Multilayered Regulation of Ethylene Induction Plays a Positive Role in Arabidopsis Resistance against *Pseudomonas syringae*[1][OPEN]

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Ethylene, a key phytohormone involved in plant-pathogen interaction, plays a positive role in plant resistance against fungal pathogens. However, its function in plant bacterial resistance remains unclear. Here, we report a detailed analysis of ethylene induction in Arabidopsis (*Arabidopsis thaliana*) in response to *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*). Ethylene biosynthesis is highly induced in both pathogen/microbe-associated molecular pattern (PAMP)-triggered immunity and effector-triggered immunity (ETI), and the induction is potentiated by salicylic acid (SA) pretreatment. In addition, *Pst* actively suppresses PAMP-triggered ethylene induction in a type III secretion system-dependent manner. SA potentiation of ethylene induction is dependent mostly on MITOGEN-ACTIVATED PROTEIN KINASE6 (MPK6) and MPK3 and their downstream ACS2 and ACS6, two type I isoforms of 1-aminocyclopropane-1-carboxylic acid synthases (ACSs). ACS7, a type III ACS whose expression is enhanced by SA pretreatment, is also involved. *Pst* expressing the *avrRpt2* effector gene (*Pst-avrRpt2*), which is capable of triggering ETI, induces a higher level of ethylene production, and the elevated portion is dependent on *SALICYLIC ACID INDUCTION DEFICIENT2* and *NONEXPRESSER OF PATHOGENESIS-RELATED GENE1*, two key players in SA biosynthesis and signaling. High-order *ACS* mutants with reduced ethylene induction are more susceptible to both *Pst* and *Pst-avrRpt2*, demonstrating a positive role of ethylene in plant bacterial resistance mediated by both PAMP-triggered immunity and ETI.

Plants have multilayered defenses to ward off invading pathogens. The first line of defense is initiated by the detection of pathogen/microbe-associated molecular patterns (PAMPs) by plant cell surface pattern recognition receptors, which triggers a robust defense response known as PAMP-triggered immunity (PTI; Ausubel, 2005; Jones and Dangl, 2006; Boller and Felix, 2009; Spoel and Dong, 2012; Zipfel, 2014). To circumvent the immune responses of plants, pathogens are capable of delivering effector proteins into plant cells through the type III secretion system (TTSS) to suppress PTI to facilitate pathogenesis (Alfano and Collmer, 2004; Boller and He, 2009; Guo et al., 2009; Feng and Zhou, 2012). As a counter measure, plants evolved to possess a second line of defense in which RESISTANCE (R) proteins mediate the recognition of pathogen-derived effectors, initiating so-called effector-triggered immunity (ETI), which is stronger than PTI in general and frequently associated with hypersensitive response cell death (Ausubel, 2005; Glazebrook, 2005; Chisholm et al., 2006; Jones and Dangl, 2006; Dodds and Rathjen, 2010). Along with PAMPs and effectors, plant endogenous elicitors known as damage-associated molecular patterns (DAMPs), such as plant-derived peptides and cell wall fragments that are released upon wounding and infection, are also capable of inducing immune responses in plants (Boller and Felix, 2009).

Plant sensing of pathogen-originated PAMPs and effectors or plant-originated DAMPs activates multiple signal transduction pathways, including mitogen-activated protein kinase (MAPK) cascades (for review, see Zhang and Klessig, 2001; Pedley and Martin, 2005; Pitzschke et al., 2009; Rodriguez et al., 2010; Tena et al., 2011; Meng and Zhang, 2013). MAPK cascades are conserved eukaryotic signaling modules composed of three sequentially acting kinases with MAPKs at the bottom tier. MAPKs require phosphorylation activation by MAPK

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kinases (MAPKKs or MKKs), and MAPKKs in turn are activated by MAPKK kinases (MAPKKKs or MEKKs) via phosphorylation (Ichimura et al., 2002; Hamel et al., 2006). Among the 20 MAPKs in Arabidopsis (*Arabidopsis thaliana*), MPK3 and MPK6 share the highest homology and have a high level of functional redundancy. They also share the same upstream MAPKKs, MKK4 and MKK5 (Ren et al., 2002, 2008; Wang et al., 2007). In a MAPK cascade, the MAPKKK(s) receive signals from the sensors/receptors either directly or indirectly, and the outputs of a MAPK cascade are determined by the phosphorylation of MAPK substrates, which can be enzymes, transcription factors, and proteins with other biochemical functions (Liu and Zhang, 2004; Andreasson et al., 2005; Bethke et al., 2009; Mao et al., 2011; Meng et al., 2013; Guan et al., 2014; Xu and Zhang, 2015b).

MAPK cascades are primary signaling modules directly downstream of sensors/receptors. Identification of the first plant MAPK substrate revealed that MPK3/MPK6 positively regulate ethylene production through the phosphorylation-mediated stabilization of ACS2 and ACS6, two 1-aminocyclopropane-1-carboxylic acid synthase (ACS) isoforms (Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010; Xu and Zhang, 2014). ACS catalyzes the committing step of ethylene biosynthesis and is rate limiting in most vegetative tissues (Kende, 1993; Wang et al., 2002; Tsuchisaka et al., 2009; Xu and Zhang, 2015a). Arabidopsis has nine functional ACS isoforms that are classified into three groups based on their sequence homology and the presence/absence of phosphorylation sites in their C termini. They are type I (ACS1, ACS2, and ACS6), type II (ACS4, ACS5, ACS8, ACS9, and ACS11), and type III (ACS7; Chae and Kieber, 2005; Yoshida et al., 2005). In addition to the phosphorylation-mediated stabilization of ACS2/ACS6 proteins, MPK3 and MPK6 are also involved in the activation of *ACS2* and *ACS6* gene expression during plant immunity via WRKY33, another MPK3/MPK6 substrate (Mao et al., 2011; Li et al., 2012). The higher rate of de novo synthesis of ACS proteins resulted from *ACS* gene activation, which, coupled with their phosphorylation-induced stabilization by MPK3/MPK6, provides a vital supply of ACS enzymes to maintain a high rate of ethylene production in response to pathogen invasion (Li et al., 2012).

