Ethylene Modulates the Role of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 in Cross Talk between Salicylate and Jasmonate Signaling[1][W][OA]

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The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play crucial roles in the signaling network that regulates induced defense responses against biotic stresses. Antagonism between SA and JA operates as a mechanism to fine-tune defenses that are activated in response to multiple attackers. In Arabidopsis (*Arabidopsis thaliana*), NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) was demonstrated to be required for SA-mediated suppression of JA-dependent defenses. Because ET is known to enhance SA/NPR1-dependent defense responses, we investigated the role of ET in the SA-JA signal interaction. Pharmacological experiments with gaseous ET and the ET precursor 1-aminocyclopropane-1-carboxylic acid showed that ET potentiated SA/NPR1-dependent *PATHOGENESIS-RELATED1* transcription, while it rendered the antagonistic effect of SA on methyl jasmonate-induced *PDF1.2* and *VSP2* expression NPR1 independent. This overriding effect of ET on NPR1 function in SA-JA cross talk was absent in the *npr1-1/ein2-1* double mutant, demonstrating that it is mediated via ET signaling. Abiotic and biotic induction of the ET response similarly abolished the NPR1 dependency of the SA-JA signal interaction. Furthermore, JA-dependent resistance against biotic attackers was antagonized by SA in an NPR1-dependent fashion only when the plant-attacker combination did not result in the production of high levels of endogenous ET. Hence, the interaction between ET and NPR1 plays an important modulating role in the fine tuning of the defense signaling network that is activated upon pathogen and insect attack. Our results suggest a model in which ET modulates the NPR1 dependency of SA-JA antagonism, possibly to compensate for enhanced allocation of NPR1 to function in SA-dependent activation of *PR* genes.

Plants have a broad spectrum of mechanisms to cope with adverse conditions such as abiotic stress (e.g. flooding and drought) and biotic stress (e.g. pathogen and insect attack). With regard to biotic stress, plants possess both physical and chemical barriers to prevent harmful attackers from causing damage. When these constitutively active layers of defense are overcome, inducible defense systems are recruited to counteract the attacker (Walters et al., 2007). The phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have emerged as key players in regulating the activation of the induced defense responses involved (Dong, 1998; Howe, 2004; Pozo et al., 2004; Grant and Lamb, 2006; Van Loon et al., 2006; Von Dahl and Baldwin, 2007; Vlot et al., 2008). Their production varies greatly, depending on the nature of the attacking pathogen or insect. The quantity, composition, and timing of the hormonal blend produced results in the activation of a specific set of defense-related genes that eventually determines the nature of the defense response that is triggered by the attacker encountered (De Vos et al., 2005; Mur et al., 2006). Other plant hormones, including abscisic acid (Mauch-Mani and Mauch, 2005; de Torres-Zabala et al., 2007; Asselbergh et al., 2008), brassinosteroids (Nakashita et al., 2003), gibberellins (Navarro et al., 2008), and auxins (Navarro

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et al., 2006; Wang et al., 2007), have also been reported to play a role in the plant's immune response, but their significance is less well studied.

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

One of the best studied examples of defense-related signal cross talk is the interaction between the SA and the JA response pathways (Kunkel and Brooks, 2002; Thaler et al., 2002; Glazebrook et al., 2003; Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Spoel and Dong, 2008). Many studies have demonstrated that endogenously accumulating SA antagonizes JA-dependent defenses, thereby prioritizing SA-dependent defenses over JA-dependent ones (Doherty et al., 1988; Peña-Cortés et al., 1993; Gupta et al., 2000; Spoel et al., 2003). As a result of the negative interaction between SA and JA signaling, activation of the SA response should render a plant more susceptible to attackers that are resisted via JA-dependent defenses and vice versa. Indeed, many examples of trade-offs between SA-dependent resistance against biotrophic pathogens and JA-dependent defense against insect herbivory and necrotrophic pathogens have been reported (Pieterse et al., 2001; Bostock, 2005; Stout et al., 2006). In Arabidopsis, Spoel et al. (2007) showed that SA-mediated defenses that are triggered upon infection by a virulent strain of the hemibiotrophic pathogen *P. syringae* rendered infected tissues more susceptible to infection by the necrotrophic pathogen *A. brassicicola* by suppressing the JA signaling pathway. Similarly, infection by the biotrophic pathogen *H. arabidopsidis* strongly suppressed JA-mediated defenses that were activated upon feeding by caterpillars of the small cabbage white *P. rapae* (Koornneef et al., 2008). Conversely, JA signaling can act antagonistically on SA-dependent defenses. For instance, *P. syringae* produces the phytotoxin coronatine, which functions as a JA mimic and suppresses effectual SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Zhao et al., 2003; Brooks et al., 2005; Cui et al., 2005; Nomura et al., 2005; Uppalapati et al., 2007). Although many reports describe an antagonistic interaction between SA- and JA-dependent signaling, synergistic interactions have been described as well (Schenk et al., 2000; Van Wees et al., 2000; Mur et al., 2006). For example, application of low concentrations of both SA and JA (10–100 μM) led to enhanced JA/ET response in the combination treatment compared with JA alone, suggesting that hormone concentration is important for the final output during plant-microbe interactions (Mur et al., 2006).

