{-1}. Fold change was calculated relative to the reference gene *At1g13320* (Czechowski *et al.*, 2005) using the $2^{-\Delta\Delta}$Ct method described previously (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). For the heat map in Figure 3, the $\log_2$ fold change relative to mock-treated Col-0 was calculated.

Table 1: List of all primers designed for genotyping and qRT-PCR in this study.

Name	Sequence (5' → 3')
Primers used for qRT-PCR:	
At1g13320 Forward	TAACGTGGCCAAAATGATGC
At1g13320 Reverse	GTTCTCCACAACCGCTTGGT
PR1 Forward	CTCGGAGCTACGCAGAACAACT
PR1 Reverse	TTCTCGCTAACCCACATGTTCA
PDF1.2 Forward	TTTGCTGCTTTCGACGCAC
PDF1.2 Reverse	CGCAAACCCCTGACCATG
VSP2 Forward	ACGGAACAGAGAAGACCGAC
VSP2 Reverse	TCTTCCACAACTTCCAACGG
WRKY18 Forward	TGCGTCCCTTCGTATGTCGCTACA
WRKY18 Reverse	AGAAGGTACAACGCAGCGCAGA
WRKY38 Forward	CCGGACAAGATCCCCTAGAT
WRKY38 Reverse	GGCTTTCCTTCTCCTGATCCT
WRKY70 Forward	GTTTGAAGATTCCGGCGATAGTC
WRKY70 Reverse	ACACGTCTCCGATCTCTTTTTTCT
Primers used for genotyping:	
NTRATDNA-Fw	TTTGTTTTCTGTGGCAGTG
NTRATDNA-Rv	CAGTGAAGCTAAGACGTTT
SALK LBb1.3	ATTTTGCCGATTTCGGAAC

Analysis of natural variation of NPR1 in Arabidopsis accessions

To study natural variation in SA/JA, 349 natural accessions of the Arabidopsis Haplotype Map (HapMap) population (http://naturalvariation.org/hapmap) were analyzed for SA-mediated antagonism of JA-responsive *PDF1.2* expression. These plants were cultivated in a growth chamber with an 8-h day and 16-h night cycle at 70% relative humidity and 21°C. Chemical induction treatments of the Hapmap population were performed by dipping leaves of 4-week-old plants in hormone solutions as described above. All leaves of the rosettes from three plants were harvested 24 h after treatment, RNA was isolated and subjected to qRT-PCR analysis as described above. For the accessions that were available, sequences for *NPR1* were downloaded from the SALK 1001 genomes project website (http://signal.salk.edu/atg1001). All acquired NPR1 protein sequences were aligned to the Col-0 NPR1 sequence using CLC Main Workbench 6.

Analysis of microarray data, expression profiling and promoter analysis

For gene expression profiling by microarray (ATH-1 full genome GeneChips (Affymetrix) preparation of samples, isolation of RNA, microarray data collection and expression profiling was performed as described by Van der Does *et al.* (2013). For analysis of differentially expressed genes, the $\log_2$-transformed expression values of the three independent biological experiments were compared between treatments using a two-sample, two-tailed Student's *t* test ($P < 0.1$; fold-change $>= 2$). Promoter analysis was performed using a collection of 403 experimentally elucidated transcription factor binding motifs (Franco-Zorrilla *et al.*, 2014; Weirauch *et al.*, 2014). The FIMO tool (Grant *et al.*, 2011) was used to scan for the presence of each motif ($P \leq 0.0001$) within the 500 bp upstream of the predicted transcription start site of each gene (TAIR version 10) on both strands. For each motif and gene list, we compared the fraction of genes in the list that contain the motif with its occurrence in all genes in the entire Arabidopsis genome. The hypergeometric distribution was used to assign a *P*-value to each fraction and $P \leq 0.05$ was considered significant.

Analysis of RNA-seq results

For RNA-seq, developmental leaf 8 was harvested from three individual mock-treated wild type Col-0 plants or SA-treated Col-0, *npr1-1,* 35S:*npr1*C82A-GFP (C82A), 35S:*npr1*C216A-GFP (C216A) plants, 5 h after SA treatment. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), including an on-column DNase treatment in accordance with manufacturer's instructions. Quality of RNA was checked by determining the RNA Integrity Number (RIN) with an Agilent 2100 bioanalyzer and RNA LabOnChip. Libraries for sequencing were prepared using

the Illumina TruSeq Stranded mRNA Sample Prep Kit, and sequenced at the Utrecht DNA sequencing facility (http://utrecht-sequencing-facility.nl) on the Illumina NextSeq500 platform with single read lengths of 75 bases. Reads were mapped to the Arabidopsis genome (TAIR version 10) using TopHat version 2.0.4 (Trapnell *et al.*, 2009) with parameter settings: 'max-intron-length 2000', 'transcriptome-mismatches 3', 'N 3', 'bowtie1', 'no-novel-juncs', 'genome-read-mismatches 3', ' p 6', 'read-mismatches 3', 'G', 'min-intron-length 40'. Aligned reads were summarized over annotated gene models (TAIR version 10) using HTseq-count version 0.5.3p9 (http://www-huber.embl.de/users/anders/HTSeq/) with parameters: 'stranded no', '-i gene_id'. Sample counts were depth-adjusted and differential expression was determined using the DESeq package with default settings (Anders and Huber, 2010). Genes with a corrected *P* value (*P* adjusted) of $\leq$ 0.05 were called as differentially expressed. All statistics associated with testing for differential gene expression were performed with R (www.r-project.org).

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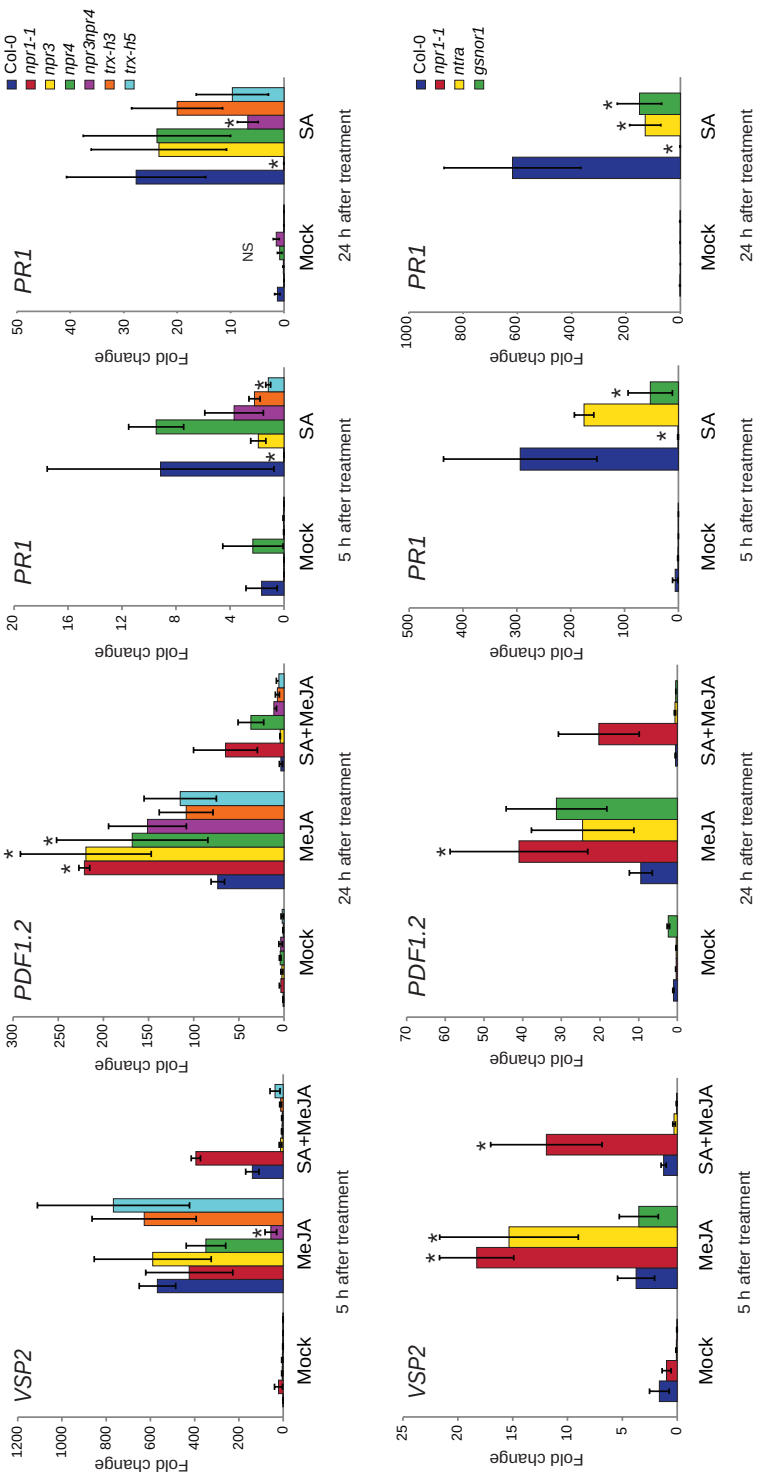
SUPPLEMENTAL DATA

Supplemental Table S1: Lists of genes induced by MeJA or SA, and MeJA-induced genes that are suppressed by SA in Col-0 and *npr1-1*.

Supplemental Table S2: Promoter binding motifs with associated *P*-value for NPR1-dependent and NPR1-independent genes.

Supplemental Table S3: Lists of genes differentially expressed between Col-0 and *npr1-1* and Col-0 and C82A as determined by RNA-seq.

Digital versions of supplemental tables are available on request.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npr1-1
npr3
npr4
npr3npr4
trx-h3
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Col-0
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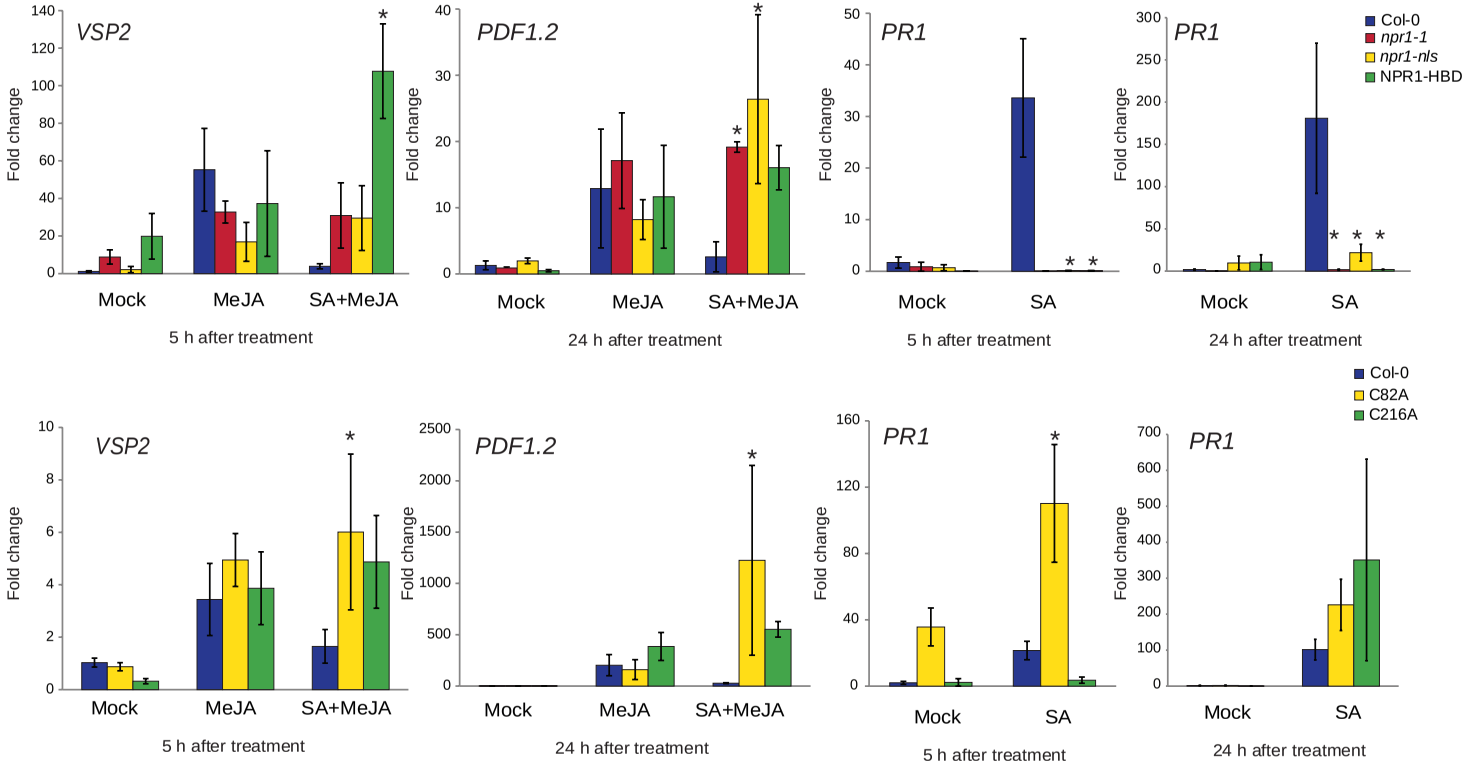
PDF1.2
VSP2
Fold change
Mock
MeJA
SA
SA+MeJA
NS
5 h after treatment
24 h after treatment



Supplemental Figure S1: **Expression of *PDF1.2*, *VSP2* and *PR1* in all analyzed mutants.** qRT-PCR analysis of *PDF1.2*, *VSP2* and *PR1* expression in 5-week-old plants treated with water (Mock), 1 mM SA and/or 0.1 mM MeJA, and harvested at 5 h (*VSP2, PR1*) and 24 h (*PDF1.2, PR1*) after treatment. An asterisks indicates a statistically significant lower or higher expression in the mutant compared to Col-0 after the indicated treatment (two-way ANOVA; N = 3;Tukey post-hoc test; $P \leq 0.05$). Error bars indicate standard error. All experiments were repeated with similar results. Results from one representative experiment are shown (one row of graphs is one experiment). The *gnsor1* mutant was only tested once.

CHAPTER 4

Jasmonate-induced oxygenases negatively regulate immunity to *Botrytis cinerea* and *Mamestra brassicae* by hydroxylation of jasmonic acid

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ABSTRACT

The plant hormone jasmonic acid (JA) plays a vital role in plant development and defense. Although much is known about biosynthesis of JA and the mode of action via its bioactive form JA-isoleucine (JA-Ile), knowledge on JA metabolism is incomplete. In particular, it is unknown what enzyme hydroxylates JA into 12-OH-JA, an inactive form of JA that accumulates after wounding and pathogen attack. Here, we report the identification of a clade of four 2-oxoglutarate/Fe(II)-dependent oxygenases in Arabidopsis that is involved in JA hydroxylation. As the expression of the encoding genes is induced by JA we named them *JASMONATE-INDUCED OXYGENASEs* (*JOXs*). Simultaneous mutation of the four genes by T-DNA insertions in a *jox4x* quadruple mutant resulted in increased defense gene expression and resistance to the necrotrophic fungus *Botrytis cinerea* and the herbivore *Mamestra brassicae*. Other JA-related phenotypes were also observed, e.g. inhibition of root and shoot growth, delayed flowering time and reduced seed set. Metabolite analysis showed that the *jox4x* mutant over-accumulates JA, and that its ability to hydroxylate JA into 12-OH-JA was strongly reduced. In contrast, plants overexpressing *JOX1* accumulated more 12-OH-JA than wild-type plants when JA was supplied exogenously. The identification of the enzymes responsible for hydroxylation of JA has revealed a missing step in JA metabolism, which is important for the inactivation of JA and thereby negatively regulates defense. It also points to a role for JA hydroxylation in fine-tuning of jasmonate responses to balance defense and growth.