In addition to ethylene, other plant hormones, such as salicylic acid (SA) and jasmonic acid (JA), also function as key secondary signaling molecules in plant immunity. Their levels/activities are modulated by plant-pathogen interaction in response to the primary signaling pathways, either positively or negatively, and such changes can profoundly impact plant immunity (Glazebrook, 2005; Broekaert et al., 2006; van Loon et al., 2006; Katagiri and Tsuda, 2010; Pieterse et al., 2012; Kazan and Lyons, 2014). At present, the regulatory pathway(s) of SA and JA biosynthesis are mostly unknown. SA plays a central role in plant defense signaling in both local and systemic immunity, mainly through its downstream components NONEXPRESSER OF PATHOGENESIS-RELATED GENE1 (NPR1; for review, see Vlot et al., 2009; An and Mou, 2011; Spoel and Dong, 2012; Fu and Dong, 2013). A connection between SA and plant MAPKs was first made with the purification and identification of salicylic acid-induced protein kinase (SIPK), the tobacco (*Nicotiana tabacum*) ortholog of Arabidopsis MPK6 (Zhang and Klessig, 1997). However, the function of SIPK activation in the SA signaling pathway is still unknown. Recently, Arabidopsis MPK3, and to a lesser extent MPK6, were shown to play pivotal roles in SA-mediated priming of plant disease resistance (Beckers et al., 2009).

Antagonistic and synergistic interactions between SA, ethylene, and JA have been reported, which adds another layer of complexity (Broekaert et al., 2006; van Loon et al., 2006; Spoel and Dong, 2008; Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). In general, it is considered that ethylene and SA are antagonistic in plant immunity (Glazebrook, 2005; Pieterse et al., 2012). Here, we report the PAMP- and effector-triggered ethylene induction and its potentiation by SA in Arabidopsis in response to *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*) infection. The potentiation effect is mostly dependent on MPK3/MPK6 and involves ACS2 and ACS6, two ACS isoforms regulated by MPK3/MPK6 at both the transcriptional and posttranslational levels (Liu and Zhang, 2004; Han et al., 2010; Li et al., 2012). In addition, *Pst* is capable of inhibiting PAMP-triggered ethylene biosynthesis in a TTSS-dependent manner, suggesting an active suppression of plant ethylene production by *Pst* effector(s). The battle between *Pst* and Arabidopsis in controlling ethylene biosynthesis suggests a positive role of ethylene in bacterial resistance. Consistent with this, we found that the loss of ethylene biosynthesis in *acs* mutants leads to pathogen susceptibility. In summary, this research highlights a novel interaction between SA and ethylene and demonstrates that ethylene is a positive regulator in Arabidopsis immunity against a bacterial pathogen.

RESULTS

Pst Actively Suppresses Ethylene Induction in Arabidopsis during PTI

Although ethylene is recognized as an important plant hormone involved in plant disease resistance, no report has analyzed in detail its induction in Arabidopsis in response to *Pst* infection. To facilitate the measurement of ethylene, we grew Arabidopsis seedlings in gas chromatography (GC) vials similar to what we used for studying the Arabidopsis-*Botrytis cinerea* interaction (Han et al., 2010; Li et al., 2012), and *Pst* inoculation was simply done by the addition of inoculum to a final concentration of optical density at 600 nm (OD_{600}) of 0.02. Ethylene accumulation in the headspace of the GC vials was monitored afterward. An advantage of this system in comparison with collecting leaves from soil-grown plants is that wounding-induced ethylene production can be avoided.

As shown in Figure 1A, ethylene gradually accumulated in the first 6 h after *Pst* inoculation and then reached a plateau. In contrast, ethylene accumulation