Pharmacological experiments with Arabidopsis revealed that transcription of JA-responsive marker genes, such as *PDF1.2* and *VSP2*, is highly sensitive to suppression by exogenous application of SA. This SA-mediated suppression of JA-responsive gene expression (hereafter referred to as SA-JA cross talk) was observed in a large number of Arabidopsis accessions, highlighting the potential significance of this phenomenon in the regulation of induced plant defenses in nature (Koornneef et al., 2008). Several lines of evidence point to a role for SA-mediated redox changes in the regulation of SA-JA cross talk (Ndamukong et al., 2007; Koornneef et al., 2008). In Arabidopsis, the redox-sensitive protein NPR1 (for NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1), an important transducer of SA-induced redox changes (Mou et al., 2003; Dong, 2004; Pieterse and Van Loon, 2004; Tada et al., 2008), was shown to be a key regulator of SA-mediated suppression of JA signaling (Spoel et al., 2003). Induction of the SA response, either by pathogen infection or by exogenous application of SA, strongly suppressed JA-responsive genes, such as *PDF1.2* and *VSP2*. However, in mutant *npr1-1* plants, this antagonistic effect was completely abolished (Spoel et al., 2003). The *npr1-1* mutant shows enhanced resistance against *Trichoplusia ni* (Cabbage looper) and *Spodoptera littoralis* (Egyptian cotton worm; Cui et al., 2002; Stotz et al., 2002), indicating that blocking the NPR1-dependent SA signaling pathway resulted in enhanced JA-dependent defenses against these insect

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

Besides SA and JA, ET has also been demonstrated to play an important role in the plant's defense response to pathogen and insect attack (Broekaert et al., 2006; Van Loon et al., 2006; Adie et al., 2007; Von Dahl and Baldwin, 2007). In addition to effects on the level of pathogen or insect resistance, ET was shown to function as an important modulator of the plant's response to other hormones, such as JA, SA, and abscisic acid (Adie et al., 2007). For instance, ET enhanced the response of Arabidopsis to SA, resulting in a potentiated expression of the SA-responsive marker gene *PATHOGENESIS-RELATED1* (*PR-1*; Lawton et al., 1994; De Vos et al., 2006). Moreover, in tobacco (*Nicotiana tabacum*), ET was shown to be essential for the onset of SA-dependent systemic acquired resistance (SAR) against *Tobacco mosaic virus* (Verberne et al., 2003). Also, the synergistic interaction between ET and JA has been well established. Many defense-related genes, such as *PDF1.2*, are regulated via a signaling pathway that requires both ET and JA (Penninckx et al., 1998; Broekaert et al., 2006; Adie et al., 2007). Similarly, the corequirement of ET and JA has been demonstrated for the onset of broad-spectrum induced systemic resistance, which is triggered after colonization of plant roots by beneficial microorganisms (Pieterse et al., 1998; Van der Ent et al., 2008; Van Wees et al., 2008), highlighting the important modulating role of ET in plant defense.

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

RESULTS

ET Modulates the NPR1 Dependency of the SA-JA Signal Interaction

In Arabidopsis, pharmacological experiments revealed that SA can antagonize the expression of JA-responsive genes, such as *PDF1.2* and *VSP2* (Spoel et al., 2003; Koornneef et al., 2008). To investigate whether ET affects this SA-JA cross talk, we analyzed the effect of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) on SA- and NPR1-dependent suppression of JA-responsive gene expression. To this end, we made use of the mutant *npr1-1*, which contains a missense mutation that alters a key ankyrin repeat in the NPR1 protein and disrupts NPR1-dependent regulation of both SA- and JA-dependent genes (Cao et al., 1997; Glazebrook et al., 2003). Twelve-day-old seedlings of wild-type accession Columbia (Col-0) and mutant *npr1-1* plants were grown on Murashige and Skoog (MS) agar medium (Murashige and Skoog, 1962) with or without increasing concentrations of ACC and 0.5 mM SA, 0.02 mM methyl jasmonate (MeJA), or a combination of both chemicals. Two days later, the expression of the SA-responsive marker gene *PR-1* and the JA-responsive marker gene *PDF1.2* was analyzed by northern-blot analysis (Fig. 1). In the absence of ACC, the single treatments of Col-0 with SA or MeJA activated *PR-1* and *PDF1.2*, respectively. In addition, the combination treatments with SA and MeJA resulted in effective SA-mediated suppression of MeJA-induced *PDF1.2* expression. As expected, neither *PR-1* induction nor *PDF1.2* suppression was apparent in the *npr1-1* mutant, supporting previous findings that SA-JA cross talk is dependent upon wild-type NPR1 function (Spoel et al., 2003). However, addition of ACC into the medium at different concentrations resulted in effective SA-mediated suppression of MeJA-induced *PDF1.2* expression in both Col-0 and *npr1-1* plants, suggesting that ET relieved the NPR1 dependency of the SA-JA signal interaction. Similar results were obtained with the JA-responsive gene *VSP2* (Supplemental Fig. S1).