INTRODUCTION

The lipid-derived plant hormone jasmonic acid (JA) is an essential signaling molecule in a wide range of processes. It plays an important role in plant defense against necrotrophic pathogens, e.g. the fungus *Botrytis cinerea* (Thomma *et al.*, 1998; De Vos *et al.*, 2005), as well as in direct and indirect defenses against herbivorous insects (Reymond *et al.*, 2004; Dombrecht *et al.*, 2007; Howe and Jander, 2008; Fernández-Calvo *et al.*, 2011). Defense responses include the production of toxic compounds, (Schweizer *et al.*, 2013) and the attraction of natural enemies (Thaler *et al.*, 2002; Van Poecke and Dicke, 2002). Moreover, JA has been implicated as a positive regulator of abiotic stress responses, contributing to cold-, salinity-, drought-, and heat-tolerance (Kazan, 2015). Several mutants in JA signaling or biosynthesis are male sterile, indicating that JA is also required for fertility (Feys *et al.*, 1994; McConn and Browse, 1996; Stintzi and Browse, 2000; Ishiguro *et al.*, 2001; Park *et al.*, 2002; Thines *et al.*, 2007). Furthermore, activation of JA responses in *Arabidopsis thaliana* (Arabidopsis) inhibits shoot and root growth, and delays flowering time (Staswick *et al.*, 1992; Zhang and Turner, 2008; Cipollini, 2010; Chehab *et al.*, 2012; Zhai *et al.*, 2015). Therefore, tight control of JA levels and downstream responses is essential for plants to respond optimally to environmental triggers, and to minimize negative effects on growth.

Under non-stressed conditions, when only low levels of JA are present, activation of JA-responsive gene expression is inhibited by JASMONATE ZIM-domain (JAZ) proteins that bind transcriptional activators of the JA pathway such as MYC2 (Chini *et al.*, 2007; Thines *et al.*, 2007). The conjugate of JA with isoleucine, JA-Ile, strongly promotes binding of JAZ repressors to the F-box protein CORONATINE INSENSITIVE1 (COI1) (Thines *et al.*, 2007; Fonseca *et al.*, 2009; Sheard *et al.*, 2010), resulting in the degradation of JAZ and the activation of JA-responsive gene expression (Devoto *et al.*, 2002; Katsir *et al.*, 2008). While JAZ proteins were initially identified as inhibitors of MYC2, they have now been shown to bind to several other transcription factors, including the MYC2-related MYC3 and MYC4 proteins (Fernández-Calvo *et al.*, 2011; Niu *et al.*, 2011), and to ETHYLENE INSENSITIVE3 (EIN3) and EIN3-LIKE1 (EIL1), which control expression of genes co-regulated by JA and ethylene (Zhu *et al.*, 2011). JAZ proteins have also been shown to repress bHLH transcription factors that act as negative regulators of JA responses (Song *et al.*, 2013), suggesting a negative feedback system where activation of the JA pathway also inhibits JA-responsive gene expression. Another means of negative feedback is the induction of *JAZ* expression by JA that accumulates after wounding (Chini *et al.*, 2007; Thines *et al.*, 2007; Chung *et al.*, 2008).

Synthesis of JA is initiated in the plastid from the precursor linolenic acid (LA),

which is oxygenated and converted into the intermediate 12-oxo-phytodienoic acid (OPDA). In the peroxisome, reduction of OPDA and three β-oxidation steps convert OPDA into JA (Wasternack and Hause, 2013). To achieve rapid production of JA, many of the JA biosynthesis genes are under positive feedback control by JA-responsive transcription factors, resulting in increased accumulation of JA once this pathway has been activated (Reymond *et al.*, 2000; Sasaki *et al.*, 2001; Pauwels *et al.*, 2008). JA is further metabolized to produce a suite of compounds. JA-isoleucine is formed by conjugation of the amino acid by the enzyme JASMONATE RESISTANT 1 (JAR1) (Staswick and Tiryaki, 2004; Suza and Staswick, 2008). Other covalent modifications are methylation resulting in methyl JA (MeJA), and hydroxylation of JA or JA-Ile (Wasternack and Strnad, 2015). The hydroxylated forms of JA and JA-Ile (12-OH-JA and 12-OH-JA-Ile), are considered inactive, and can be further modified by carboxylation, sulfonation, or glucosylation (Gidda *et al.*, 2003; Seto *et al.*, 2009; Heitz *et al.*, 2012). Conversion of bio-active JA molecules into inactive forms is an important mechanism to control bio-active JA levels and downstream responses.

The hydroxylated form of JA-Ile accumulates in Arabidopsis after wounding and *B. cinerea* infection (Glauser *et al.*, 2008; Aubert *et al.*, 2015). Compared to JA-Ile, 12-OH-JA-Ile only weakly promotes COI1-JAZ assembly *in vitro*, and treating plants with 12-OH-JA-Ile does not lead to activation of JA-responsive gene expression (Koo *et al.*, 2011; Aubert *et al.*, 2015). JA-Ile is hydroxylated by two cytochrome P450s, CYP94B3 and CYP94B1 (Kitaoka *et al.*, 2011; Koo *et al.*, 2011; Koo *et al.*, 2014). CYP94C1, another cytochrome P450, carboxylates 12-OH-JA-Ile into 12-COOH-JA-Ile (Heitz *et al.*, 2012). The *cyp94b3* and *cyp94b1 cyp94b3* mutants accumulate high levels of JA-Ile. Conversely, overexpression of *CYP94B1* or *CYP94B3* in plants results in low JA-Ile and increased 12-OH-JA-Ile levels. Consequently, these overexpression lines display reduced JA-mediated inhibition of root and shoot growth and are more susceptible to caterpillar feeding (Koo *et al.*, 2011; Koo *et al.*, 2014).

The hydroxylated form of JA, 12-OH-JA, has been identified in several plant species, including Arabidopsis, maize, tomato and rice, and accumulates after wounding and *B. cinerea* infection (Glauser *et al.*, 2008; Miersch *et al.*, 2008; Seto *et al.*, 2009; Aubert *et al.*, 2015). Recently, the rice blast fungus *Magnaporthe oryzae* was shown to attenuate plant defense by releasing a monooxygenase that hydroxylates JA into 12-OH-JA (Patkar *et al.*, 2015). A plant enzyme that hydroxylates JA has not yet been identified. It was hypothesized that members of the CYP94 family could be responsible for hydroxylation of JA (Koo and Howe, 2012). However, none of the enzymes that have been described so far display JA-hydroxylase activity (Heitz *et al.*, 2012). Two amidohydrolases that cleave the isoleucine group of JA-Ile and 12-OH-JA-Ile can produce 12-OH-JA (Widemann *et al.*, 2013), but in the double mutant

that no longer produces these enzymes, 12-OH-JA was still detected, suggesting that direct hydroxylation of JA contributes to the accumulation of 12-OH-JA.

Besides the cytochrome P450 enzymes, members of the 2-oxoglutarate Fe(II)-dependent (2OG) oxygenase family in Arabidopsis catalyze numerous oxidative reactions (Kawai *et al.*, 2014). Many members of this family are involved in hormone biosynthesis, including that of gibberellin (GA) and ethylene, and in the production of secondary metabolites, e.g. flavonoids and glucosinolates (Farrow and Facchini, 2014; Kawai *et al.*, 2014). Interestingly, several 2OG oxygenases were shown to hydroxylate and inactivate hormones; e.g. two different groups of 2OG oxygenases inactivate GA by hydroxylating either the bioactive C19-GA or a precursor of bioactive GA (Schomburg *et al.*, 2003; Rieu *et al.*, 2008). More recently, the active form of auxin was reported to be hydroxylated and inactivated in rice by the 2OG oxygenase DIOXYGENASE FOR AUXIN OXIDATION (DAO) (Zhao *et al.*, 2013), while SA 3-HYDROXYLASE (S3H) was shown to hydroxylate salicylic acid (SA) to control its levels during senescence (Zhang *et al.*, 2013a). Since inactivation of hormones via hydroxylation by 2OG oxygenases is common in plants, we hypothesized that 2OG oxygenases could function as JA-hydroxylases. Here, we describe the identification of a family of four 2OG oxygenases that are induced by JA, which we named JASMONATE-INDUCED OXYGENASEs (JOXs). Using genetic and metabolic approaches we demonstrate a role for JOXs in the hydroxylation of JA to 12-OH-JA. Our data suggest that the JOX proteins act as negative regulators of JA-mediated defense responses to the fungus *B. cinerea* and the insect herbivore *Mamestra brassicae* by inactivating JA.

RESULTS

Four JASMONATE-INDUCED OXYGENASES group in a distinct clade in Arabidopsis

The gene encoding the SA hydroxylase S3H, which has also been called DMR6-LIKE OXYGENASE 1 (DLO1), and the paralogous gene encoding 2OG oxygenase DOWNY MILDEW RESISTANT 6 (DMR6) are highly upregulated after treatment with SA or the SA-analog BTH (Zhang *et al.*, 2013a; Zeilmaker *et al.*, 2015). We therefore hypothesized that JA-induced 2OG oxygenase genes could encode JA hydroxylases. To identify families of JA-induced oxygenases, we first constructed a phylogenetic tree of 93 Arabidopsis proteins that contain two conserved 2OG oxygenase Pfam domains. These are Pfam domain PF03171 (2OG-Fe(II) oxygenase superfamily) present in 108 proteins, and Pfam domain PF14226 (non-haem dioxygenase in

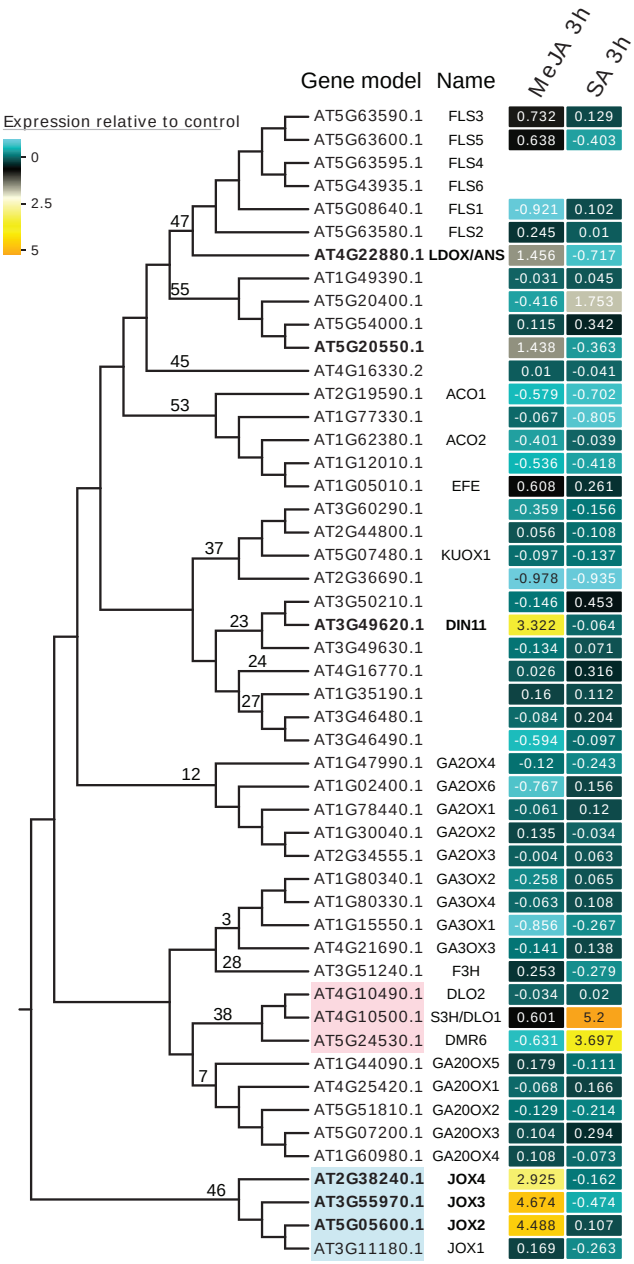


Figure 1: Phylogenetic tree of SA- and JA-induced and related 2OG oxygenases of Arabidopsis. The cladogram shows the relatedness of 50 2OG oxygenases selected from the phylogram in Figure S1. For each protein model, the name (when available) is supplied. For each clade, the number which was assigned to it by Kawai *et al.* (2014) is indicated. The heat map indicates the $\log_2$ fold change of the corresponding genes in Arabidopsis seedlings 3 h after MeJA or SA treatment. Indicated in pink is clade 38, which contains SA-induced *S3H/DLO1*. 2OG oxygenases induced by MeJA are indicated in bold. Clade 46 containing the *JOX* genes induced by MeJA is indicated in blue.

morphine synthesis N-terminal) present in 95 proteins. PF03171 locates to the C-terminal half, and PF14226 to the N-terminal half of typical 2OG oxygenases. Phylogenetic clustering of the 93 proteins revealed clear families (Figure S1), that largely overlapped with clades defined by Kawai *et al.* (2014). Projection of gene expression data showed that JA- and SA-induced 2OG oxygenases were present in a cluster of 50 proteins (indicate by the arrow in Figure S1) that contains 14 2OG oxygenase clades as defined by Kawai *et al.* (2014). A close up of the tree containing these 50 proteins is shown in Figure 1, with the clade number defined by Kawai *et al.* (2014) indicated for each clade.

Publically available data confirmed the SA-induced expression of *S3H/DLO1* (At4g10500) and *DMR6* (At5g24530) that are present in Kawai clade 38 together with *DLO2* (At5g10490), which is non-responsive to SA (Fig. 1, indicated in pink). Expression of six genes encoding 2OG oxygenases present in our tree was induced more than 2-fold at 3h after MeJA treatment (indicated in bold; Fig. 1). Of two weakly induced genes, At5g20550 is a gene of unknown function, whereas LDOX (At4g22880) encodes an enzyme involved in anthocyanin biosynthesis. Expression of *LDOX* has been described to be controlled by a MYB/bHLH complex and induced by JA (Shan *et al.*, 2009). A third MeJA-induced gene is *DIN11* (At3g49620), which was described as a senescence-associated gene involved in the response to viral infection (Fernández-Calvino *et al.*, 2015). Strikingly, three closely related genes, of yet unknown function were strongly induced by MeJA. These genes are At2g38240, At3g55970 and At5g05600. The fourth member of this clade, At3g11180, was not found to be induced by MeJA in publically available data. This clade, indicated in light blue in Figure 1, corresponds to clade 46 as defined by Kawai *et al.* (2014). Because of the high induction by JA of the three genes, the members of this clade were considered good candidates for JA-hydroxylases.