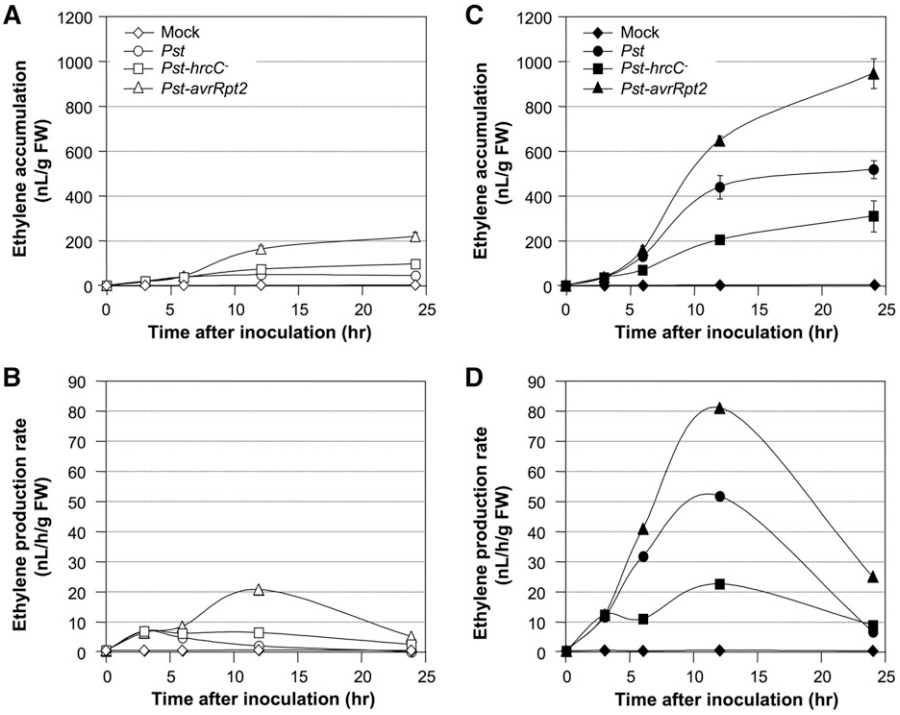

Figure 1. Bacterial PAMP- and effector-triggered ethylene induction and its potentiation by SA in Arabidopsis. A, Fourteen-day-old seedlings grown in GC vials were inoculated with *Pst, Pst-hrcC*$^-$, or *Pst-AvrRpt2* (final OD_{600} = 0.02). Mock inoculation was used as a control. Ethylene accumulations in the headspace were determined at the indicated times. B, Replot of the data in A as the rates of ethylene production. Ethylene production rates were calculated as the average rates of ethylene production in the intervals of the two adjacent time points. C, Twelve-day-old seedlings grown in GC vials were treated with SA (final concentration of 100 μM). Two days later, they were inoculated with *Pst, Pst-hrcC*$^-$, or *Pst-avrRpt2* (final OD_{600} = 0.02). Mock inoculation was used as a control. Ethylene accumulations in the headspace were determined at the indicated times. D, Replot of the data in C as the average rates of ethylene production in the intervals of the two adjacent time points. All data were all collected side by side. Error bars indicate SD (n = 3). FW, Fresh weight.

continued to increase in seedlings inoculated with *Pst-hrcC*$^-$, a *Pst* strain carrying a deletion mutation in *hrcC* gene that cannot deliver effectors into plant cells. After the conversion of ethylene accumulation to the average rates of ethylene production between the two adjacent time points (Fig. 1B), it became obvious that, within the first 3 h, ethylene induction rates were similar in Arabidopsis inoculated with *Pst* and *Pst-hrcC*$^-$. After that, the ethylene production rate started to decline in *Pst*-inoculated plants, while that in *Pst-hrcC*$^-$-inoculated Arabidopsis stayed at a relatively high level, which is consistent with the scenario that both *Pst* and *Pst-hrcC*$^-$ can trigger the PAMP-induced defense responses but only *Pst* can deliver effector proteins to suppress plant immunity and facilitate the pathogenesis process. As a result, we conclude that *Pst* is able to actively suppress ethylene induction by delivering effectors into Arabidopsis cells during PTI.

avrRpt2 ETI Is Associated with a High Level of Ethylene Induction

Seedlings inoculated with *Pst* expressing the *avrRpt2* effector gene (*Pst-avrRpt2*) produced higher levels of ethylene. In the first 3 h, the ethylene production rate was similar to those inoculated with either *Pst* or *Pst-hrcC*$^-$ (Fig. 1, A and B). However, there was no sign of effector-mediated suppression of ethylene induction after 6 h; instead, the ethylene production rates surpassed that induced by *Pst-hrcC*$^-$ and continued to increase to a much higher level. The high-level ethylene production is likely a result of avrRpt2 effector-triggered activation of defense responses (i.e. ETI). In the end, seedlings inoculated with *Pst-avrRpt2* produced much more ethylene than those inoculated with *Pst-hrcC*$^-$ or *Pst* (Fig. 1, A and B). In *resistance to P. syringae2 (rps2)* mutant seedlings that lack the R protein to sense the avrRpt2 effector, *Pst-avrRpt2*-induced ethylene was much lower, at a level similar to that in seedlings inoculated with *Pst* (Fig. 2A), confirming that the higher ethylene production is the result of an effector-triggered response.

To demonstrate directly the induction of ethylene by the avrRpt2 effector, we utilized dexamethasone (DEX)-inducible promoter-driven *avrRpt2* (*GVG-avrRpt2* [*GVG* encodes a hybrid transcription factor consisting of one domain each from GAL4, VP16, and GR]) transgenic plants (McNellis et al., 1998). As shown in Figure 2B, treatment of *GVG-avrRpt2* seedlings with