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

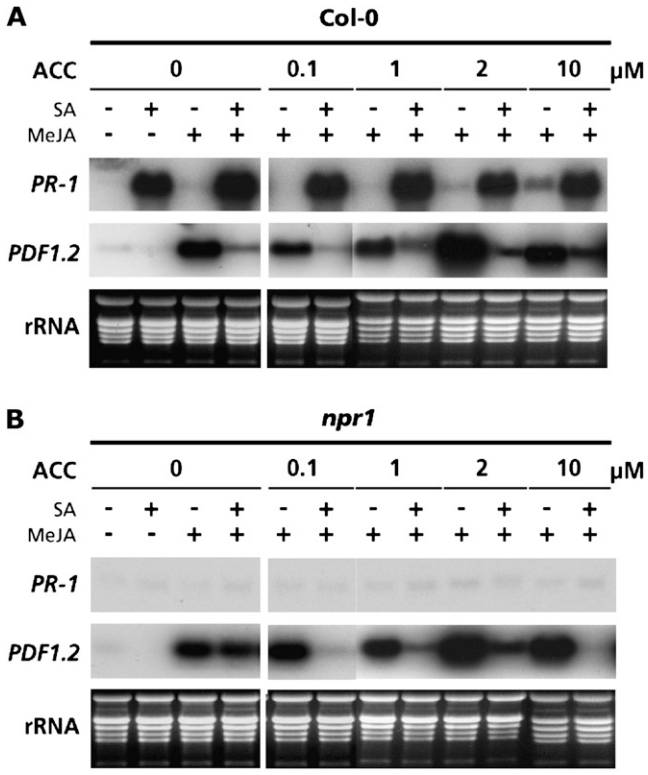

Figure 1. ACC modulates the NPR1 dependency of SA-JA cross talk in Arabidopsis seedlings. Northern-blot analysis of *PR-1* and *PDF1.2* mRNA levels in Col-0 and *npr1-1* seedlings that were treated with SA, MeJA, or a combination of both chemicals in the absence or presence of the ET precursor ACC. Pharmacological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

that ET affects the dependency of SA-mediated suppression of JA-responsive gene expression on wild-type NPR1 function. The observation that neither ACC nor ET treatment bypassed the NPR1 dependency of SA-induced *PR-1* expression (Figs. 1 and 2) indicates that NPR1 has a dual role in the suppression of JA-dependent genes on the one hand and in the activation of SA-dependent gene expression on the other.

Modulation of the NPR1 Dependency of SA-JA Cross Talk by ET Is EIN2 Dependent

To test if the modulation of the NPR1 dependency of SA-JA cross talk by ET is governed by the ET signaling pathway, we performed cross talk experiments with an *npr1-1/ein2-1* double mutant (Clarke et al., 2000). Because the *ein2-1* mutation completely blocks the ET signaling pathway (Alonso et al., 1999) and *PDF1.2* expression requires an intact response to both JA and ET (Penninckx et al., 1998), we performed these experiments with the JA-responsive marker gene *VSP2*, which is similarly sensitive to the antagonistic effect of SA (Spoel et al., 2003; Koornneef et al., 2008). Five-week-old Col-0, *ein2-1*, *npr1-1*, and *npr1-1/ein2-1* plants were treated with SA, MeJA, or a combination of both chemicals in the absence or presence of ACC. In the absence of ACC, MeJA-induced expression of *VSP2* was effectively suppressed by SA in Col-0 and *ein2-1* but not in the *npr1-1* and *npr1-1/ein2-1* backgrounds (Fig. 3), confirming the critical role of wild-type NPR1 in SA-JA cross talk under low-ET conditions. In ACC-treated plants, the NPR1 dependency of SA-JA cross talk was again relieved, as demonstrated by the SA-mediated suppression of MeJA-induced *VSP2* expression in the *npr1-1* background. Compared with *npr1-1* plants, however, SA-JA cross talk remained blocked upon ACC treatment of the *npr1-1/ein2-1* double mutant. These data indicate that the modulation of the NPR1 dependency of SA-JA cross talk by ET is dependent upon EIN2 and is thus regulated by the ET signaling pathway.

Abiotic Induction of Endogenous ET Relieves the NPR1 Dependency of SA-JA Cross Talk

To test the biological relevance of the effect of ET on the role of NPR1 in SA-JA cross talk, we performed SA-JA cross talk experiments under abiotic conditions in which Arabidopsis produces enhanced levels of ET. To this end, 5-week-old plants were placed in trays with open or closed lids. As shown in Figure 4A, Col-0 and *npr1-1* plants grown in trays with closed lids showed a typical hyponastic response, which is a phenomenon demonstrated to be mediated by ET (Millenaar et al., 2005). Mutant *ein2-1* did not display this hyponastic response, confirming the ET dependency of this phenomenon. Besides the ET-dependent hyponastic response, plants grown in trays with closed lids produced more ET (Fig. 4B) and accumulated enhanced transcript levels of the ET-responsive genes *ERS2* and *EBF2* (Fig. 4C; Millenaar et al., 2005; Van der Ent et al., 2008), indicating that growth of the plants in closed trays results in enhanced ET signaling. To investigate the effect of endogenously produced ET on the NPR1 dependency of SA-JA cross talk, plants grown in trays with open or closed lids were treated with SA, MeJA, or a combination of both chemicals and harvested 24 h later for northern-blot analysis of *PR-1* and *PDF1.2* gene expression. Figure 4D shows that the antagonistic effect of SA on MeJA-induced expression of *PDF1.2* is blocked in *npr1-1* mutant plants when grown in trays with open lids (basal ET signaling). However, when the cross talk experiment was performed with plants grown in trays with closed lids (enhanced ET signaling), the level of SA-mediated suppression of *PDF1.2* in *npr1-1* plants was similar to that observed in Col-0 plants. Hence, abiotic induction of the ET response relieves the dependency of SA-JA cross talk on wild-type NPR1 function.