To further study the JA-responsiveness of the four 2OG oxygenase genes of this clade, we treated five-week-old plants with MeJA and measured gene expression using qRT-PCR at 0, 1, 2, and 6 h after treatment. MeJA induced expression of all four genes (Fig. 2A). We thus named the four genes *JASMONATE-INDUCED OXYGENASEs* (*JOXs*). The expression of *JOX1* (At3g11180), which was not reported as being induced by MeJA in the publically available data, was highly induced 1 h after MeJA treatment, and was slightly higher than in mock-treated plants at 2-6 h after MeJA treatment. The expression patterns of *JOX2* (At5g05600) and *JOX3* (At3g55970) were quite similar: induction was low at 1 h and 2 h after treatment, but high at 6 h after treatment. Expression of *JOX4* (At2g38240) was significantly induced at all time points, but showed a different temporal behavior: it was highly induced at 1 h, lower at 2 h and high again at 6 h after treatment (Fig. 2A). We next tested if induced expression of the *JOX* genes is dependent on the JA receptor

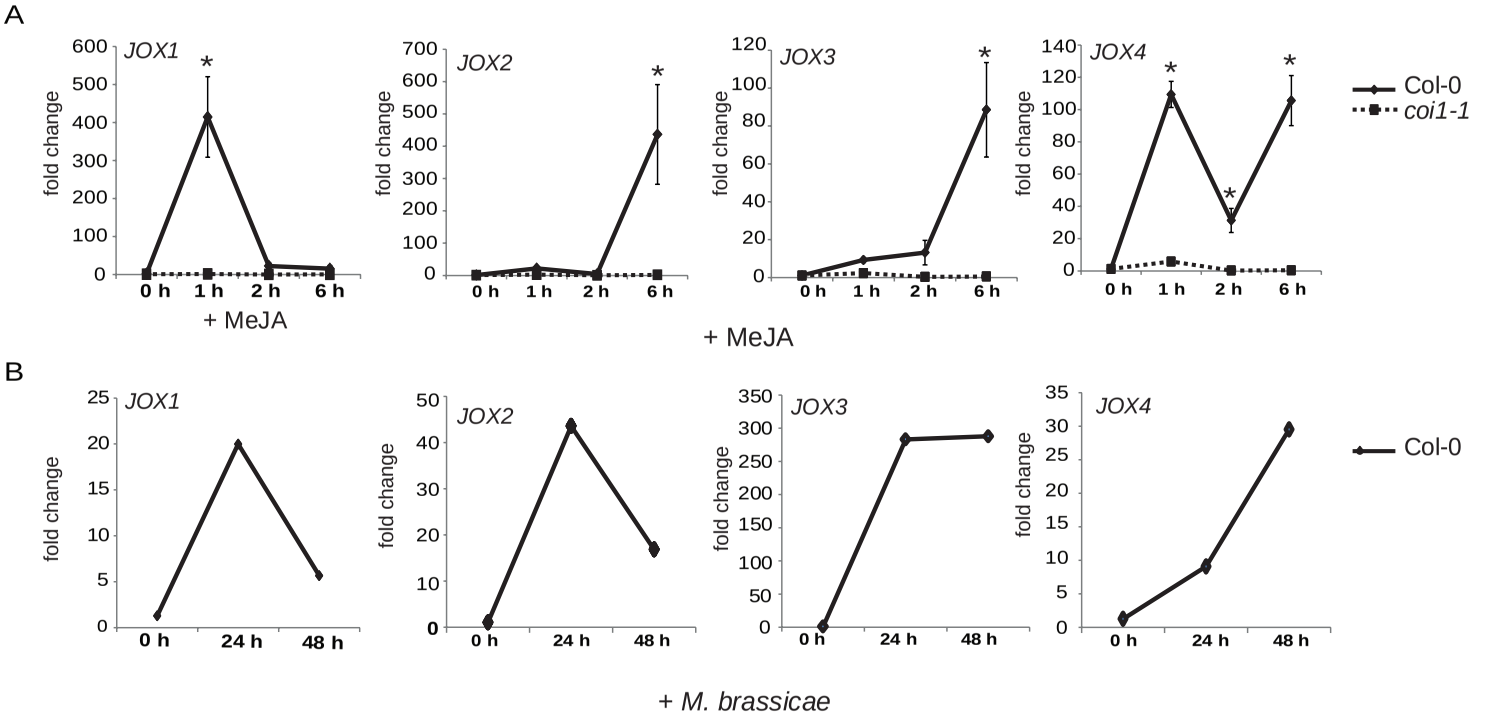


Figure 2: Expression of *JOX* genes after MeJA treatment or *M. brassicae* feeding. (A) qRT-PCR analysis of expression of *JOX1, JOX2, JOX3* and *JOX4* in response to MeJA treatment in Col-0 and *coi1-1*. Shown is the expression (fold change) at 0 h, 1 h, 2 h, or 6 h after MeJA treatment, relative to that in mock-treated plants at the same time. An asterisk indicates a significant higher expression compared to mock-treated plants ($P \leq 0.05$; two-way ANOVA). **(B)** Expression (fold change) of *JOX1, JOX2, JOX3* and *JOX4* in Col-0 wild-type plants after 24 h or 48 h of feeding by *M. brassicae* relative to plants that were not exposed to *M. brassicae.* The *M. brassicae* experiment was repeated once and therefor no statistics have been done on these results.

COI1, as expression of the majority of MeJA-regulated genes is known to be COI1-dependent (Devoto *et al.*, 2005). All four *JOX* genes were no longer induced by MeJA treatment in the *coi1-1* mutant (Fig. 2A), indicating that they are activated through the general COI1-dependent JA-response pathway.

Finally, we measured expression of the *JOX* genes after feeding by the herbivore *M. brassicae.* Four first-instar caterpillars were placed on each plant, and allowed to feed for up to two days. Damaged leaves were harvested after 24 and 48 hours from separate plants. Expression of all four *JOX* genes was induced in plants that were fed on by caterpillars compared to non-infested plants (Fig. 2B). Expression of *JOX1* was significantly induced after 24 hours of feeding, *JOX2* and *JOX3* were significantly induced at both 24 and 48 hours, while *JOX4* showed a low but clear induction of expression in response to *M. brassicae*. Our data demonstrate that the four *JOX* genes are induced by caterpillar feeding, which is known to activate the JA pathway (Reymond *et al.*, 2004), and by exogenous application of MeJA in a COI1-dependent manner.

JOXs act redundantly as negative regulators of JA responses

To study the function of the *JOX*-encoded 2OG oxygenases, we selected T-DNA insertion lines for each gene. The knockdown of each gene was confirmed by RT-PCR. To investigate if the JOX proteins could play a role in JA-dependent processes, we studied associated phenotypes in the four *jox* mutants. No clear phenotype was visible in any of the single mutants, as they were as susceptible to the necrotrophic fungal pathogen *B. cinerea* as wild type (data not shown). Since the four genes could act redundantly, a quadruple mutant *jox1 jox2 jox3 jox4* mutant (hereafter *jox4x*) combining the four insertions was generated, and JA-related phenotypes were analyzed in this mutant. We inoculated five-week-old wild-type and *jox4x* mutant plants with *B. cinerea* and measured the size of necrotic lesions after 3 days. As shown in Figure 3A, the lesions caused by infection of *B. cinerea* were significantly smaller in the *jox4x* mutant compared to lesions on Col-0 leaves ($P \leq 0.001$), indicating that *jox4x* is more resistant to *B. cinerea*. Immune responses to *B. cinerea* are regulated by the JA and ethylene signaling pathways that activate expression of defense-related genes such as *PLANT DEFENSIN1.2* (*PDF1.2*). To understand the molecular basis of this increased resistance, we measured expression of *PDF1.2* and *OCTADECANOID-RESPONSIVE ARABIDOPSIS59* (*ORA59*), which encodes an ERF transcription factor that activates *PDF1.2* expression. Already before infection, expression of the two genes was higher in the *jox4x* mutant compared to Col-0; *ORA59* was expressed 15-fold higher, while expression of *PDF1.2* was increased 9000-fold (Fig. 3B). During infection with *B. cinerea ORA59* and *PDF1.2* levels strongly increased and were significantly higher in the *jox4x* mutant than in Col-0 ($P \leq 0.001$) (Fig. 3B). Our

results show that these two defense-related genes are strongly upregulated in the *jox4x* mutant, which could explain the observed increased resistance of *jox4x* to *B. cinerea*.

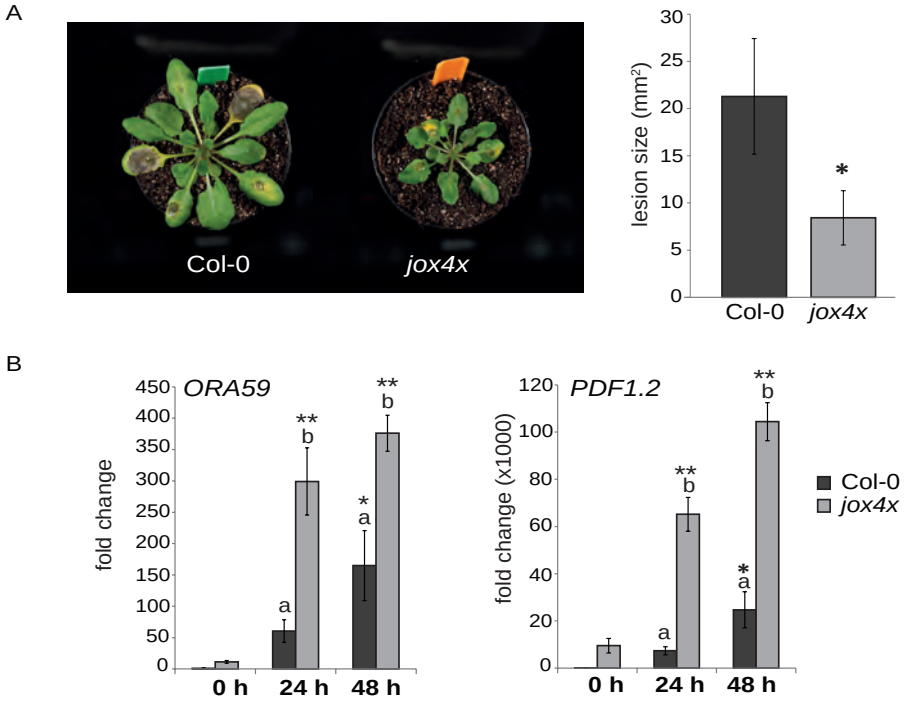


Figure 3: Simultaneous knock-down of four *JOX* genes results in increased resistance to *B. cinerea*. **(A)** Resistance of Col-0 and *jox4x* to *B. cinerea* infection as seen by disease symptoms in photograph (left) and size of necrotic lesions caused by *B. cinerea*. **(B)** Expression of the JA-responsive genes *ORA59* and *PDF1.2* before infection (0h) and after 24 or 48 hours of *B. cinerea* infection, relative to uninfected plants. Different letters indicate significant differences between genotypes. An asterisk indicates a significant higher expression compared to mock-treated plants (two-way ANOVA, Tukey post-hoc test; * $P \leq 0.05$; ** $P \leq 0.001$).

Defense against necrotrophic pathogens or against insect herbivores is thought to be regulated by two separate JA-regulated pathways, which often act antagonistically (Anderson *et al.*, 2004; Vos *et al.*, 2013b). For example, a mutant in transcription factor gene *MYC2* was increased susceptible to herbivorous insects but more resistant against *B. cinerea* (Lorenzo *et al.*, 2004; Dombrecht *et al.*, 2007). To see if the increased resistance we observed in *jox4x* plants was specific to necrotrophic pathogens or also effective against chewing herbivores, we measured the performance of the generalist caterpillar *M. brassicae*. Caterpillars were allowed to feed on wild-type and *jox4x* plants for eight days, after which they were weighed. As can be seen in Figure 4A, caterpillars that fed on *jox4x* were smaller than those that fed on

Col-0. The average weight of caterpillars was significantly lower when fed on *jox4x* than on wild-type Col-0 plants ($P \leq 0.001$; Fig. 4B), suggesting defense against herbivores is also upregulated in *jox4x* plants. The fact that the *jox4x* mutant is more resistant to both a necrotrophic pathogen and an herbivore suggests that the JOX proteins negatively regulate general JA-dependent defense.

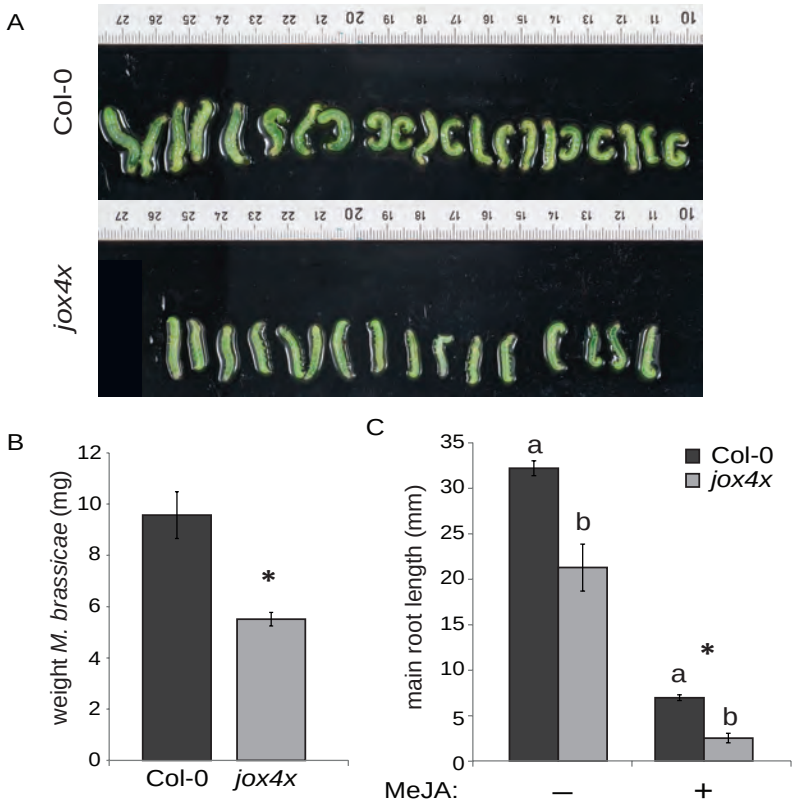


Figure 4: Increased resistance to *M. brassicae* and JA-sensitivity to root growth inhibition of *jox4x*
(A) Growth of *M. brassicae* caterpillars on Col-0 and *jox4x* plants. Caterpillar fresh weight was measured and caterpillars were photographed after 8 days of feeding. An asterisk denotes a significant difference between Col-0 and *jox4x* (*t* test, $P \leq 0.001$). **(B)** Root growth inhibition assays performed with Col-0 and *jox4x*. Seedlings were grown for 15 days on ½ MS medium with or without 50 μM MeJA. Different letters indicate significant differences between genotypes after each treatment. An asterisk indicates a significant growth inhibition compared to seedlings grown without MeJA. ($P \leq 0.05$; two-way ANOVA).