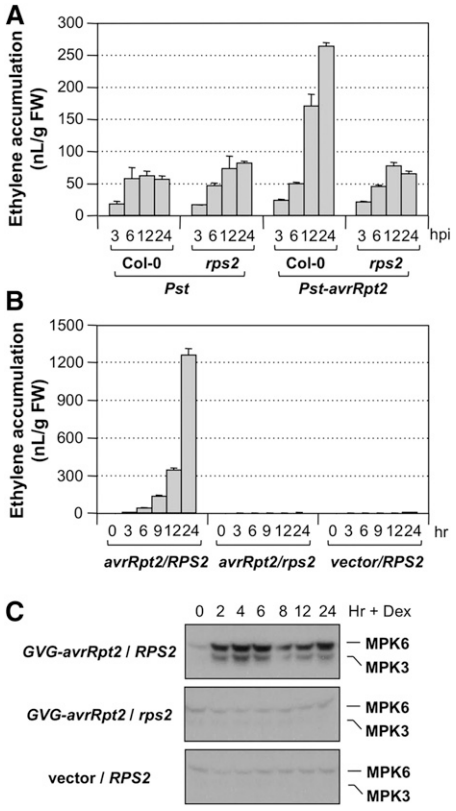

Figure 2. *RPS2*-dependent induction of ethylene biosynthesis in Arabidopsis triggered by the avrRpt2 effector. A, Higher levels of ethylene induction in Arabidopsis inoculated with *Pst-avrRpt2* are dependent on the presence of *RPS2*, the corresponding R gene. Fourteen-day-old wild-type (Col-0) and *rps2* mutant seedlings grown in GC vials were inoculated with *Pst* or *Pst-avrRpt2* (final OD_{600} = 0.02). Ethylene accumulations in the headspace were determined at the indicated times. Error bars indicate SD (n = 3). FW, Fresh weight; hpi, hours post inoculation. B, Fourteen-day-old steroid-inducible *avrRpt2* transgene (*GVG-avrRpt2*) in the wild-type Col-0 background (*avrRpt2/RPS2*), *GVG*-avrRpt2 in the *rps2* mutant background (*avrRpt2/rps2*), and steroid-inducible vector control were treated with DEX (2 μM). Ethylene accumulations in the headspace were determined at the indicated times. Error bars indicate SD (n = 3). Seedlings were collected at the indicated times for in-gel kinase assays. C, *RPS2*-dependent activation of MPK3/MPK6 by the avrRpt2 effector. MAPK activities in samples collected in B were determined using 10 μg of total proteins by the in-gel kinase assay with myelin basic protein (MBP) as a substrate.

DEX strongly induced ethylene biosynthesis. In the absence of *RPS2*, no induction of ethylene was observed. As another control, we also tested a vector control transgenic line and observed no ethylene production either. As a result, we can conclude that, in the absence of PTI, effector by itself is sufficient to trigger strong ethylene production. An in-gel kinase assay revealed the activation of MPK6 and MPK3 in *GVG-avrRpt2* transgenic seedlings after DEX treatment (Fig. 2C). No activation of MPK3 and MPK6 was observed in *GVG-avrRpt2 rps2* or vector control transgenic seedlings after DEX treatment, demonstrating the specificity of MPK3/MPK6 activation in the ETI.

SA Potentiates *Pst*-Induced Ethylene Production, a Process Dependent on *NPR1*

SA is known to enhance many defense responses triggered by *Pst* (Vlot et al., 2009; An and Mou, 2011; Fu and Dong, 2013). As a result, we examined ethylene production in SA-pretreated seedlings. In this experiment, the seedlings were treated with SA (100 μM) for 2 d before *Pst* inoculation. The normal SA response in the liquid-cultured seedling system was validated by the high-level *PATHOGENESIS-RELATED1* (*PR1*) gene induction (Supplemental Fig. S1). Remarkably, pretreatment of seedlings with SA greatly enhanced the ethylene production induced by *Pst* inoculation (Fig. 1C). The maximal accumulation of ethylene in *Pst*-inoculated seedlings was approximately 50 nL g^{-1} fresh weight without SA pretreatment (Fig. 1A), whereas the ethylene induction in SA-pretreated seedlings reached greater than 500 nL g^{-1} fresh weight (Fig. 1C; the data in Fig. 1 were all collected side by side), an increase of about 10-fold.

SA pretreatment not only enhanced *Pst*-induced ethylene production but also promoted ethylene biosynthesis induced by *Pst-hrcC*$^{-}$ and *Pst-avrRpt2* (Fig. 1, C and D), although the fold enhancement was not as high, at approximately 3-fold and approximately 4-fold for *Pst-hrcC*$^{-}$ and *Pst-avrRpt2*, respectively. SA treatment alone only weakly induced ethylene production (Supplemental Fig. S2), with a rate about twice the basal level without any treatment, which is very low in comparison with the pathogen-induced (Fig. 1, C and D, mock versus *Pst* inoculated) or flg22-induced (Supplemental Fig. S2) ethylene production. This provides strong support that increased ethylene biosynthesis after SA pretreatment is a potentiation (also known as priming) effect rather than a direct contribution to ethylene production.

SA potentiation of *Pst*-induced ethylene production was dependent on *NPR1*. In the *npr1* mutant, *Pst*-induced ethylene production was similar to that in the wild-type (Columbia-0 [Col-0]) control. However, SA-potentiated ethylene induction was greatly reduced (Fig. 3), demonstrating that, similar to other SA-enhanced defense responses, SA-mediated potentiation of ethylene induction in response to *Pst* is also dependent on *NPR1*. In *NahG* transgenic plants expressing the bacterial salicylate hydroxylase (encoded by *NahG* gene) that converts SA to catechol, therefore effectively removing SA, the enhancement effect of SA pretreatment was also greatly inhibited (Fig. 3). These results demonstrate that (1) the liquid-cultured seedlings have a normal SA response and (2) the potentiation effect we observed is a bona fide SA-mediated response.