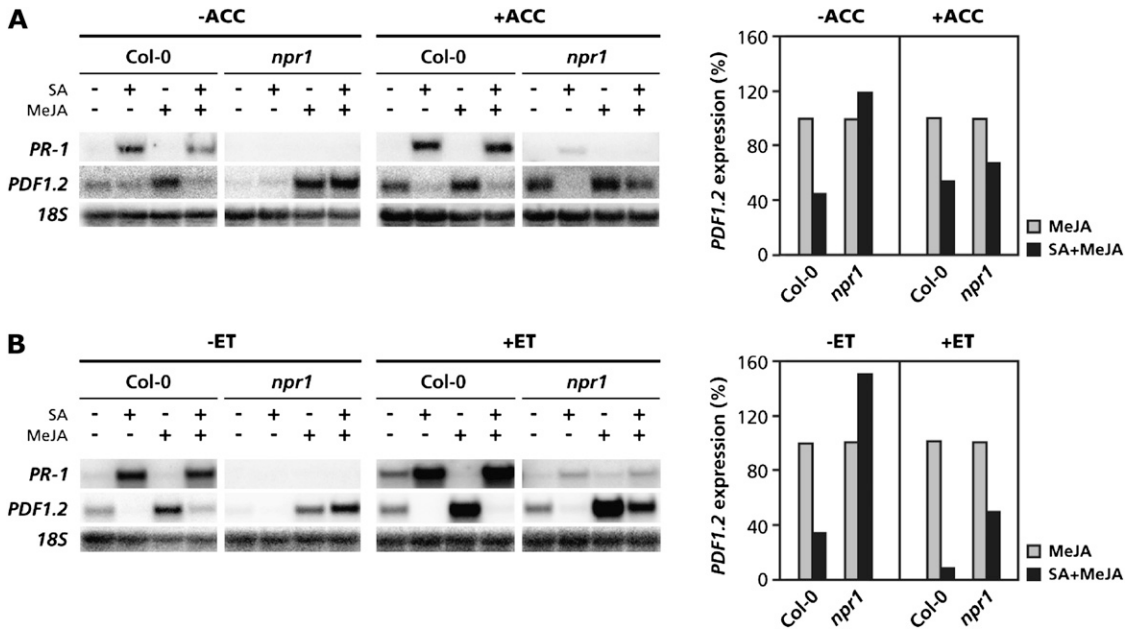

Figure 2. ACC and gaseous ET enable SA-JA cross talk in the absence of NPR1 in 5-week-old Arabidopsis plants. Northern-blot analysis of *PR-1* and *PDF1.2* transcript levels in 5-week-old Col-0 and *npr1-1* plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals in the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

Attacker-Induced ET Enables NPR1-Independent SA-JA Cross Talk

Next, we wanted to investigate whether ET produced during a plant-attacker interaction affects the NPR1 dependency of SA-JA cross talk. To this end, we made use of two JA-inducing attackers: the necrotrophic fungal pathogen *A. brassicicola* and the herbivorous insect *F. occidentalis*. *A. brassicicola* stimulates the biosynthesis of both JA and ET, while *F. occidentalis* induces only JA production (De Vos et al., 2005). Inoculation of Col-0 and *npr1-1* plants with *A. brassicicola* indeed resulted in a strong increase in the production of ET, whereas infestation with thrips had no effect (Fig. 5A).

To investigate the NPR1 dependency of SA-mediated suppression of JA-responsive gene expression during both Arabidopsis-attacker combinations, Col-0 and *npr1-1* plants were infested with *F. occidentalis* or infected with *A. brassicicola* 24 h prior to SA treatment. Twenty-four hours later, leaf material was harvested for northern-blot analysis of *PR-1* and *PDF1.2* transcript levels. Figure 5B shows that *F. occidentalis* and *A. brassicicola* both induced the expression of *PDF1.2* and that this expression was strongly suppressed by SA in Col-0 plants. In the *npr1-1* mutant, this SA-mediated suppression of *PDF1.2* transcription was not observed when the JA response was activated by the non-ET-inducer *F. occidentalis*, indicating that in this plant-attacker combination SA-JA cross talk is NPR1 dependent. However, when the JA response was activated by the JA- and ET-inducer *A. brassicicola*, *PDF1.2* transcription was suppressed in the *npr1-1* mutant background. These results indicate that attacker-induced ET largely overrules the NPR1 dependency of SA-JA cross talk and hence potentially affects the outcome of the defense response that is induced upon attack by multiple invaders.