Besides being involved in defense against necrotrophic pathogens and herbivores, JA is implicated in many other processes, including growth and development. In accordance with this, the size of the *jox4x* quadruple mutant was consistently smaller than wild-type plants (Fig. 3A). We also investigated whether root growth was affected in the *jox4x* mutant, as this has been extensively used as a measure of JA-sensitivity.

We found that on media without MeJA, roots of the *jox4x* were shorter than those of Col-0 (Fig. 4C). When grown on media containing 50 μM MeJA, roots of *jox4x* were significantly shorter than those of Col-0 ($P \leq 0.01$; Fig. 4C), that were already strongly reduced in length. This suggests that the *jox4x* mutant is hypersensitive to JA. Flowering time was delayed by 6 days in the *jox4x* mutant compared to Col-0 under short-day conditions. Moreover, the mutant produced fewer seeds than wild-type Col-0 (Supplemental Figure S2). These results support the idea of a negative role of JOX in JA-mediated development and growth. In conclusion, all phenotypes observed in the quadruple *jox4x* mutant are reminiscent of plants with high JA levels and activated JA responses.

The *jox4x* mutant accumulates JA and has reduced turnover of JA into 12-OH-JA

The observation of JA-related phenotypes in the *jox4x* quadruple mutant would fit a putative role for the JOX proteins as JA-hydroxylases. Loss of hydroxylation of JA would result in higher JA levels and lower levels of the inactive 12-OH-JA (Fig. 3A). To test this idea, we measured the accumulation of JA and JA-related metabolites in the *jox4x* mutant. We chose to use wounded plants because wounding is known to result in accumulation of JA and 12-OH-JA (Glauser *et al.*, 2008). Five-week-old plants were wounded by stabbing the leaves five times across the mid-vein with a pin. Leaves were harvested at three hours after wounding and jasmonates were extracted. Accumulation of JA, JA-Ile, 12-OH-JA, and 12-OH-JA-Ile was measured with LC/MS. For comparison, the basal accumulation of JA and JA-derivatives was measured in non-wounded leaves of Col-0 and *jox4x* plants.

In plants that had not been wounded, JA levels were about 5x higher in the *jox4x* mutant than in Col-0 (Fig. 5B). Three hours after wounding, the basal JA levels were tripled to 11 ng/g FW in wild-type plants. In the *jox4x* mutant, JA rose to 170 ng/g FW (Fig. 5B). Thus, as predicted from the phenotypes of the *jox4x* mutant, JA accumulates to high levels in this mutant before and after wounding. As can be seen in Figure 5C, JA-Ile, 12-OH-JA, 12-OH-JA-Ile were not detected in plants that had not been wounded. After wounding, JA-Ile, 12-OH-JA and 12-OH-JA-Ile accumulated to low but detectable levels in Col-0. In the *jox4x* mutant, levels of these compounds were all slightly higher. The increased accumulation of JA in the *jox4x* mutant suggests that in wild-type plants the JOX proteins have a negative effect on the accumulation of JA, possibly via hydroxylation of JA. To further study this, *JOX1* (At3g11180) was overexpressed in Col-0 background (*JOX1* OX). In accordance with our hypothesis, JA levels were slightly lower than in Col-0 under basal conditions when *JOX1* was overexpressed. Moreover, JA levels did not increase after wounding in this line (Fig. 5B). The levels of the other JA-derivatives were very low or not detectable in this line (Fig. 5C).

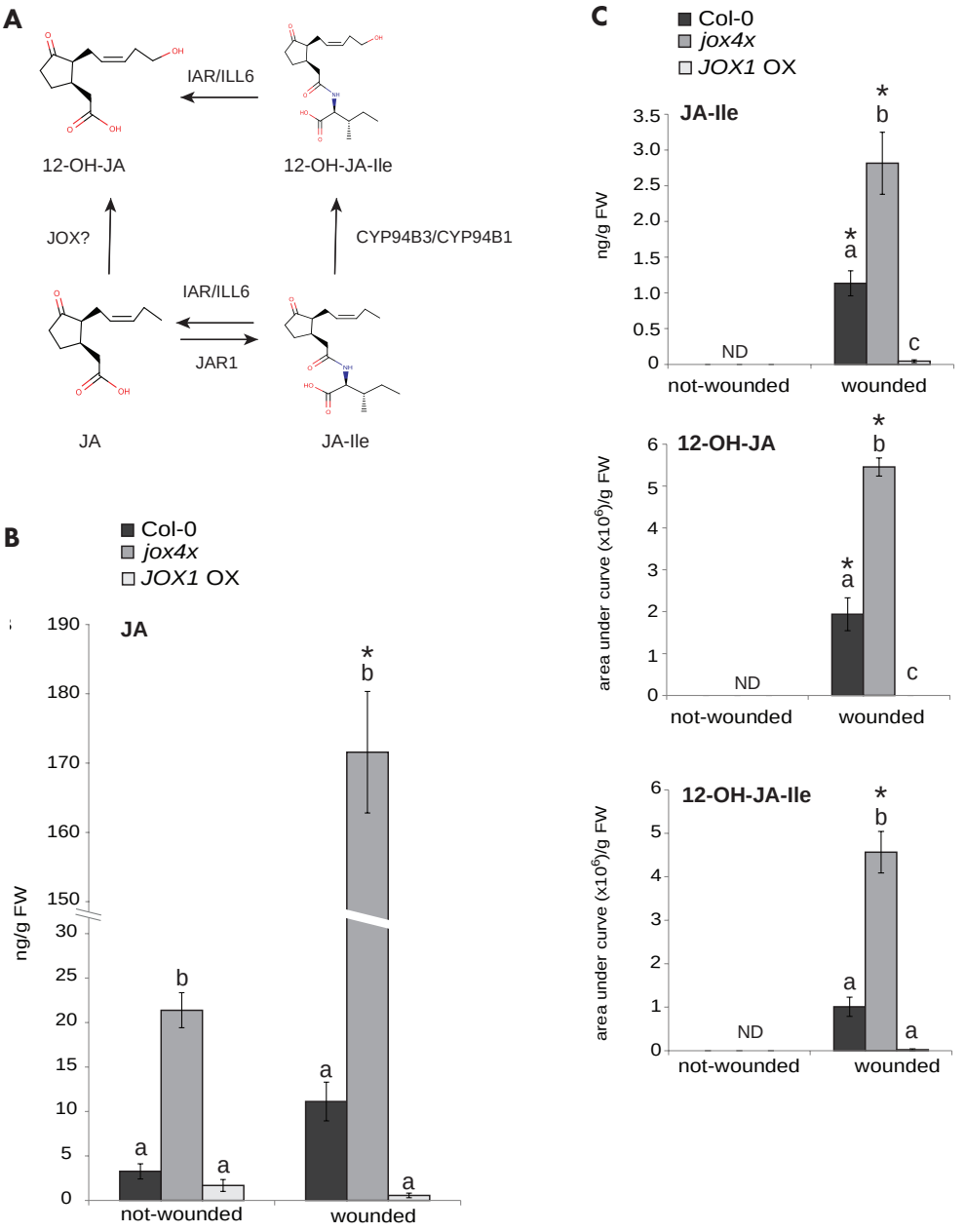


Figure 5: The *jox4x* mutant accumulates JA and derived metabolites (A) Scheme of JA and three JA-derived compounds: JA-Ile, 12-OH-JA, and 12-OH-JA-Ile. Enzymes that catalyze the conversions are indicated: JAR1 conjugates isoleucine to JA to form JA-Ile. CYP94B3 and CYP94C1 hydroxylate JA-Ile to 12-OH-JA-Ile. IAR and ILL6 can hydrolyze the Ile from JA-Ile or from 12-OH-JA-Ile, forming JA or 12-OH-JA, respectively. The hypothesized hydroxylation of JA by JOX is indicated with a question mark. **(B, C)** JA levels (B) and 12-OH-JA, JA-Ile, and 12-OH-JA-Ile levels (C), in not-wounded and wounded leaves (3 h after mechanical damage) in Col-0, *jox4x* and *JOX1* OX plants. Each data point represents the mean

of four biological replicates. Error bars indicate standard error. JA levels were calculated by correcting for the internal standard of JA, and dividing by leaf weight. JA-Ile levels were calculated by using a standard curve. For 12-OH-JA and 12-OH-JA-Ile, the area under the curve for each compound (parent ion/daughter ion: 12-OH-JA: 225/59; 12-OH-JA-Ile: 338/130) was recorded and corrected for recovery of JA and leaf weight. Different letters indicate statistically significant differences between genotypes at the same treatment. An asterisk indicates that wounding significantly induced the compound (two-way ANOVA; Tukey post-hoc test; $P \leq 0.05$).

Unexpectedly, the levels of 12-OH-JA were also higher in *jox4x* in response to wounding and were about 3x increased compared to Col-0 (Fig. 5C). However, this was lower than the 14x increase in JA levels. If JOXs indeed hydroxylate JA, we would expect low levels of 12-OH-JA in the *jox4x* mutant. However, 12-OH-JA could still be generated in *jox4x* mutant via JA-Ile. JA can be converted into JA-Ile, followed by hydroxylation into 12-OH-JA-Ile. As a last step, the Ile group can be cleaved of 12-OH-JA-Ile by ILL6 and IAR3 to produce 12-OH-JA (Figure 5A). These aminohydrolases have been shown to generate 12-OH-JA upon wounding (Widemann *et al.*, 2013). The observation that 12-OH-JA levels followed the same pattern as JA-Ile and 12-OH-JA-Ile levels after wounding supports the idea that 12-OH-JA is formed via this route (Fig. 5C). In addition, in the line that overexpresses *JOX1* we expected increased 12-OH-JA to accumulate, however, 12-OH-JA was not detected in this line (Fig. 5C). We hypothesize that JA levels in this OX line are so low, that increased hydroxylation is not seen.

To overcome the difference in JA levels, we aimed to equalize them in wild-type, *jox4x* and *JOX1* OX plants to investigate if similar levels of JA would lead to differences in 12-OH-JA levels in the different genotypes. For this, we treated plants with JA, by immersing the leaves in a 100 μM JA solution, and harvested material for analysis of jasmonates after 3 hours. As in the previous experiment, JA levels were higher in untreated *jox4x* leaves than in untreated Col-0 (data not shown). In JA-treated leaves, JA levels were similar to Col-0 in *jox4x*, and lower in the *JOX1* OX line. JA-Ile levels were between 1 and 1.5 ng/g FW in all genotypes (Fig. 6). Strikingly, the level of 12-OH-JA was lower in the *jox4x* mutant. In contrast, in the *JOX1* overexpression line, the levels of 12-OH-JA were higher than in Col-0 (Fig. 6). This indicates that the conversion of JA into 12-OH-JA is clearly reduced in the *jox4x*, and enhanced in the *JOX1* OX line. The 12-OH-JA-Ile levels were similar in *jox4x* to wild type, and lower in the *JOX1* OX. This suggests that the reduced 12-OH-JA levels we measured in *jox4x*, are not caused by decreased 12-OH-JA-Ile levels, but rather by a reduced direct hydroxylation of JA (Fig. 5A). We conclude that the *jox4x* mutant accumulates JA under basal conditions, and shows reduced activity to form 12-OH-JA after treatment with JA. In contrast, a line overexpressing *JOX1* builds up less JA, and accumulates more of the hydroxylated form when treated with JA. We propose that the JOX proteins catalyze the hydroxylation of JA, thereby inactivating JA, resulting in negative regulation of downstream immunity and other responses.

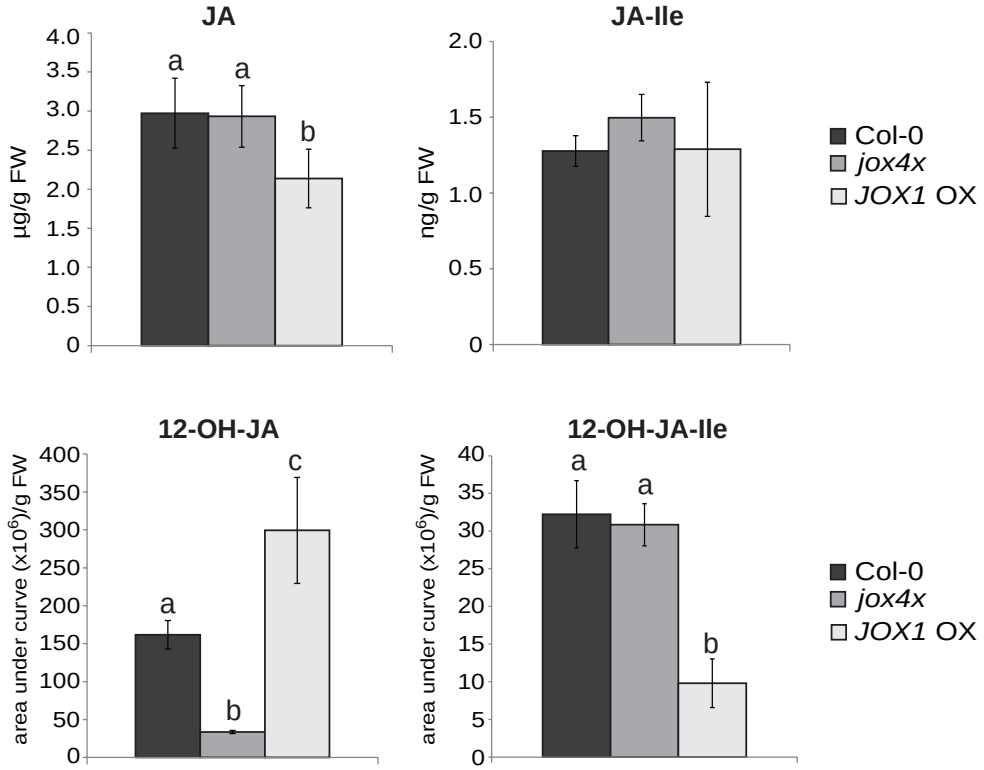


Figure 6: The *jox4x* mutant has reduced turnover of JA into 12-OH-JA. Accumulation of JA, 12-OH-JA, JA-Ile, and 12-OH-JA-Ile in plants treated for 3 hours with 100 µM JA. Each data point represents the mean of four biological replicates. Error bars indicate standard error. JA levels were calculated by correcting for the internal standard of JA, and dividing by the leaf weight. JA-Ile levels were calculated by using a standard curve. For 12-OH-JA and 12-OH-JA-Ile, the area under the curve for each compound (parent ion/daughter ion: 12-OH-JA: 225/59; 12-OH-JA-Ile: 338/130) was recorded and corrected for recovery of JA and leaf weight. Different letters indicate statistically significant differences between genotypes (two-way ANOVA; Tukey post-hoc test; $P \leq 0.05$).