The Potentiation of Ethylene Induction by SA Is Associated with MAPK Activation

The MPK3/MPK6 cascade plays important roles in ethylene induction in response to *B. cinerea* at both the transcriptional and posttranslational levels (Han et al.,

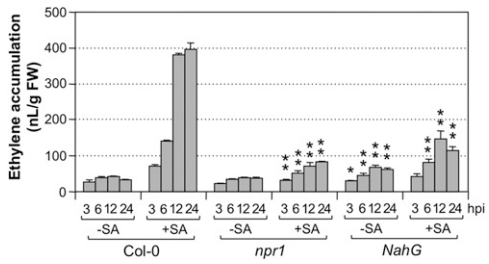

Figure 3. Potentiation of *Pst*-induced ethylene biosynthesis by SA pretreatment is dependent on functional *NPR1*, and the *NahG* transgene abolishes this SA effect. Twelve-day-old wild-type (Col-0), *npr1*, or *NahG* Arabidopsis seedlings grown in GC vials were treated with SA (+SA; final concentration of 100 μM) or a solvent of SA stock solution (−SA). Two days later, they were inoculated with *Pst* (final OD_{600} = 0.02). Ethylene accumulations in the headspace were determined at the indicated times. Error bars indicate SD ($n = 3$). Student's *t* test was used to compare mutants and the wild type at the same time point after the same treatment (*, $P \leq 0.05$; and **, $P \leq 0.01$). FW, Fresh weight; hpi, hours post inoculation.

2010; Li et al., 2012). As a result, we examined the activation of MAPKs in seedlings inoculated with *Pst*, *Pst-hrcC*$^-$, and *Pst-avrRpt2* with and without SA pretreatment. We did not observe a major difference in the MAPK activation in *Pst*-, *Pst-hrcC*$^-$-, or *Pst-avrRpt2*-inoculated seedlings (Fig. 4; Supplemental Fig. S3A). In *mpk6* and *mpk3* single mutants, their corresponding kinase activity bands disappeared (Supplemental Fig. S3A), demonstrating that the two larger MAPK activity bands are MPK6 and MPK3, respectively. The smallest and the weaker MAPK activity is likely to be MPK4 based on its size (Xu et al., 2014). It is also clear that, in the absence of MPK3, MPK6 activation was higher, a compensatory effect that we reported before (Wang et al., 2008). The lack of suppression of PAMP-triggered MAPK activation suggests that *Pst* does not possess effector(s) that directly target MPK3/MPK6 activation, and the suppression of ethylene production in Arabidopsis (Fig. 1) by *Pst* is likely a result of the suppression of other components in the ethylene induction process.

Seedlings pretreated with SA showed heightened MAPK activation, especially MPK3, after *Pst*, *Pst-hrcC*$^-$, or *Pst-avrRpt2* inoculation (Fig. 4; Supplemental Fig. S3). MPK4 activation was also stronger. MPK3 has been shown to play a key role in SA-induced priming (Beckers et al., 2009). To determine the role of MPK3 and MPK6 in *Pst*-induced ethylene production and its enhancement by SA pretreatment, we examined ethylene induction in *mpk3*, *mpk6*, and an *mpk3 mpk6* double mutant rescued with an MPK6 variant ($MPK6^{YG}$) that is sensitized to a 4-amino-1-*tert*-butyl-3-(1′-naphthyl) pyrazolo[3,4-d]pyrimidine (NA-PP1) inhibitor (genotype *mpk3 mpk6* P_{MPK6}:$MPK6^{YG}$, *MPK6SR* for short; Xu et al., 2014). We first compared ethylene production in Col-0, *mpk3* single mutant, and *mpk6* single mutant after *Pst* inoculation with and without SA pretreatment. As shown in Figure 5A, loss of function of *MPK6* slightly reduced ethylene biosynthesis, while no major difference was observed in the *mpk3* mutant. In *MPK6SR* line 45 without NA-PP1 treatment, we observed normal SA potentiation of ethylene induction. In contrast, application of NA-PP1 almost completely blocked the potentiation effect of SA (Fig. 5B), demonstrating that MPK3/MPK6 are required for the full potentiation effect of SA. We also observed that, in the absence of SA pretreatment, *Pst*-induced ethylene production was higher in seedlings with NA-PP1 treatment. Since this was observed in both Col-0 and *MPK6SR* seedlings, we assumed that it might be caused by a nonspecific inhibition of some other unknown target(s) by NA-PP1. However, a stronger effect of NA-PP1 was observed in *MPK6SR* seedlings. Examination of another independently rescued *mpk3 mpk6* double mutant line (*MPK6SR* line 58) gave similar results (Supplemental Fig. S4).

The Potentiation of *Pst*-Induced Ethylene Production by SA Is Dependent on *ACS2*, *ACS6*, and *ACS7*

We next determined the contribution of different ACS isoforms using various *acs* mutants. In the *acs2* single mutant, no significant reduction in ethylene induction was observed after *Pst* inoculation. Ethylene induction was slightly lower, but significant, in *asc6* single, *acs2 acs6* double, and *acs2 acs6 acs4 acs5 acs9* quintuple mutants, and there was no significant difference between these four genotypes, suggesting that *ACS6* also contributes to *Pst*-triggered ethylene biosynthesis. Ethylene induction was much lower in the *acs2 acs6 acs4 acs5 acs9 acs7* sextuple mutant in comparison with the *acs2 acs6 acs4 acs5 acs9* quintuple mutant (Fig. 6, −SA groups), demonstrating that *Pst*-induced ethylene production in Arabidopsis without SA pretreatment was mostly dependent on *ACS7*. The residual ethylene induction suggests the involvement of *ACS8*, since additional mutation of *acs1* and *acs11* in the *acs2 acs6 acs4 acs5 acs9 acs7* mutant background did

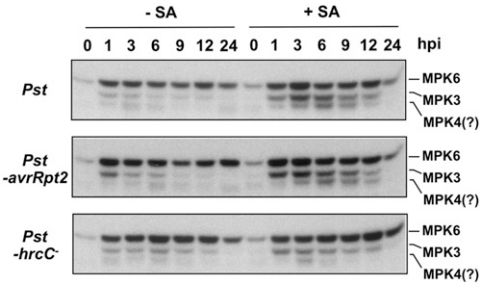

Figure 4. SA pretreatment enhances MPK3/MPK6 activation by *Pst*. Twelve-day-old wild-type (Col-0) Arabidopsis seedlings grown in GC vials were treated with dimethyl sulfoxide (DMSO) solvent (−SA) or SA (+SA; final concentration of 100 μM). Two days later, they were inoculated with *Pst*, *Pst-hrcC*$^-$, or *Pst-avrRpt2* (final OD_{600} = 0.02). Seedlings were collected at the indicated times. MAPK activities were determined using 10 μg of total proteins by the in-gel kinase assay with MBP as a substrate. hpi, Hours post inoculation.