NPR1 Is Not Required for SA-Mediated Suppression of JA-Dependent Resistance against ET-Inducing Attackers

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

In the thrips resistance assays, Col-0 and *npr1-1* plants were pretreated with 1 mM SA, 0.1 mM MeJA, or a combination of both. Twenty-four hours later, leaf discs from this material were taken and infested with *F. occidentalis*. Two days later, the level of thrips

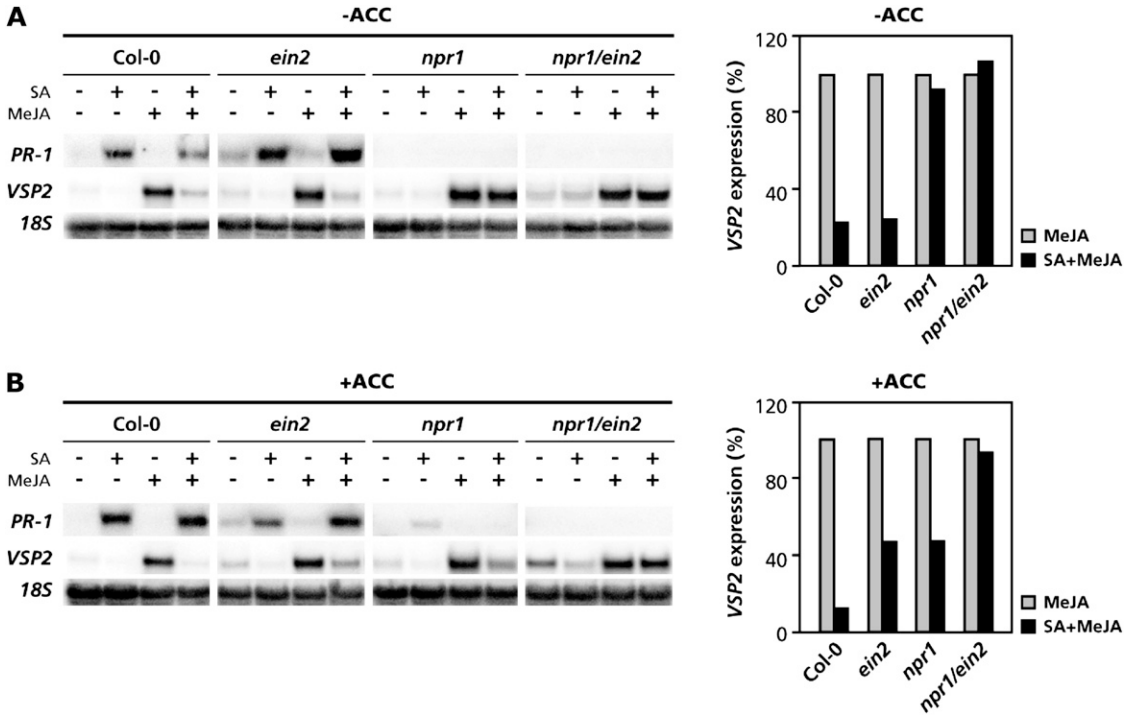

Figure 3. NPR1-independent SA-JA cross talk facilitated by ET is EIN2 dependent. Northern-blot analysis of *PR-1* and *VSP2* transcript levels in 5-week-old Col-0, *ein2-1*, *npr1-1*, and *npr1-1/ein2-1* plants that were treated with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals in the absence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

resistance was determined by measuring the level of feeding scar damage that was inflicted by thrips feeding (Abe et al., 2008). As shown in Figure 6A, SA treatment had no significant effect on the basal level of thrips resistance in Col-0 plants. However, MeJA-treated Col-0 plants showed a significantly reduced area of feeding scars, indicating that MeJA treatment enhanced the level of resistance to thrips feeding. Col-0 plants treated with both SA and MeJA showed a basal level of thrips resistance that was not significantly different from that in control plants, suggesting that SA suppressed the level of MeJA-induced resistance against thrips feeding. In mutant *npr1-1* plants, MeJA and SA/MeJA treatments both led to a significant increase in the level of thrips resistance, indicating that the SA-mediated suppression of MeJA-induced resistance to *F. occidentalis* is controlled by NPR1.

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

DISCUSSION

ET Modulates the Role of NPR1 in Cross Talk between SA and JA Signaling

Cross talk between defense signaling pathways is thought to play an important role in the regulation and fine-tuning of the defense responses that are activated upon pathogen and insect attack. The antagonism between SA and JA signaling emerged as one of the