DISCUSSION

Hormone inactivation by 2OG oxygenase-mediated hydroxylation

Inactivation of hormones by hydroxylation is an important mechanism to prevent over-accumulation of active hormones, thereby inhibiting inappropriate activation of their downstream signaling pathways. Here, we provide evidence that members of the JOX 2OG oxygenase family contribute to negative regulation of JA responses by converting JA into the inactive 12-OH-JA form. The four characterized *JOX* genes, *JOX1, JOX2*, *JOX3* and *JOX4* are induced by MeJA treatment and by caterpillar feeding, and their JA-responsive expression requires the JA receptor COI1. Plants in

which the four genes were mutated (*jox4x*) exhibited phenotypes indicative of high JA levels, i.e. enhanced defense gene expression and resistance to both *B. cinerea* and the herbivore *M. brassicae*. We show that the *jox4x* mutant has a higher basal JA level than wild-type plants, which is strongly increased after wounding. In addition, when treated with exogenous JA, hydroxylation of JA into 12-OH-JA was strongly reduced in the *jox4x* mutant compared to Col-0, while a line overexpressing *JOX1* accumulated almost twice as much of the hydroxylated form as the wild type. The hormones GA and SA have also been shown to be hydroxylated by 2OG oxygenases closely related to the JOXs. In Figure 1, clade 12 containing GA2OXs are enzymes hydroxylating GA, and S3H (clade 38) was shown to hydroxylate SA (Rieu *et al.*, 2008; Zhang *et al.*, 2013a). It is striking to see that many members of the 2OG oxygenase family have evolved to inactivate different plant hormones.

Clade 46 of the 2OG oxygenases as defined by Kawai *et al.* (2014) consists of four Arabidopsis members encoded by the genes At3g11180, At5g05600, At3g55970 and At2g38240, which we named *JOX1*, *JOX2*, *JOX3,* and *JOX4*, respectively. Because we did not see increased resistance to *B. cinerea* in the single mutants, and expected redundancy in the function of the JOXs, we generated a quadruple mutant. It is consequently not clear yet which of the four enzymes can indeed hydroxylate JA. Only for JOX1 we can state that it is an active JA-hydroxylase as overexpression of *JOX1* resulted in increased accumulation of 12-OH-JA. We are currently testing if also JOX2, JOX3 and JOX4 have JA hydroxylating activity in transgenic plants. In addition, assays with recombinant JOX enzymes will show if they can hydroxylate JA and related compounds *in vitro*. These experiments are necessary before we can definitely establish the JOX enzymes as JA-hydroxylases.

JA metabolism

Plants convert JA to derivatives that remain biologically active, or become reduced active or inactive. In total, 12 different JA-derived compounds have been identified (Wasternack and Strnad, 2015). The enzyme JAR1 conjugates JA to isoleucine, generating the bioactive JA-Ile (Staswick and Tiryaki, 2004; Suza and Staswick, 2008; Fonseca *et al.*, 2009). In recent years, the mechanism of hydroxylation of JA-Ile has been elucidated. Researchers from different labs have identified that the enzymes CYP94B3, CYP94B1 and CYP94C1 hydroxylate and subsequently carboxylate of JA-Ile in Arabidopsis. Our *jox4x* mutant shows similar phenotypes as the JA-Ile hydroxylase mutants, e.g. enhanced expression of JA-responsive genes, increased resistance to an herbivore and to a necrotrophic fungus and increased sensitivity to JA-dependent inhibition of root growth (Fig. 3; Fig. 4). This suggests that hydroxylation of JA by the JOX proteins contributes to inactivation of the active JA signal to a similar extent as hydroxylation of JA-Ile.

A model depicting the role of JOX in JA accumulation and JA-mediated responses in the *jox4x* mutant and *JOX1* OX line is summarized in Figure 7. In the *jox4x* mutant, the partial loss of JA hydroxylation leads to higher JA levels in unwounded plants compared to Col-0 (Fig. 5B). In response to wounding, JA-Ile levels are also higher in *jox4x*, possibly because JA and JA-Ile levels are in equilibrium (Fig. 5C). As JA-biosynthesis genes are JA-responsive, it is likely that this then leads to increased biosynthesis and thus to even more accumulation of JA (Fig. 7). Supporting the idea that JA biosynthesis is upregulated in *jox4x*, we detected that OPDA levels were about 4-5 x times higher in the *jox4x* mutant than in Col-0 (data not shown). In the *JOX1* OX line, presumably more JA is turned over into its inactive form, so that JA and coupled JA-Ile levels are reduced (Fig. 5B and 5C). Reduced JA-Ile leads to a decreased expression of JA-biosynthesis genes, further reducing the amount of JA present in the plant.

Activation of JA-dependent gene expression relies on the degradation of JAZ repressor proteins. The conjugate JA-Ile is considered the biologically active form as its binding to COI1–JAZ complexes leads to the degradation of JAZ repressor proteins and activation of JA-induced gene expression (Chini *et al.*, 2007; Thines *et al.*, 2007; Fonseca *et al.*, 2009). JA-Ile was shown to promote binding of COI1 to JAZ1, JAZ2, JAZ3, JAZ6, JAZ10 and JAZ12. In contrast, other forms of JA that are considered active, such as JA itself and MeJA, did not promote the interaction of COI1 and JAZ1, JAZ3 or JAZ9 (Thines *et al.*, 2007; Katsir *et al.*, 2008; Fonseca *et al.*, 2009; Shyu *et al.*, 2012). However, for exogenous MeJA to activate JA-responses it first needs to be metabolically converted to JA and subsequently JA-Ile in plants (Tamogami *et al.*, 2008; Wu *et al.*, 2008).

In untreated *jox4x*, we did not detect any JA-Ile without wounding, but we observed several JA-responsive phenotypes, most notably a high basal expression of *ORA59* and *PDF1.2* (Fig. 3B). Possibly, JA-Ile is turned over so quickly that we could not detect it in our samples. Alternatively, it is possible that the unconjugated form of JA could trigger some JA-dependent responses. While the *jar1* mutant is affected in several JA-mediated phenotypes, wound-induced expression of *JAZ5*, *JAZ7*, and *MYC2* was comparable to wild-type plants (Staswick *et al.*, 1998; Chung *et al.*, 2008). It has been suggested that the low amounts of JA-Ile that remain in *jar1* still activate JA-responsive genes (Suza and Staswick, 2008). It has also been speculated that other derivatives of JA or JA itself can trigger gene activation. Interestingly, MeJA-induced expression of *ORA59* and wound-induced expression of *PDF1.2* was not affected in the *jar1* mutant (Devoto *et al.*, 2005; Suza and Staswick, 2008). We are currently investigating if JA by itself could be activating these genes in the *jox4x*. Alternatively, the increased levels of OPDA could influence several phenotypes, as OPDA has been shown to act as a signaling compound independent of JA signaling (Wasternack and Strnad, 2015).

Hydroxylation of JA is essential to balance jasmonate levels

In this study, we identified JOXs as negative regulators of JA responses that contribute to hydroxylation and inactivation of JA. By controlling trade-offs between defense and growth related processes, JA can trigger plants to temporally prioritize immune responses over growth. Our data shows that it is imperative that plants balance JA levels by controlling JA biosynthesis and metabolism. Increased levels of JA do lead to increased defense against a necrotrophic pathogen and a caterpillar, but this could be detrimental for the plant in conditions where there are no attackers, as the plants are smaller and flower later, producing fewer seeds. The fact that expression of the *JOX* genes is COI1-dependent, suggest that JOX enzymes are involved in a JA-responsive system for the inactivation of JA that prevents untimely or exaggerated responses. The identification of JOXs as JA hydroxylases adds an important component to the knowledge on JA metabolism.

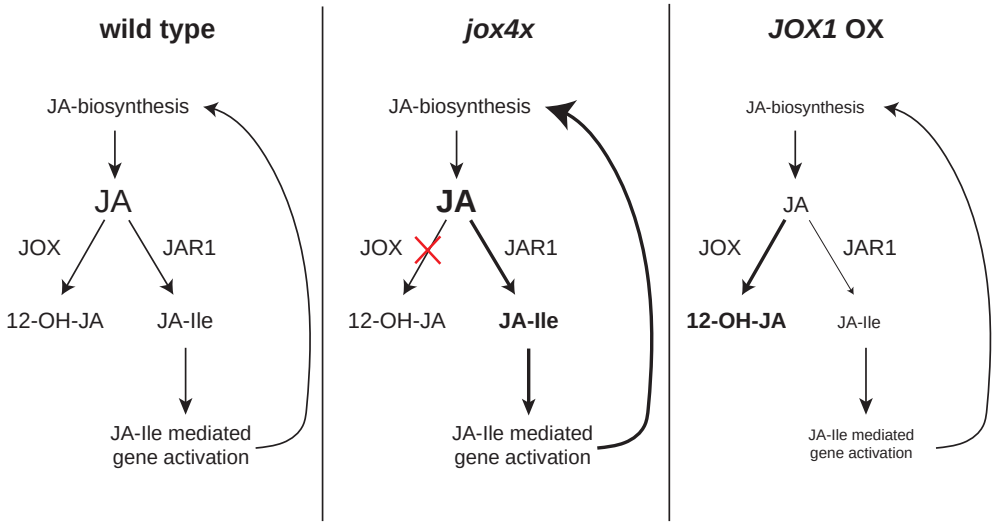


Figure 7: JOXs are negative regulators of JA responses by reducing bioactive JA levels (A) In wild-type plants, activation of the JA pathway leads to JA-responsive gene expression and other JA responses by conversion of JA to the bioactive form JA-Ile by JAR1. JOX inactivate JA by metabolizing it into 12-OH-JA and thus prevent over-accumulation of JA and JA-Ile. **(B)** In the *jox4x* mutant, the loss of hydroxylation results in higher levels of JA and, consequently, higher levels of JA-Ile. JA-responsive gene expression and JA responses are enhanced. As JA-responsive gene expression also activates JA-biosynthesis genes, a positive feedback loop enhances accumulation of JA. **(C)** Overexpression of *JOX1* leads to increased hydroxylation and inactivation of JA. Levels of active JAs (JA and JA-Ile) are strongly reduced, thereby decreasing JA responses and JA biosynthesis.

MATERIALS AND METHODS

Phylogenetic analysis and data mining

A set of Arabidopsis genes encoding 2OG-oxygenases containing both the Pfam domains PF03171 (2OG-Fe(II) oxygenase superfamily) and PF14226 (non-haem dioxygenase in morphine synthesis N-terminal) was selected from TAIR10 using Biomart (plant.ensemble.org/biomart). For each gene the longest protein model was selected for phylogenetic analysis at phylogeny.fr (Dereeper *et al.*, 2008), using MUSCLE (default settings), Gblocks (least stringent settings) and PhyML (using the approximate Likelihood-Ratio Test). Phylograms/cladograms were generated with the obtained tree file, and visualized/decorated using Evolview (Zhang *et al.*, 2012a). Publically available microarray data for the 93 selected 2OG oxygenases was obtained from Genevestigator V3 (Hruz *et al.*, 2008). For Figure 1, $\log_2$ values of fold changes for a subset of 50 2OG oxygenase genes in Arabidopsis seedlings at 3 h after MeJA treatment (Exp. ID AT-00110) or 3 h after SA treatment (Exp. ID AT-00113) relative to untreated controls were used (Goda *et al.*, 2008).

Plant material and growth conditions

Arabidopsis thaliana seeds were sown on river sand. Two weeks after germination, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture (5:12 v/v) that had been autoclaved twice for 45 min with a 24-h interval. Plants were cultivated in a growth chamber with a 10-h day and 14-h night cycle at 70% relative humidity and 21˚C. T-DNA insertion lines *jox1* (At3g11180; SAIL_131_D01), *jox2* (At5g05600; GK-870-C04), *jox3* (At3g55970; SAIL_861_E01) and *jox4* (At2g38240; SAIL_268_B05) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). A quadruple mutant *jox4x* was obtained by crosses between homozygous T-DNA insertion lines in subsequent generations, followed by self-fertilization to obtain homozygous lines. Knockdown of expression of each *JOX* was confirmed with RT-PCR. Primers used for genotyping and RT-PCR are described in Table 1. As *coi1-1* is male sterile, seeds heterozygous for the mutation were grown, and plants homozygous for the *coi1* mutation (JA-insensitive) were selected on ½ MS plates containing 50 μM MeJA.

The coding sequence of At3g11180 was amplified from Col-0 and cloned into the pENTR vector using Gateway® cloning (Invitrogen) and then cloned into the pB7WG2 Gateway® compatible binary vector under control of the 35S promoter. Binary vectors were transformed into *A. tumefaciens* strain C58C1 containing pGV2260, which was used for transformation of Arabidopsis Col-0 using the floral dip method (Clough and Bent, 1998). Transformants were selected by spraying with BASTA, and resistant T1 plants were transplanted for seed production.

To measure root growth, plants were grown on ½ MS plates with or without 50 μM MeJA for 15 days. Photographs were taken and root length was measured using ImageJ image processing software (Abramoff *et al.*, 2004). To measure flowering time of plants, the days were counted until the first flower appeared. Seeds were harvested per plant and the weight of seeds per plant was measured.

Pathogen and insect inoculation

B. cinerea inoculations were performed with strain B05.10 on five-week old plants as previously described (Van Wees *et al.*, 2013). On each plant 5 μL spore suspension of 10^5 conidia/mL was applied on five leaves. Inoculated plants were kept under 100% relative humidity until lesion size was determined. For measurement of lesion size, photographs were taken of detached leaves, and the lesion size was determined with FIJI image processing software (Schindelin *et al.*, 2012).

M. brassicae eggs were provided by the Entomology department at Wageningen University where *M. brassicae* was reared as described (Pangesti *et al.*, 2015). To measure *M. brassicae*-induced gene expression, four first instar caterpillars were placed on five week old plants. At indicated time points, two leaves were harvested for RNA extraction. To measure caterpillar performance, one first instar caterpillars was placed on a five-week-old plant. Caterpillars were allowed to feed for eight days on the plants, after which they were weighed.