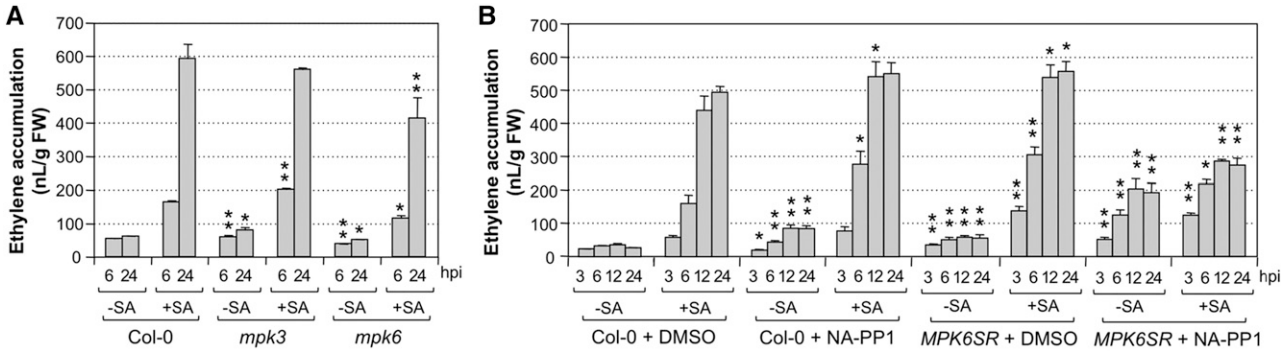

Figure 5. Arabidopsis MPK3 and MPK6 play essential and overlapping roles in *Pst*-induced ethylene biosynthesis and SA potentiation. A, Twelve-day-old wild-type (Col-0), *mpk3*, and *mpk6* seedlings grown in GC vials were treated with DMSO solvent (−SA) or SA (+SA; final concentration of 100 μM). Two days later, *Pst* was inoculated (final OD_{600} = 0.02), and ethylene accumulation in the headspace of the GC vials was measured. B, Twelve-day-old wild-type (Col-0) and chemical genetically rescued *mpk3 mpk6* double mutant (*MPK6SR* line 45) seedlings grown in GC vials were treated with SA (+SA; final concentration of 100 μM) or DMSO, the solvent of SA stock solution (−SA). Two days later, they were inoculated with *Pst* (final OD_{600} = 0.02) after pretreatment with NA-PP1 (+NA-PP1; 5 μM final concentration) or solvent control (+DMSO) for 30 min. Ethylene accumulations in the headspace were determined at the indicated times. Error bars indicate SD (n = 3). Student's t test was used to compare mutants and the wild type at the same time point after the same treatment (*, $P \leq 0.05$; and **, $P \leq 0.01$). FW, Fresh weight; hpi, hours post inoculation.

not further reduce the ethylene induction, and *acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11* octuple mutant seedlings with only functional *ACS8* still produced very low levels of ethylene. Alternatively, the residual ethylene production observed in octuple *acs* mutant seedlings could come from the bacteria. It was reported that *P. syringae* pathovars produce low levels of ethylene (Weingart and Volksch, 1997). Indeed, we found that *Pst* (with and without *avrRpt2*) produced low levels of ethylene (Supplemental Fig. S5). Without Arabidopsis seedlings, *Pst* does not grow much in Murashige and Skoog (MS) medium. As a result, we measured ethylene production by *Pst* of different concentrations from OD_{600} of 0.02 to 0.5. In the presence of Arabidopsis seedlings, *Pst* could grow from the initial concentration of 0.02 after inoculation to approximately 0.5 within 24 h. If we assume that *Pst* grew in a linear rate, its average concentration should be approximately 0.25. As shown in Supplemental Figure S5, GC vials with *acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11* seedlings still accumulated more ethylene than the vials with only 0.25 OD_{600} *Pst*, suggesting that the octuple mutant seedlings still produced a small amount of ethylene. One caveat is that, in the presence of Arabidopsis seedlings, *Pst* might produce more ethylene due to its interaction with plants, which makes it difficult to draw a definitive conclusion about the involvement of ACS8. Either way, the contribution of ACS8 should be very small in comparison with ACS2, ACS6, or ACS7.