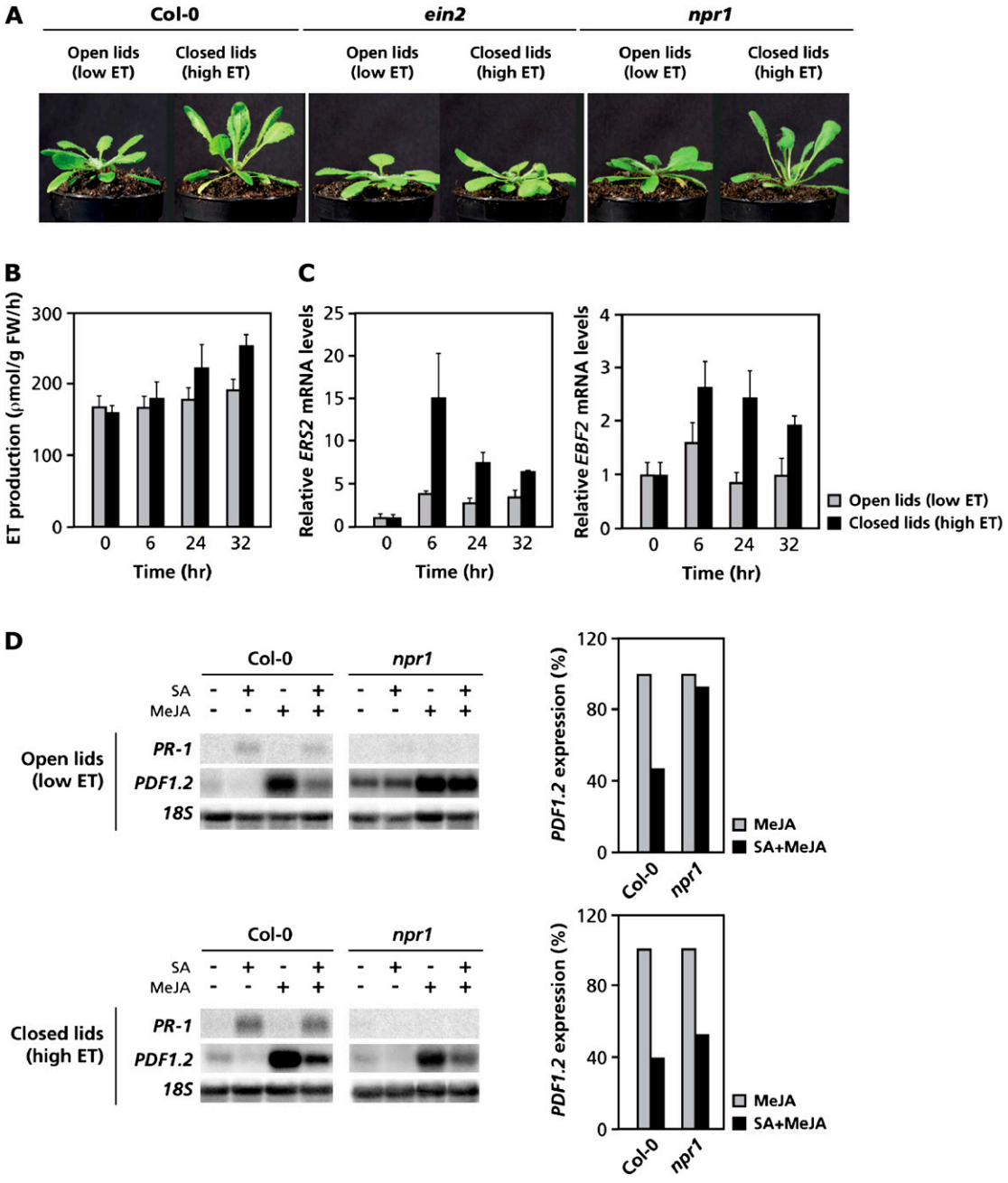

Figure 4. Abiotic induction of ET relieves the NPR1 dependency of SA-JA cross talk. To enhance the ET response in Arabidopsis plants in a biological manner, 5-week-old plants were placed in trays with the lids open (low ET) or closed (high ET). A, Arabidopsis Col-0 and *npr1-1* plants grown for 24 h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