Chemical treatment, RNA extraction and qRT-PCR

Five-week-old plants were dipped in a solution containing 100 μM MeJA (from a stock solution of 100 mM MeJA dissolved in 96 % ethanol (Serva, Brunschwig Chemie, Amsterdam, the Netherlands), and 0.015% Silwet (Van Meeuwen Chemicals BV, Weesp, the Netherlands). For control treatment, plants were dipped in a 0.015% Silwet solution containing the same volume of 96% ethanol. To measure MeJA-induced gene expression two leaves per plant were harvested at indicated times after treatment and frozen in liquid nitrogen. RNA was extracted as described for vegetative tissues by Oñate-Sánchez and Vicente-Carbajosa (2008). RNA that was used for qRT-PCR was pretreated with DNAse I (Fermentas, St. Leon-Rot, Germany) to remove genomic DNA. RevertAid H minus Reverse Transcriptase (Fermentas) was used to convert DNA-free total RNA into cDNA using an oligo-dT primer. PCR reactions were performed in optical 384-well plates with a ViiA 7 realtime PCR system (Applied Biosystems, Carlsbad, CA, USA), using SYBR® Green to monitor the synthesis of double-stranded DNA. Primers used to determine expression levels are found in Table 1. A standard thermal profile was used: 50° C for 2 min, 95° C for 10 min, 40 cycles of 95° C for 15 s and 60° C for 1 min. Amplicon dissociation curves

were recorded after cycle 40 by heating from 60 to 95° C with a ramp speed of 1.0° C min^{-1}. Fold change was calculated relative to the reference gene At1g13320 (Czechowski *et al.*, 2005) using the $2^{-\Delta\Delta}$Ct method described previously (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Expression after JA-treatment was calculated relative to expression of the gene in mock-treated plants at the same time point.

Table 1: List of all primers designed for genotyping and qRT-PCR in this study.

Name	Sequence (5' → 3')
Primers used for genotyping:	
At3g11180 SAIL_131_D01 Fw	GCCTCAACGATCAAGAACAAG
At3g11180 SAIL_131_D01 Rv	GTTGAGGGCACTTTGGGTAA
At5g05600 GK_870C04 Fw	CTCATCCCCATGCTTTCATC
At5g05600 GK_870C04 Rv	CGAGGAGAAATATGAGATTCAACA
At3g55970 SAIL_861_E01 Fw	GAACCAGCTCCTCATGCTTT
At3g55970 SAIL_861_E01 Rv	GGGTTCACGATCACTCTGTG
At2g38240 SAIL_268_B05 Fw	ATGGCTGGGTCACCATTAAA
At2g38240 SAIL_268_B05 Rv	CAAGGACCCTTTTGCCTTATC
SAIL LB (for SAIL_268_B05)	TAGCATCTGAATTTCATAACCAATCTCGATACAC
SAIL LB modified (for SAIL_131_D01 and SAIL_861_E01)	TCTGAATTTCATAACCAATCTCG
GABIKAT LB08409	ATATTGACCATCATACTCATTGC
At5g05600 GK_870C04 Rv-2 (for insert PCR)	CCACGTTTTTCAAACATGACG
Primers used for qRT-PCR:	
at5g05600 qRT-PCR F	CTCATCCCCATGCTTTCATC
at5g05600 qRT-PCR R	TCCGAGTTCACTATCACTCTATGC
at3g11180 qRT-PCR F	CGTGGATCACTGTCAATCCT
at3g11180 qRT-PCR R	CGATGTTCCACGCTCTTGTA
at5g55970 qRT-PCR F	GAACCAGCTCCTCATGCTTT
at5g55970 qRT-PCR R	GGGTTCACGATCACTCTGTG
at2g38240 qRT-PCR F	ATGGCTGGGTCACCATTAAA
at2g38240 qRT-PCR R	GTTCCACGCTTTTGTAAATTCC

Quantification of jasmonates by LC-MS/MS

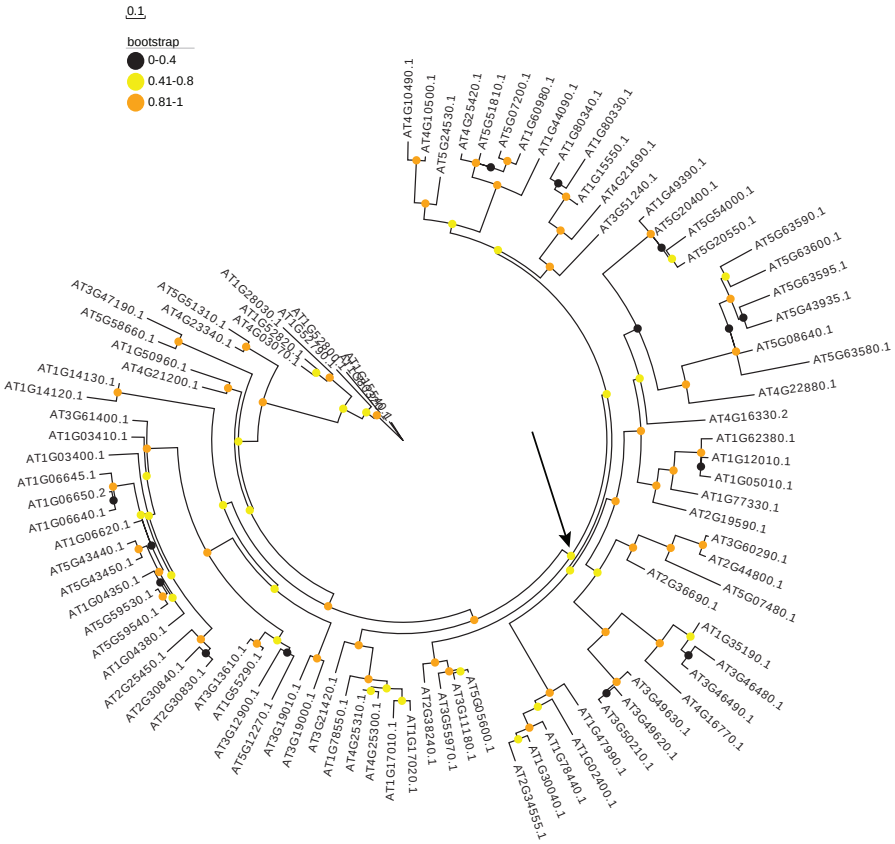
For quantification of JA and JA-derivatives in plants after wounding, leaves were damaged across the mid-vein by stabbing five times with a pin. Wounded and non-wounded leaves (from separate plants) were harvested in four biological replicates, at indicated time points, and frozen. Frozen leaf material was ground and homogenized in 1 mL ethyl acetate containing D_5-JA and D_6-SA as internal standards, and further processed and analyzed by LC-MS/MS as described (Alba *et al.*, 2015). For JA-treated leaves, four leaves were soaked in 100 µM JA (Sigma) and 0.015% Silwet for 3h, after which leaves were frozen. Frozen leaf material was ground and homogenized in 1 mL 90% MeOH and further processed as described

(Widemann *et al.*, 2013) and analyzed by LC-MS/MS as described (Alba *et al.*, 2015). For all JA-derivatives measured we used D_5-JA to estimate the recovery rate. JA-Ile was quantified using a standard curve generated by running a serial deletion of pure JA-Ile.

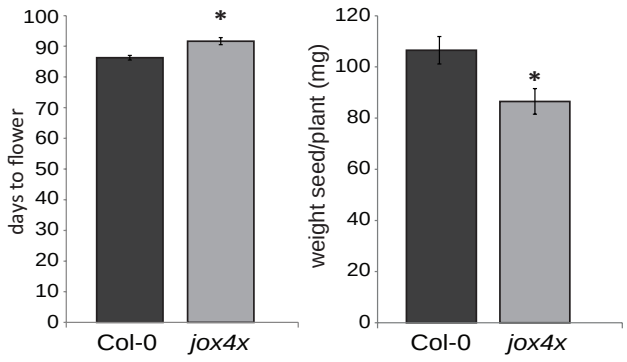
ACKNOWLEDGMENTS

We are grateful to Léon Westerd for rearing of *M. brassicae* and providing eggs and to Hans van Pelt for taking photographs of plants and caterpillars. This work was supported by the European Research Council (ERC Advanced Investigator Grant no. 269072 to C.M.J. Pieterse).

SUPPLEMENTAL DATA



Supplemental Figure S1: Phylogenetic tree of 93 proteins that contain both Pfam domain PF03171 (2OG-Fe(II) oxygenase superfamily) that is in the C-terminal half, and Pfam domain PF14226 (non-haem dioxygenase in morphine synthesis N-terminal) that is in the N-terminal half of most 2OG oxygenases. The confidence of each branch point is indicted by the bootstrap values that are grouped in three classes (see color legend). The arrow indicates the cluster of 50 proteins that was selected for gene expression analysis and is visualized in main Figure 1.



Supplemental Figure S2: The *jox4x* mutant flowers later and produces less seed. Flowering time and seed production (weight per plant) in Col-0 and *jox4x* plants. Flowering time was measured as the day, measured from germination, on which the first open flower was visible. Seeds were harvested per plant, and weighed. An asterisk indicates a significant difference between Col-0 and *jox4x* (*t* test, $P \leq 0.01$).

CHAPTER 5

Summarizing discussion

The activation of defense responses, in which salicylic acid (SA) and jasmonic acid (JA) are essential signaling molecules, is imperative to fight off attackers and survive disease. However, the induction of defenses is at the expense of plant growth (Huot *et al.*, 2014). Resources that are allocated to resistance processes can no longer be used for growth or reproduction. Furthermore, the activation of defense can have autotoxicity costs as induced secondary metabolites that are repellent for attackers may be toxic for the plant as well (Vos *et al.*, 2013a). The costs of activation of the SA signaling pathway are evident from the dwarf phenotype of mutants that constitutively express SA-inducible defenses (*cpr* mutants) (Bowling *et al.*, 1994). In general, plants that overaccumulate SA, such as plants that overexpress *EDS5,* which encodes an SA transporter, or the double mutant of SA-inactivating enzyme S3H and DMR6, show stunted growth phenotypes (Ishihara *et al.*, 2008; Zeilmaker *et al.*, 2015). Similarly, a mutant with constitutively activated JA responses is smaller and has shorter roots (Ellis and Turner, 2001). JA has been shown to inhibit cell growth by inhibiting mitosis, and delays the switch from the mitotic cycle to the endoreduplication cycle (Zhang and Turner, 2008; Noir *et al.*, 2013). Moreover, activation of JA represses expression of photosynthesis genes (Attaran *et al.*, 2014) and delays flowering (Zhai *et al.*, 2015).

Hormonal crosstalk between defense hormones and growth-promoting hormones such as auxin, gibberellins (GA), brassinosteroids (BR), and cytokinins is implicated in fine-tuning the growth versus defense trade-off. Suppression of defense by growth hormones allows the plant to prioritize growth over defense when (rapid) growth is essential for survival, for example in seedlings that are emerging from the soil or in plants that are in fierce competition for light (De Wit *et al.*, 2013; Lozano-Durán *et al.*, 2013). Conversely, activation of JA and SA pathways can suppress growth hormone signaling pathways to (temporarily) prioritize defense responses. For instance, SA was shown to inhibit the auxin signaling pathway through stabilization of AUX/IAA repressor proteins (Wang *et al.*, 2007). Moreover, JA was shown to antagonistically act on GA, auxin and BR (Huot *et al.*, 2014).

In order to minimize the negative effects of induction of defenses, it is essential for plants to control activation of defense hormonal signaling pathways in a time- and cost-efficient manner. Plants have evolved elaborate mechanisms to strictly regulate the production, activity and shutdown of hormonal signaling pathways. Activation of transcription is in many of these pathways regulated by the removal of repressors via ubiquitin-mediated degradation. Hormone-dependent degradation of repressor proteins by the 26S proteasome ensures that transcription factors are not active until the hormones accumulate. In the case of the JA signaling pathway, JA-Ile promotes the binding of JAZ repressors proteins to the E3 ligase SCF^{COI1} complex, which triggers their degradation. A strikingly similar mechanism is used in the

auxin, GA, and strigolactone pathways (Shabek and Zheng, 2014). Moreover, the ubiquitin-proteasome system plays important roles in ethylene and abscisic acid (ABA) signaling (Kelley and Estelle, 2012). In SA signaling, NPR3 and NPR4 have been shown to bind SA, and target NPR1 for degradation by the proteasome in a SA-dependent manner (Fu *et al.*, 2012; Furniss and Spoel, 2015).

Metabolism of hormones is also strictly controlled. Biosynthesis of both JA and SA are repressed until danger signals are recognized that induce expression of biosynthesis genes (Campos *et al.*, 2014; Seyfferth and Tsuda, 2014). Inactivation of the produced hormones is essential to prevent overaccumulation of active forms. SA and JA may both undergo several chemical modifications, e.g. glucosylation, methylation and conjugation with amino acids (Dempsey *et al.*, 2011; Wasternack and Strnad, 2015), of which some are inactive forms of the hormones. The conjugates of SA with sugars, i.e. SA 2-*O*-β-D-glucoside (SAG) or salicyloyl glucose ester (SGE) are inactive and stored in the vacuole (Dean *et al.*, 2003). However the major inactive forms of SA are likely hydroxylated forms of SA, i.e. 2,3 dihydroxybenzoic acid (DBHA) and 2,5-DHBA (Bartsch *et al.*, 2010). The 2-oxoglutarate/Fe(II)-dependent (2OG) oxygenase S3H hydroxylates SA to 2,3-DHBA. Interestingly, the hydroxylated form of JA and of its bioactive form JA-Ile have also been described to be inactive. In Chapter 4 of this thesis, we describe the identification of four 2OG oxygenases that are involved in the hydroxylation and inactivation of JA.

Indirect costs from activation of the SA and JA signaling pathways may arise from a changed physiology of the plant that affects interactions with other (beneficial or harmful) organisms and competing plants (Vos *et al.*, 2013a). Besides a trade-off between growth and defense, there is in this way a trade-off in defense against biotrophic pathogens and against necrotrophic pathogens or herbivores. Seemingly, plants can activate resistance against one type of attacker or another, but not to all at the same time. This phenomenon is largely ascribed to the crosstalk that exists between the SA and JA signaling pathways (Vos *et al.*, 2013a). The benefits for the plant of this type of crosstalk are less obvious. Possibly, the suppression of unnecessary defenses that are ineffective against the attacker that is encountered has evolved as a means of the plant to reduce allocation costs (Thaler *et al.*, 2012). Preliminary evidence for this hypothesis was provided recently, when Vos and colleagues showed that pre-infection with *Hyaloperonospera arabidopsidis* enhanced susceptibility to subsequent infection with *Botrytis cinerea*, but did not increase the fitness costs compared to the single attacker (Vos *et al.*, 2015). Another hypothesis is that the interactions between the SA- and JA- pathways are advantageous because they enable the plant to fine-tune its defense responses and adjust defense to each attacker encountered. The complex and interconnected gene regulatory networks that have been identified in the response against pathogens, which contain factors from both JA- and SA-signaling pathways, seem to support this idea (Kim *et al.*,

2014; Lewis *et al.*, 2015). Increasing application of systems biology approaches to hormone signaling pathways will assist in understanding this aspect of hormonal crosstalk.

Attack by one single pathogen, which is still the way that most plant-microbe interactions are studied in the laboratory, is probably quite rare in nature. Plants are likely being attacked by more than one attacker at the same time, while their roots are in contact with soil that contains a great diversity of microbes (Berendsen *et al.*, 2012). The complexity of these kind of systems is something we are just beginning to be able to investigate and understand. In the research described in this thesis, we used a pharmacological approach to study the molecular mechanisms underlying the antagonistic interactions between the SA and JA signaling pathways. This is a reductionist method, in which (combinations of) hormones are applied exogenously to activate their respective signaling pathways. While in this way a large part of the complexity described above is not studied, this type of research is instrumental in identifying signaling hubs that are important in SA/JA crosstalk. This contributes to understanding the complexity of hormonal signaling pathways and ultimately helps to comprehend the role SA/JA crosstalk plays in plant defense signaling. The main goal of the study described in this thesis was to investigate the molecular mechanisms that execute SA-mediated suppression of JA-responsive gene expression. For this, we studied the role of SA-induced ERF repressors (Chapter 2) and the function of SA signaling hub NPR1 (Chapter 3) in SA-mediated suppression of JA-responsive genes.