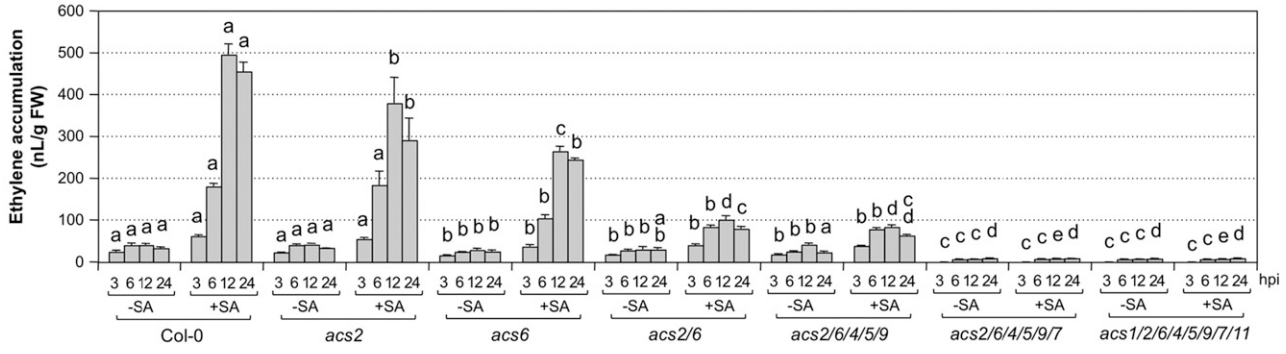

Figure 6. SA potentiated ethylene induction in various *acs* mutants after *Pst* infection. Twelve-day-old wild-type (Col-0) seedlings and high-order *acs* mutants were treated with DMSO solvent (−SA) or SA (+SA; final concentration of 100 μM). Two days later, *Pst* was inoculated (final OD_{600} = 0.02), and ethylene accumulation in the headspace of the GC vials was measured at the indicated times. Error bars indicate SD (n = 3). One-way ANOVA was performed to compare *acs* mutants and the wild type at the same time point after the same treatment. Lowercase letters above the columns indicate statistically different groups. The two genotypes are considered to produce different amounts of ethylene when two or more time points are significantly different. The allele numbers are omitted for easy labeling. They are *acs1-1*, *acs2-1*, *acs4-1*, *acs5-2*, *acs6-1*, *acs7-1*, *acs9-1*, and *acs11-1*. FW, Fresh weight; hpi, hours post inoculation.

In contrast, SA potentiation of ethylene biosynthesis is mostly dependent on *ACS2* and *ACS6*. In *acs2* and *asc6* single mutants, the enhanced ethylene production by SA pretreatment was reduced, and an additive effect was observed in the *acs2 acs6* double mutant (Fig. 6, +SA groups). Instead of a 10-fold enhancement in the wild type, the *acs2 acs6* double mutant only had an approximately 2-fold increase. There was no significant change in ethylene induction in the *acs2 acs6 acs4 acs5 acs9* quintuple mutant in comparison with the *acs2 acs6* double mutant, suggesting that *ACS4*, *ACS5*, and *ACS9* played minimal roles in the process. Additional mutation of the *ACS7* gene in the *acs2 acs6 acs4 acs5 acs9* background reduced ethylene production further to a very low level that was no longer responsive to SA pretreatment. Again, the very low-level ethylene accumulation in *acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11* seedlings inoculated with *Pst* could come either from *Pst* or Arabidopsis seedlings as a result of functional *ACS8*, as discussed above. In summary, we found that *ACS7* is involved in *Pst*-induced ethylene induction, while *ACS2* and *ACS6* are more important to the SA-potentiated induction of ethylene biosynthesis.

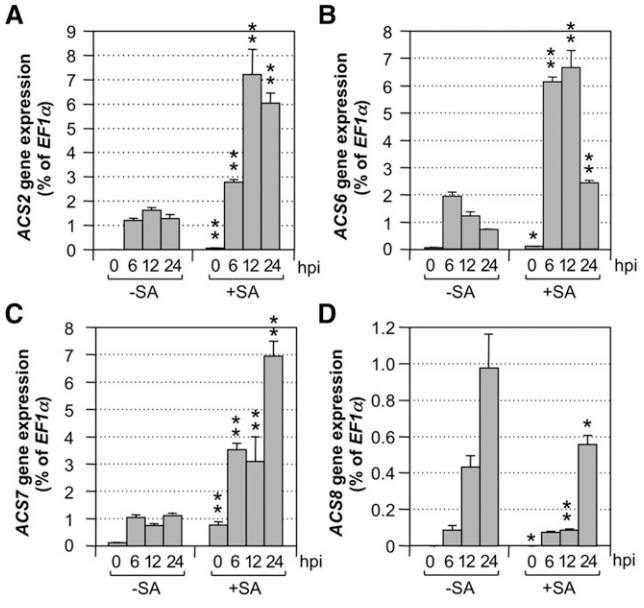

Figure 7. SA potentiation of ethylene induction is associated with enhanced *ACS2*, *ACS6*, and *ACS7* gene activation. Twelve-day-old wild-type (Col-0) seedlings grown in GC vials were treated with DMSO solvent (−SA) or SA (+SA; final concentration of 100 μM). Two days later, *Pst* was inoculated (final OD_{600} = 0.02), and seedlings were collected at the indicated times for total RNA preparation. After reverse transcription, transcript levels of *ACS2* (A), *ACS6* (B), *ACS7* (C), *and ACS8* (D) genes were quantified by real-time PCR. Expression of the *ACS* genes was calculated as the percentage of *ELONGATION FACTOR-1α* (*EF1α*) transcript. Error bars indicate SD (n = 3). Student's t test was used to compare plants treated with SA (+SA) and DMSO solvent (−SA) at the same time point after the same treatment (*, $P \leq 0.05$; and **, $P \leq 0.01$). hpi, Hours post inoculation.