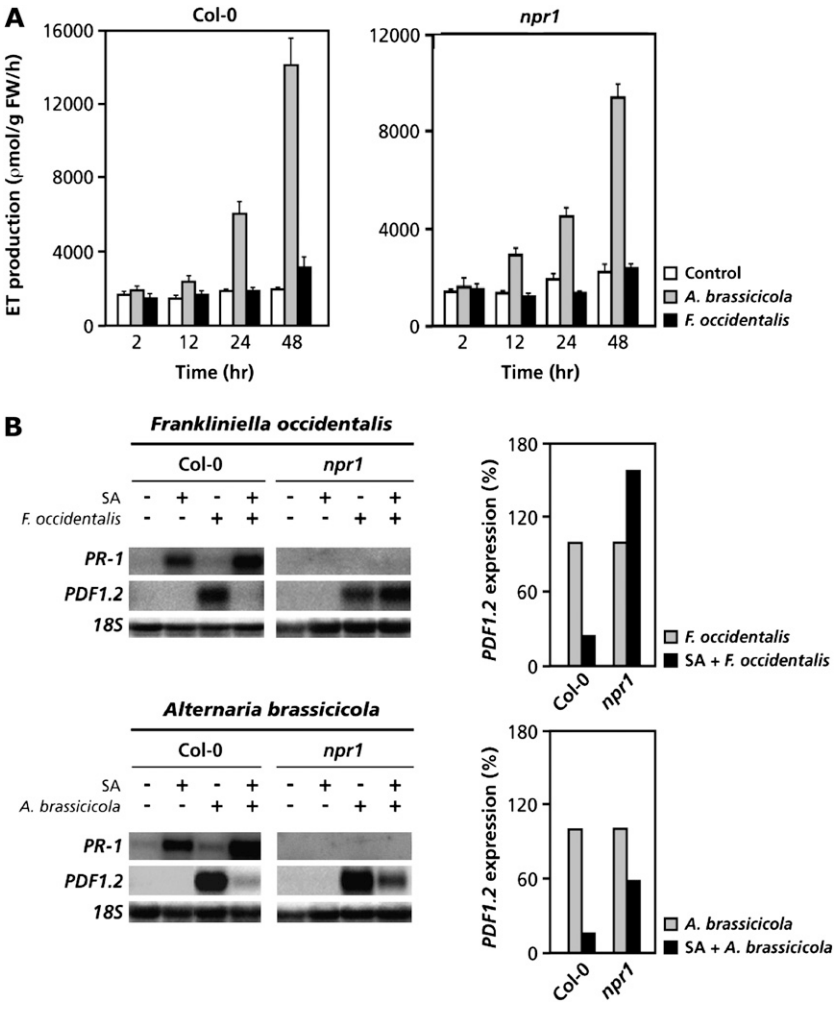

Figure 5. Attacker-induced ET enables NPR1-independent SA-JA cross talk. A, ET production in Col-0 and *npr1-1* after infection with the necrotrophic fungus *A. brassicicola* or infestation with larvae of the western flower thrips *F. occidentalis*. FW, Fresh weight. B, Northern-blot analysis of *PR-1*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

most prominent of all signal interactions studied to date (Koornneef and Pieterse, 2008; Spoel and Dong, 2008). Pharmacological experiments revealed that the suppression of JA-responsive genes such as *PDF1.2*, *VSP2*, and *LOX2* by SA is regulated by NPR1 (Spoel et al., 2003). Following a whole-genome transcript profiling approach to identify Arabidopsis genes that are sensitive to SA-JA cross talk, we recently identified 258 MeJA-responsive genes of which the expression was significantly affected by SA (A. Koornneef and C.M.J. Pieterse, unpublished data). Sixty percent of the JA-responsive genes that were suppressed by SA displayed this suppression in an NPR1-dependent manner, demonstrating that NPR1 is involved in the SA-mediated down-regulation of a large number of MeJA-responsive genes. Because ET is an important modulator of plant defense and a major constituent of the blend of defense signals that is produced during many plant-attacker interactions (Broekaert et al., 2006; Van Loon et al., 2006; Adie et al., 2007; Von Dahl and Baldwin, 2007), we investigated the effect of ET on the SA-JA signal interaction. Here, we demonstrate that ET strongly affects the requirement of wild-type NPR1 in the antagonistic effect of SA on JA-dependent defenses. Exogenous application of the ET precursor ACC or gaseous ET (Figs. 1–3), as well as endogenously produced ET during induction of the hyponastic response (Fig. 4) or pathogen attack (Fig. 5), bypassed the NPR1 dependency of SA-JA cross talk. Experiments in the mutant *ein2-1* background showed that this ET effect is EIN2 dependent and thus mediated through the ET signaling pathway (Fig. 3). These findings indicate that the final outcome of the SA-JA signal interaction during the complex interaction of plants with their attackers can be shaped by ET. Indeed, the antagonistic effect of SA on MeJA-induced resistance against feeding by ET-noninducing thrips was controlled by NPR1. By contrast, SA-mediated suppression of JA-dependent resistance against the JA- and ET-inducing necrotroph *A. brassicicola* functioned independently of NPR1 (Fig. 6), highlighting the modulating role of ET in the SA-JA signal interaction. In Figure 7, we present a schematic model of the interplay between SA, JA, ET, and NPR1 in the Arabidopsis-attacker interactions studied.

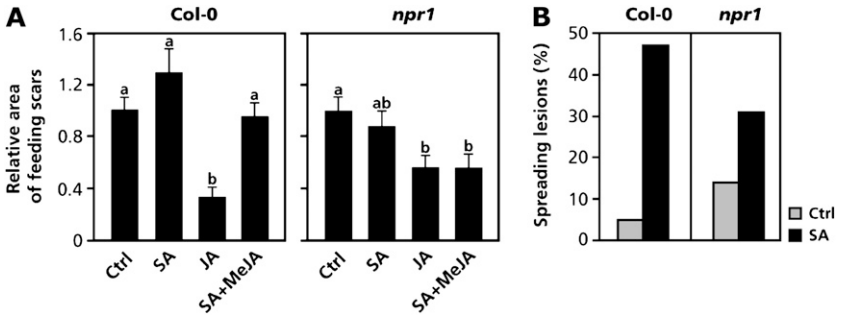

Figure 6. Antagonistic effect of SA on JA-dependent resistance against *F. occidentalis* and *A. brassicicola* in Col-0 and *npr1-1*. A, *F. occidentalis* resistance assay with 3-week-old Col-0 and *npr1-1* plants that were pretreated for 24 h with 1 mM SA, 0.1 mM MeJA, or a combination of both chemicals.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