NOVEL JA-INACTIVATING ENZYMES

JA has been shown to be modified to MeJA, JA-Ile, 12-OH-JA and several other metabolites (Wasternack and Strnad, 2015). JA is conjugated to isoleucine by the enzyme JAR1 (Staswick and Tiryaki, 2004; Suza and Staswick, 2008). This bioactive JA-Ile molecule activates JA-dependent gene expression by promoting the interaction between JAZ and COI1, which results in degradation of JAZ (Chini *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008). Conversion of bio-active JA molecules into inactive forms is another mechanism to control the level of active JA molecules and downstream responses. In the last five years, researchers from different labs have identified cytochrome P450 hydroxylases that catalyze the ω-oxidation of JA-Ile. CYP94B3 and CYP94B1 hydroxylate JA-Ile, while CYP94C1 carboxylates 12-OH-JA-Ile to 12-COOH-JA-Ile (Kitaoka *et al.*, 2011; Koo *et al.*, 2011; Heitz *et al.*, 2012; Koo *et al.*, 2014). Overaccumulation of JA-Ile in *cyp94* mutants resulted in enhanced wound-induced expression of JA-responsive genes and increased sensitivity to JA-mediated root growth inhibition (Kitaoka *et al.*, 2011; Heitz *et al.*, 2012). The

hydroxylated and carboxylated form of JA-Ile are less active than JA-Ile, as they do not promote binding of COI1 to JAZ as JA-Ile does (Aubert *et al.*, 2015).

In Chapter 4, we identified the enzymes involved in ω-oxidation of JA. We showed that four members of the 2-oxoglutarate/Fe(II)-dependent oxygenase family convert JA into the inactive 12-OH-JA form. As the genes were induced by exogenous JA, we named them *JASMONATE-INDUCED OXYGENASEs* (*JOX*). Using a quadruple mutant of the four *JOX* genes (*jox4x*), we show that JOXs are involved in the conversion of JA into 12-OH-JA in plants. In the *jox4x* mutant, exogenous JA was less converted into 12-OH-JA. Moreover, JA accumulated to high levels in healthy untreated *jox4x* plants, whereas JA levels in wild-type Arabidopsis plants were low. After wounding, JA accumulated in wild-type plants, and JA increased to levels 17x higher in *jox4x*. These results show that the function of the JOX enzymes is to remove JA under basal conditions and after wounding. Failure to do so results in high levels of JA, and the consequences of this are clear from the phenotypes of *jox4x*: defense-related genes *PDF1.2* and *ORA59* are highly expressed, and the mutant is more resistant to *B. cinerea* and *Mamestra brassicae*. However, the costs for this increased resistance on growth are evident: Plants of the *jox4x* mutant are smaller, have shorter roots, flower later and produce fewer seeds than wild-type plants. These defects in growth and development show that it is imperative for plants to keep JA levels at a low level to minimize negative effects on growth. Our identification of JOX as JA-hydroxylases add an important component to the JA metabolism pathway.

In uninfected *jox4x* plants, expression of *ORA59* and *PDF1.2,* which are part of the ERF branch of the JA pathway, is high compared to their expression in wild-type plants. We have also tested expression of genes that are controlled by MYC transcription factors (MYC-branch), such as *VSP2* and several JA-biosynthesis genes. We found that in basal conditions, these are only slightly higher expressed in the *jox4x* mutant than in wild-type plants. Interestingly, this indicates that the high levels of JA regulate these two types of genes differently. The phenotype of the *jox4x* mutant enables us to test the regulation of these genes in more detail. As the branches are co-regulated by different hormones, ethylene and ABA respectively, one of the first explanations that can be considered is that in our system or experimental conditions, there is more ethylene and less ABA present. However, we found that ABA levels in the *jox4x* mutant were similar to those in wild-type plants. Ethylene measurements have not yet been done. Other explanations can be that the MYC branch is more stringently controlled, for example by negative regulators, such as repressive bHLH transcription factors (Sasaki-Sekimoto *et al.*, 2013), or that crosstalk between the ERF and MYC branch is influencing gene expression in the *jox4x* mutant.

Finally, we found that while JA levels were higher in unwounded *jox4x* plants, JA-Ile levels were not. Possibly, JA-Ile was turned over so quickly that it was not detected. However, it is also possible that JA is not converted into JA-Ile without further stimulation, and that high JA levels are able to induce (part of) JA-responsive gene expression without conversion into JA-Ile. Interestingly, MeJA-induced expression of *ORA59* was not affected in the *coi1-16* mutant, and wound-induced expression of *PDF1.2* was not affected in the *jar1* mutant (Devoto *et al.*, 2005; Suza and Staswick, 2008). This suggests that these genes could possibly be activated in a manner independent of JA-Ile and COI1-mediated degradation of JAZ. Chemical or genetic inhibition of JAR1 in the *jox4x* mutant in future studies could help to understand if this is indeed the case.

Hormone inactivation in hormonal crosstalk

The existence of JA-inactivating enzymes provides potential for crosstalk with other hormones. An increase of JA hydroxylation would result in reduced activation of the JA signaling pathway, and would thus be a potential point for interference by, for example, the SA signaling pathway. Activation of *JOX* expression by SA would be an efficient way to suppress JA. We investigated this, but did not find any evidence for this hypothesis. First of all, expression of the four *JOX* genes was not induced by SA. Secondly, SA was able to suppress expression of *VSP2* in the *jox4x* mutant. Possibly, hormone inactivation is employed in hormonal crosstalk between other hormones. For example, the 2OG oxygenase that functions as an auxin oxidase (At1g14120) is induced by JA treatment (publically available microarray data). This is an interesting topic for future studies.

MOLECULAR MECHANISMS OF SALICYLIC ACID/JASMONIC ACID CROSSTALK

At the start of the research described in this thesis, significant progress had been made in the identification of the site of action in the JA pathway where that SA targets. Although SA can suppress expression of JA biosynthesis genes, SA-mediated suppression of JA-dependent gene expression was shown to be independent of JA biosynthesis (Leon-Reyes *et al.*, 2010b). Using the JA-receptor mutant *coi1-1* ectopically expressing *ERF1* to constitutively express downstream JA-responsive genes, SA was shown to suppress ERF1-activated *PDF1.2* expression independently of JA-receptor COI1 (Van der Does *et al.*, 2013). The GCC-box, which is the binding site for ERF transcription factors, was next shown to be sufficient for SA/JA crosstalk. This indicates that SA could target ERF transcription factors that act as transcriptional activators of the JA pathway. SA was then shown to interfere with

the stability of ORA59, the transcription factor that activates *PDF1.2* expression (Van der Does *et al.*, 2013).

In Chapter 2, we provide evidence for a role of novel synthesized factors in SA/JA crosstalk by showing that *de novo* protein synthesis is required for the suppression of *PDF1.2*. We then investigated the hypothesis that SA could target the GCC-box by induction of negative regulators. SA-induced proteins could act as transcriptional repressors by occupying cis-regulatory elements in the promoters of JA-responsive genes and hence compete with JA-regulated transcriptional activators, leading to repression of transcription of JA-inducible genes. Because of the importance of the GCC-box in SA/JA crosstalk, we investigate members of the ERF transcription factor family, that are known to bind the GCC-box (Franco-Zorrilla *et al.*, 2014). We specifically focused on ERFs that contain the EAR transcriptional repressor motif. We generated knock-out mutants in 17 *ERF*s, and studied MeJA-induced induction and SA/MeJA-mediated suppression of *PDF1.2* and *VSP2*. All tested *erf*s showed a wild-type level of SA-mediated suppression of these two genes, indicating that the analyzed ERFs are not involved in SA/JA crosstalk or act redundantly. A mutant in the general co-repressor TPL also did not affect SA-mediated suppression of JA-induced *PDF1.2* and *VSP2* expression. This together makes it unlikely that ERF suppressors play a role in SA/JA crosstalk.

Interestingly, we did find an effect of knocking out several *ERFs* on MeJA-induced expression of *PDF1.2* and *VSP2*. We confirm the function of ERF4 as a suppressor of MeJA-induced *PDF1.2*, and show that *at-erf1*, *erf13*, *erf112* and *dreb2a* also have higher *PDF1.2* expression than Col-0 after MeJA treatment. In addition, the mutants *aterf-1, erf112* and *erf5* displayed a significantly enhanced expression of *VSP2* upon MeJA treatment. This shows that these transcription factors directly or indirectly repress these genes in response to MeJA. This also indicates that expression after MeJA treatment alone, which sometimes is used to study SA/JA crosstalk, is not a good measure for SA-mediated suppression of JA-induced genes. Instead, the combination treatment of SA and MeJA is necessary. We further provide evidence for a role for ERF104, RAP2.5, HRE2 and ERF5 as positive regulators of *PDF1.2* expression and for ERF8, DREB2A and RAP2.6, RAP2.6L and CEJ1/DEAR1 in activation of *VSP2*.

Our investigation in Chapter 2 shows that induction of repressive ERFs is not important in SA/JA crosstalk. This indicates that targeting of transcriptional activators that bind to the GCC-box is probably essential. For ORA59 the degradation by SA has been shown (Van der Does *et al.*, 2013). We have tried to study this transcription factor to understand more about the mechanism by which it is targeted. One of our aims was to find (SA-induced) factors that could interact with ORA59 and target it for degradation. Proteomics studies of tagged ORA59 were therefor initiated.

However, this research line was unsuccessful due to technical difficulties with the cell culture overexpressing ORA59. Future investigations could focus on identifying more positive regulators of the JA pathway that are targeted by SA. A candidate for this is MYC2, which is a master regulator of JA-responsive gene expression (Kazan and Manners, 2013). Phosphorylation-mediated degradation of MYC2 is important in its function as transcriptional activator and repressor (Zhai *et al.*, 2013). Very recently, MYC2, MYC3 and MYC4 protein levels were shown to be reduced after application of *Pieris brassicae* egg extract, a treatment that induces activation of the SA pathway in Arabidopsis plants (Schmiesing *et al.*, 2016). This indicates that SA could target two important transcription factors in the JA pathway for degradation. Future studies with ORA59 or MYC interactors would be informative to understand how these transcription factors are affected by SA. Finally, more potential targets of SA-mediated degradation could be identified by investigating which JA-responsive genes are subject to crosstalk, understanding which transcription factors control their positive regulation by promoter analysis and mutant or overexpressor studies, and investigating the stability of these positive actors.

Another major gap in the knowledge of mechanisms of SA/JA crosstalk is the role of NPR1. NPR1 is an indispensable regulator of SA-induced defense gene expression and defense, and has been shown to be essential for suppression of JA-marker genes as well. Previously, NPR1 was suggested to function in SA/JA crosstalk in the cytosol (Spoel *et al.*, 2003). However, in Chapter 3 we describe that in adult plants nuclear localization is required for suppression of *PDF1.2* and *VSP2* after SA-treatment. We showed that *PDF1.2* and *VSP2* are not suppressed after SA and MeJA treatment in two different mutants that express a cytosolic version of NPR1, i.e. NPR1-HBD and *npr1nls*. This indicates that, at least in adult plants, NPR1 is required in the nucleus to carry out its role in SA/JA crosstalk. This is a major shift in the understanding of the function of NPR1 in SA/JA crosstalk. As NPR1 suppresses JA-responsive genes in the nucleus, its activity in SA/JA crosstalk could consist of either targeting positive transcription factors directly, or induction of targets that then suppress JA-responsive gene expression.

In Chapter 3 we provide preliminary evidence that NPR1 could act by activating the expression of genes that then have a role in suppression of JA-responsive genes. We showed that two lines that express a Cys-mutated NPR1 protein (C82A and C216A), are impaired in suppression of *PDF1.2* and *VSP2*. Using RNAseq, we identify 97 genes that are differentially expressed in C82A compared to wild-type plants after SA-treatment. We investigated if these 97 genes contain candidates for a role in SA/JA crosstalk and found that four SA-induced NPR1-targets, namely *WRKY18*, *WRKY38*, *WRKY53* and *WRKY70* are lower expressed in this mutant. The lower expression of WRKY18 and WRKY38 was confirmed in plants that were treated with

the combination treatment of SA and MeJA in C82A, C216A and *npr1-1*. The reduced expression of these targets could thus have an effect on SA-mediated suppression of *PDF1.2* and *VSP2*.

A role for SA-induced WRKY transcription factors in suppression of JA-responsive genes has been shown before by others for the transcription factors WRKY46, WRKY53, WRKY62 and WRKY70 (Li *et al.*, 2004; Mao *et al.*, 2007; Hu *et al.*, 2012). Future research should focus on the role of WRKYs in SA/JA crosstalk. Besides the WRKYs that were already described to function in SA/JA crosstalk, we identify WRKY18 and WRKY38 as potential candidates for suppression of JA responses. The next challenge is to understand how WRKYs repress JA-responsive gene expression. Possibly this is directly via the W-box that was shown to be overrepresented in JA-induced genes that are suppressed by SA (Van der Does *et al.*, 2013). How WRKY transcription factors are recruited to JA-dependent promoters is a major question. In this context it is interesting that many WRKYs interact with VQ-motif containing proteins (Jing and Lin, 2015). The VQ-motif containing protein JAV1 was shown to suppress JA-responses, and interacts with WRKY28 and WRKY51 (Hu *et al.*, 2013). WRKY51 has also been implicated in suppression of JA–responses (Gao *et al.*, 2011). Other interesting interaction partners of WRKY transcription factors are histone deacetylases (HDAs). Deacetylation of histones is correlated with gene repression. HDA19 interacts with WRKY38 and WRKY62 and represses their transcription-activating activity (Kim *et al.*, 2008). Future research on the interactions of WRKY transcription factors will help to elucidate the mechanism of their repression of JA responses.

A function of NPR1 in the nucleus also opens up possibilities for NPR1 to function in targeting of positive transcriptional regulators. In Chapter 3 we showed that the GCC-box is overrepresented in genes that are suppressed by SA in a NPR1-dependent manner. This could mean that NPR1 targets positive transcriptional regulators of JA-responses that bind to the GCC-box. Interaction studies of NPR1 can show if it can bind these positive transcriptional regulators. Alternatively, other interactions of NPR1 may be important in SA/JA crosstalk. TGA transcription factors TGA2, TGA6 and TGA6 are known to interact with NPR1 and are important for suppression of *PDF1.2* (Leon-Reyes *et al.*, 2010a). TGAs were further shown to bind to the *as-1* element in the promoter of *ORA59* and could regulate both induction of *ORA59* by ACC and suppression by SA (Zander *et al.*, 2014). It would be interesting to test the role of NPR1 as a TGA-interactor in this suppression. Possibly, NPR1 helps to switch the TGA transcription factors from activator to repressors depending on the promoter context.