Dual Role of NPR1

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

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

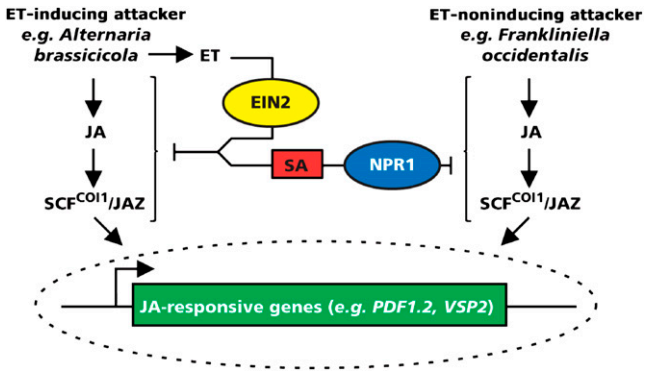

Figure 7. Working model illustrating the role of ET in modulating the NPR1 dependency of SA-JA cross talk. Attack of Arabidopsis by the necrotrophic fungus *A. brassicicola* and the herbivorous insect *F. occidentalis* results in the biosynthesis of JA and the activation of the JA signaling pathway in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

that misses the C-terminal domain with the nuclear localization signal (Dong, 2004), was only affected in SA-dependent gene expression, suggesting that the cytoplasmatic function of NPR1 plays a role in the control of JA- and ET-dependent responses. In agreement with this, the antagonistic effect of SA on JA-responsive gene expression was much less affected in *npr1-3* than in *npr1-1* (Supplemental Fig. S2). These results suggest a model in which the cytosolic function of NPR1 plays a role in SA-JA cross talk and can be bypassed by ET and in which the nuclear function of NPR1 plays a role in the activation of SA-responsive genes and can be stimulated by ET.

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

Interaction between ET and NPR1

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

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

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

MATERIALS AND METHODS

Plant Material

Seeds of Arabidopsis (*Arabidopsis thaliana* accession Col-0), mutants *npr1-1*, *npr1-3* (Cao et al., 1994), *ein2-1* (Alonso et al., 1999), and double mutant *npr1-1/ein2-1* (Clarke et al., 2000) were sown in quartz sand. After 2 weeks, seedlings were transferred to 60-mL pots containing a sand/potting soil mixture that was autoclaved twice for 20 min (Pieterse et al., 1998). Plants were cultivated in a growth chamber with an 8-h-day (24°C)/16-h-night (20°C) cycle at 70% relative humidity for another 3 weeks. Plants were watered every other day and received half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) containing 10 mM Sequestreen (CIBA-Geigy) once per week. For experiments with in vitro-grown plants, seedlings were grown on plates containing MS medium, pH 5.7, supplemented with 20 g L^{-1} Suc and 0.8% (w/v) plant agar. In all experiments, 5-week-old plants were used, except in the experiment presented in Figure 1, in which 12-d-old seedlings grown on MS agar medium were used, as described by Spoel et al. (2003).

Alternaria brassicicola Assays

For induction of JA-responsive gene expression and ET production, Col-0 plants were inoculated with *Alternaria brassicicola* strain MUCL 20297 as described previously (De Vos et al., 2005). Briefly, the fungus was grown on potato dextrose agar for 2 to 3 weeks at 22°C. Spores were collected as described by Broekaert et al. (1990). Five-week-old plants were inoculated with 5-μL drops of 50% potato dextrose broth containing 5×10^5 spores mL^{-1}. For assessing the effect of SA on the level of resistance against *A. brassicicola*, leaves of 5-week-old plants were pressure infiltrated with a solution of 10 mM $MgSO_4$ supplemented with or without 1 mM SA (Spoel et al., 2007). After 24 h, the treated leaves were inoculated with *A. brassicicola* by applying a 3-μL drop of 50% potato dextrose broth containing 1×10^6 spores mL^{-1}. At 4 d after inoculation, the percentage of leaves with spreading lesions was assessed.

Frankliniella occidentalis Assays

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

Chemical Treatments

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

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

ET Measurements

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

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

RNA Extraction and Northern-Blot Analysis

For RNA extraction, at least five plants per treatment were harvested at the time points indicated. RNA isolation was performed as described previously by Van Wees et al. (2000). For RNA-blot analysis, 15 mg of RNA was denatured using glyoxal and dimethyl sulfoxide (Sambrook et al., 1989), electrophoretically separated on a 1.5% agarose gel, and blotted onto Hybond-N^+ membranes (Amersham) by capillary transfer. The buffers used for electrophoresis and blotting were 10 and 25 mM sodium phosphate (pH 7.0), respectively. RNA blots were hybridized with probes for *PR-1*, *PDF1.2*, and *VSP2* as described previously by Pieterse et al. (1998). To check for equal loading, ribosomal RNA (rRNA) bands were stained with ethidium bromide or the blots were stripped and hybridized with a probe for *18S* ribosomal RNA. The Arabidopsis Genome Initiative numbers for the genes studied are At2g14610 (*PR-1*), At5g44420 (*PDF1.2*), and At5g24770 (*VSP2*). After hybridization with [α-^{32}P]dCTP-labeled probes, blots were exposed for autoradiography. Signal intensities of *PDF1.2* or *VSP2* mRNA on the northern blots were quantified using a Bio-Rad Molecular Imager FX with Quantity One software (Bio-Rad). The *PDF1.2* and *VSP2* mRNA levels of the MeJA treatment were set to 100% and compared with *PDF1.2* and *VSP2* mRNA levels of the rest of the treatments. All gene expression analyses were repeated with similar results.

Quantitative Real-Time PCR

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

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. ACC modulates the NPR1 dependency of SA-mediated suppression of MeJA-induced *VSP2* expression in Arabidopsis seedlings.

Supplemental Figure S2. Differential effects of SA-mediated suppression of MeJA-responsive *PDF1.2* expression in mutants *npr1-1* and *npr1-3*.

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