Furthermore, in Chapter 3 we show that redox-mediated modifications of the NPR1 protein are not essential for SA-mediated suppression of JA-responsive genes.

As redox changes were associated with SA/JA crosstalk, it is possible that redox regulation of other factors is important. Possibly, TGA transcription factors are redox regulated in response to SA to switch from transcription activators to suppressors of *ORA59* expression. Their interaction with redox transmitters GRXs points in this direction (Ndamukong *et al.*, 2007; Zander *et al.*, 2012).

Interestingly, in Chapter 3 we describe that a set of 37 JA-induced genes was still suppressed by SA in the *npr1-1* mutant. This indicates that only part of the JA-responsive pathway is suppressed in an NPR1-dependent manner. High-density time series RNA-seq of Col-0 and *npr1-1* would be instrumental to identify more genes that are affected by crosstalk in an NPR1-dependent or –independent manner. In Chapter 3, RNA-seq identified 5499 genes that were differentially expressed in *npr1-1* compared to Col-0 5 h after SA treatment. In SA-treated *npr1-1* plants, several JA-dependent genes are increased expressed compared to Col-0, suggesting that SA suppresses these genes in an NPR1-dependent manner in Col-0. However, as we did not include a MeJA-treatment in our analysis, we do not have enough information at the moment to identify all MeJA-induced and SA-suppressed genes in Col-0 in this study. In our research group, RNA-seq of hormone-treated wild-type plants in a high-density time series has been performed and is being analyzed. Once MeJA-induced and SA-suppressed genes have been identified in these experimental conditions in wild-type plants, future RNA-seq of SA and MeJA-treated different *npr1* mutants will greatly increase our knowledge on NPR1 targets in JA-responsive gene expression. It would also be interesting to investigate the NPR1-independent mechanism of SA-induced suppression of JA-responsive genes. Possibly SA-induced NPR1-independent WRKY transcription factors, such as WRKY41 that was shown before to suppress *PDF1.2* (Higashi *et al.*, 2008), can play a role in this process.

Previous studies on SA/JA crosstalk have shown that suppression of the JA-responsive pathway by SA is predominantly regulated at the level of gene transcription. In this thesis, the function of transcriptional regulators in this process, such as ERF transcription factors and co-regulator NPR1, was studied. A major challenge for the future is now to elucidate the mechanisms by which these transcriptional regulators control suppression of JA-dependent defense gene expression. Using proteomics studies to identify interactors of important signaling hubs in SA/JA crosstalk, and combining transcriptome data with ChIP-seq or DNase-seq studies to identify transcription factors that are occupying DNA sites after different hormone stimuli can provide more detailed knowledge on this. Moreover, future investigations can focus on additional mechanisms that regulate gene expression which have not been studied in relation to SA/JA crosstalk yet. Two exciting options are Mediator subunits, which were described to be targeted by an effector to influence hormonal signaling (Caillaud *et al.*, 2013) and microRNAs (Curaba *et al.*, 2014).

According to the U.N., food production should double in 2050 to feed the growing world population. A major challenge is to grow crops that are resistant to major plant diseases without negative effects on growth or yield. Knowledge on crosstalk between hormonal signaling pathways can contribute to this. The research described in this thesis improves the knowledge on several components of the SA and JA signaling pathways, and describes how they may contribute to the SA-mediated suppression of JA responses. The importance of these factors should be studied in different plant-microbe interactions or in agricultural context and could help in understanding how plants respond to different attackers at the same time. As SA/JA crosstalk also influences defense against these different attackers, this knowledge may then be used to develop strategies for plants that are resistant to multiple attackers at the same time. We further identified the JOX proteins that are new negative regulators of the JA pathway. Knocking out these genes in crop species may create plants that are more resistant to JA-inducing attackers. Future studies into the mechanism of JOX may help to separate their effects on defense and growth, and this can be used to improve both resistance and yield in crops.

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Samenvatting

Planten hebben gedurende hun leven te maken met verschillende micro-organismen en insecten. Deze interacties kunnen gunstig zijn voor de plant, zoals bijvoorbeeld de wisselwerking met wortelbacteriën die de plant helpen voedingsstoffen uit de grond te halen. Veel interacties zijn echter nadelig voor de plant. Zo kunnen micro-organismen, zoals bacteriën en schimmels, plantenweefsel ziek maken en doden. Ook kunnen vraatzuchtige insecten zich voeden met de bladeren of wortels van de plant. Tegen deze aanvallers moet de plant zich zo goed mogelijk verdedigen. Net als bij mensen worden verschillende processen in de plant gereguleerd door hormonen. Twee planthormonen die belangrijk zijn in de afweer tegen ziekteverwekkers zijn salicylzuur (SA) en jasmonzuur (JA). SA is met name belangrijk in het activeren van afweer tegen ziekteverwekkers die zich voeden met levend plantenmateriaal (biotrofe pathogenen). JA speelt een belangrijke rol in de afweer tegen schimmels of bacteriën die zich voeden met dood plantmateriaal (necrotrofe pathogenen), en tegen insecten, zoals rupsen.

Ophoping van deze hormonen in de plant leidt tot productie van antimicrobiële eiwitten of toxische stoffen die aanvallers afschrikken. Als er iets mis gaat in dit afweersysteem, bijvoorbeeld in een mutant die geen SA herkent, of in een mutant die geen JA meer kan maken, is de plant vatbaarder voor schadeveroorzakers en ziekteverwekkers.

Activatie van de afweer heeft niet alleen positieve effecten, maar ook negatieve gevolgen, bijvoorbeeld op plantengroei en -ontwikkeling. SA en JA beïnvloeden deze processen namelijk óók. Planten die het hormonaal gereguleerde afweersysteem van hormonen continu aan hebben staan, hebben een zogenaamd dwergfenotype. Het is voor de plant dan ook belangrijk dat deze routes alleen aangeschakeld worden als dat echt nodig is, namelijk als er een direct gevaar is van een aanvaller. Planten hebben verschillende mechanismen om de productie en activiteit van SA en JA te beheersen. Op het moment dat de plant aangevallen wordt, worden genen met een rol in SA- of JA-biosynthese actief, zodat het specifieke hormoon wordt geproduceerd. SA leidt tot de productie van antimicrobiële PR-eiwitten, via de regulator NPR1. De signaleringsroute van JA leidt tot activatie van de afweergenen *PDF1.2* en *VSP2*.

Er zijn synergistische en antagonistische interacties tussen de hormoonsignaleringsroutes van SA en JA. Dit wordt ook wel hormoon-“crosstalk” genoemd. De interacties tussen de twee routes stellen de plant in staat om de verschillende reacties goed op elkaar af te stemmen en zo efficiënt mogelijk te reageren. Beide routes kunnen elkaar negatief beïnvloeden, maar het negatieve effect van SA op de JA-route is het sterkst. Een belangrijk doel van het onderzoek beschreven in dit proefschrift was

aantonen welke moleculaire mechanismen ten grondslag liggen aan de onderdrukking van de JA signaleringsroute door de SA signaleringsroute. Bij het onderzoek is gebruik gemaakt van de modelplant *Arabidopsis thaliana*.

Voorafgaand onderzoek heeft laten zien dat het effect van SA op de JA-signaleringsroute voornamelijk plaatsvindt op het niveau van genexpressie. De staat van het onderzoek in dit gebied is besproken in hoofdstuk 1. Hoe activering van de SA-route de expressie van JA-afhankelijke genen weet te verminderen is nog niet duidelijk. Uit een analyse van de promotors van genen die worden geïnduceerd door JA en onderdrukt door SA, bleek dat het GCC-box-promotorelement belangrijk was voor SA/JA crosstalk. Van ERF-transcriptie factoren is bekend dat zij de GCC-box kunnen binden en kunnen leiden tot activatie of onderdrukking van genen met zo'n GCC-box in hun promotor. In hoofdstuk 2 selecteerden we van de 122 ERFs in Arabidopsis, alleen degenen waarvan de expressie door SA geïnduceerd wordt en degenen die coderen voor een ERF-eiwit met een EAR-domein. Van dit domein is bekend dat het een rol speelt bij de repressie van genen. Na selectie bleven er negentien genen over en van zeventien van deze genen hebben we mutanten getest.

Geen van de geteste mutanten was verstoord in de SA-geïnduceerde repressie van JA-afhankelijke genexpressie. De expressie van *PDF1.2* en *VSP2* na behandeling met SA en JA was in alle mutanten namelijk onderdrukt vergeleken met een behandeling met alleen JA. We onderzochten daarnaast een mutant in TOPLESS (TPL), een eiwit dat een remmende werking heeft op de JA-signaleringsroute en samenwerkt met verschillende ERF repressieve transcriptie factoren. Mutatie van TPL had ook geen effect op de onderdrukking van JA-afhankelijke genexpressie door SA. We concluderen daarom dat het onwaarschijnlijk is dat TPL of een van de ERFs een rol spelen in SA/JA-crosstalk, en verwerpen daarmee een belangrijke hypothese in dit onderzoek.

In hoofdstuk 3 onderzoeken we de rol van NPR1 in de onderdrukking van JA-geïnduceerde genexpressie door SA. NPR1 is een belangrijke regulator in de activatie van SA-geïnduceerde genexpressie en afweer. Eerder was al aangetoond dat NPR1 belangrijk is voor de onderdrukking van de genen *PDF1.2* en *VSP2* door SA. In hoofdstuk 3 hebben we een vergelijking gemaakt tussen alle JA-geïnduceerde genen die onderdrukt worden door SA in het Arabidopsisecotype Col-0 planten en in de mutant *npr1-1*. Van de 59 JA-geïnduceerde genen die onderdrukt worden door SA in Col-0, worden er 21 niet meer onderdrukt in de *npr1-1*-mutant. Verder onderzochten we natuurlijke variatie in NPR1, door de aminozuur volgorde in dit eiwit in verschillende ecotypes van Arabidopsis te vergelijken. We onderzochten vervolgens of de ecotypes wel of geen SA-geïnduceerde onderdrukking van *PDF1.2* laten zien.

Om te begrijpen hoe NPR1 in de cel leidt tot onderdrukking van JA-responsieve genen, hebben we hierna SA/JA crosstalk getest in verschillende mutanten die de locatie, activiteit of stabiliteit van het NPR1 eiwit in de cel beïnvloeden. Verder hebben we twee mutanten van NPR1 getest waar één van de cysteïne-aminozuren gemuteerd is. Deze mutanten blijken niet verstoord te zijn in de SA-geïnduceerde expressie van het gen *PR1*, maar wel in repressie van de JA-responsieve genen *PDF1.2* en *VSP2*. Dit maakt deze mutanten bijzonder. Het stelt ons namelijk in staat de functies van NPR1 in SA-geïnduceerde genexpressie en in de onderdrukking van JA-geïnduceerde genexpressie uit elkaar te halen. We hebben in deze mutant alle genen getest die differentieel tot expressie komen door al het RNA te sequensen (RNAseq). Hieruit bleek dat er 32 SA-geïnduceerde en NPR1-afhankelijke genen zijn, die lager tot expressie komen in deze mutant. De lagere expressie van deze genen zou de oorzaak kunnen zijn dat in de mutant *PDF1.2* en *VSP2* niet onderdrukt worden na SA-behandeling en zijn dus nieuwe kandidaten voor een rol in SA/JA crosstalk. Verder onderzoek kan uitwijzen of deze genen betrokken zijn bij de onderdrukking van JA-geïnduceerde genexpressie door SA.

Behalve SA/JA-crosstalk is er in dit proefschrift ook gekeken naar een andere invloed op de JA-signaleringsroute. Remming van deze route gebeurt namelijk ook door de afbraak of uitschakeling van het actieve JA-molecuul. Dit kan gebeuren door hydroxylering van JA, een reactie waarbij een hydroxylgroep wordt toegevoegd en 12-OH-JA ontstaat. Het enzym dat deze reactie katalyseert was tot nu toe nog niet bekend. Bij andere plantenhormonen, zoals SA, auxine en gibberelline, wordt deze reactie uitgevoerd door leden van een familie van oxygenases in Arabidopsis. Daarom onderzochten we in hoofdstuk 4 de expressie van 50 genen van deze familie na JA-behandeling en laten zien dat een tak van vier leden van deze familie geïnduceerd wordt door JA. Deze vier genen hebben we JASMONATE-INDUCED OXYGENASES (JOX) genoemd. We laten zien dat gelijktijdige mutatie van deze vier genen leidt tot fenotypes van de plant die duiden op een verhoogd niveau van JA, zoals een verhoogde afweer tegen de nectrotrofe schimmel *Botrytis cinerea* en de rups *Mamestra brassicae*. Met een analyse van de metabolieten in de mutant vonden we dat er inderdaad veel meer JA ophoopt in deze mutant dan in wildtype planten. Bovendien lieten de metaboliet analyses zien dat de mutant exogeen JA minder omzet in 12-OH-JA. In dit hoofdstuk wordt dus bewijs geleverd dat de JOX-enzymen JA hydroxyleren naar 12-OH-JA. Bovendien laten we zien dat hydroxylering van JA belangrijk is om de balans tussen afweer en groei stabiel te houden.

Het werk beschreven in dit proefschrift heeft tot nieuwe inzichten geleid in de moleculaire mechanismen die ten grondslag liggen aan de interacties tussen de SA- en JA- signaaltransductieroutes. De identificatie van de JOX-enzymen als JA-

hydroxylases draagt bij aan de kennis over JA metabolisme. Deze kennis over fundamentele processen in de plant helpt ons de afweerrespons van de plant beter te begrijpen, geeft een goede basis voor verder onderzoek, en kan uiteindelijk het ontwikkelen van resistentere gewassen bevorderen.

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About the author

Lotte Caarls was born on January 14, 1986 in Heemstede. She finished her secondary education at the Haags Montessori Lyceum in The Hague in 2005. In 2006 she started with the bachelor Biology at the University of Amsterdam. She obtained her Bachelor of Science in 2009, after which she enrolled in the master Integrative Plant Sciences. During this master, two internships were carried out. The first at the University of Amsterdam in the group of Christa Testerink, working on the role of Snf1-related protein kinases in root system architecture during salt stress. For the second internship, she moved to California to work in the lab of Prof. Michelmore at the University of California in Davis. Here she studied the role of NPR1 in different *Arabidopsis* accessions. During her master, she was also admitted to the Talent Program of the Experimental Plant Science (EPS) graduate school, in which she wrote and defended a PhD proposal. In 2011 she graduated *cum laude*. In January 2012 she started as a PhD candidate at the Plant Microbe Interactions group at the Utrecht University. Here, the research which was described in this thesis was carried out under supervision of Dr. Saskia van Wees and Prof. Corné Pieterse. During this time, she also was an active member of the PhD council of both the PhD Programme Environmental Biology (of the Graduate school of Life Sciences Utrecht) and of the graduate school EPS.

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