The Potentiation of Ethylene Induction by SA Is Associated with Enhanced *ACS* Gene Expression

Without SA pretreatment, *Pst*-induced ethylene production was associated with *ACS2*, *ACS6*, *ACS7*, and *ACS8* gene activation (Fig. 7), which is similar to *B. cinerea*-induced ethylene production (Li et al., 2012). SA pretreatment further enhanced the induction of *ACS2*, *ACS6*, and *ACS7*. Interestingly, SA pretreatment did not enhance *ACS8* expression (Fig. 7D), which is consistent with the finding that *ACS8* is not involved in SA-enhanced ethylene production (Fig. 6). As a result, SA-potentiated ethylene induction by *Pst* is likely a result of, at least partially, the enhanced gene activation of *ACS2*, *ACS6*, and *ACS7*. The higher level of *ACS2*/*ACS6* expression in conjunction with MPK3/MPK6-mediated phosphorylation stabilization of ACS2/ACS6 proteins could lead to high cellular ACS activity levels and ethylene production. Pathway(s) that regulate *ACS7* expression are unclear at present. Despite lacking a potential phosphorylation site in the C terminus, ACS7 was recently shown to be phosphorylated by a calcium-dependent protein kinase (CPK) activity at the N terminus, and phosphorylation could also lead to protein stabilization and ethylene production (Huang et al., 2013).

Even without SA pretreatment, we observed high levels of *ACS2* and *ACS6* gene activation at approximately 185- and 33-fold, respectively, at 6 h post inoculation, in response to *Pst* inoculation in Arabidopsis (Fig. 7, −SA groups). However, there is little/no contribution from *ACS2* and *ACS6* to *Pst*-induced ethylene production (Fig. 6, −SA groups). Using the in-gel kinase assay, we also detected similar levels of MPK3/MPK6 activation after *Pst* infection (Fig. 4; Supplemental Fig. S3A, top). Both should have led to an increase in ACS activity from ACS2/ACS6 isoforms and higher ethylene production. The lack of ACS2/ACS6 contribution suggests that *Pst* effector(s) might target these two ACS isoforms and impede their function either directly or indirectly.

The Elevated Ethylene Biosynthesis during ETI Is Dependent on ACS2/ACS6 Isoforms and SA Biosynthesis and Signaling

While ethylene induction by *Pst* in seedlings without SA pretreatment was mostly dependent on *ACS7* (Fig. 6), ethylene induction by *Pst-avrRpt2* without SA pretreatment was dependent on *ACS2* and *ACS6* in addition to *ACS7* (Fig. 8, −SA groups). In the absence of *ACS2* and *ACS6* (*acs2 acs6* double mutant), the level and kinetics of ethylene induction were almost identical to those induced by *Pst* (Figs. 6 and 8, −SA groups), suggesting that avrRpt2-triggered ethylene production is mostly dependent on *ACS2* and *ACS6*. Ethylene induction by *Pst-avrRpt2* in seedlings pretreated with SA was dependent on the same set of *ACS* genes (e.g. *ACS2*, *ACS6*, and *ACS7*) based on mutant analysis (Fig. 8, +SA groups). Again, the contribution of *ACS8* to ethylene induction in response to *Pst-avrRpt2* inoculation

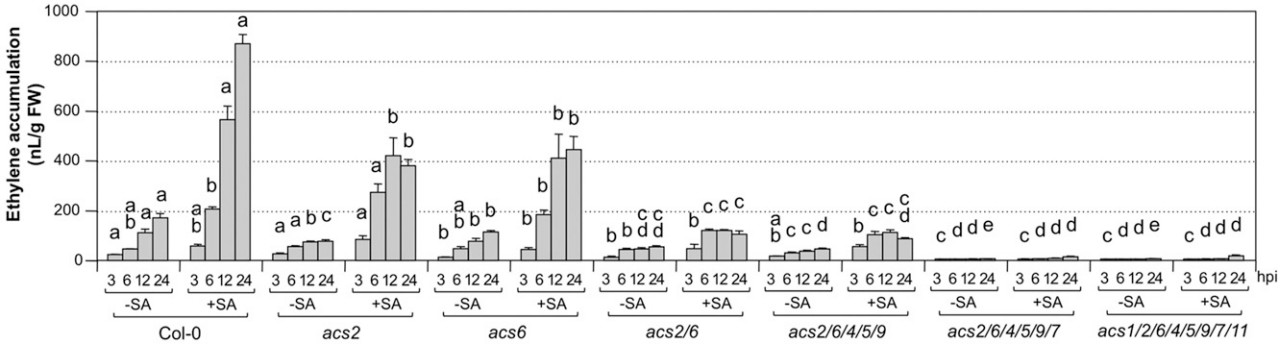

Figure 8. SA potentiated ethylene induction in high-order *acs* mutants after *Pst-avrRpt2* inoculation. Twelve-day-old wild-type (Col-0) seedlings and *acs* mutants generated in Dr. A. Theologis's laboratory were treated with DMSO solvent (−SA) or SA (+SA; final concentration of 100 μM). Two days later, *Pst-avrRpt2* was inoculated (final OD_{600} = 0.02), and ethylene accumulation in the headspace of the GC vials was measured at the indicated times. Error bars indicate SD (n = 3). One-way ANOVA was performed to compare *acs* mutants and the wild type at the same time point after the same treatment. Lowercase letters above the columns indicate statistically different groups. The two genotypes are considered to produce different amounts of ethylene when two or more time points are significantly different. The allele numbers are omitted for easy labeling. They are *acs1-1*, *acs2-1*, *acs4-1*, *acs5-2*, *acs6-1*, *acs7-1*, *acs9-1*, and *acs11-1*. hpi, Hours post inoculation.

is minimal, if any. The low-level ethylene production by *Pst-avrRpt2* (Supplemental Fig. S5) prevented us from drawing a definitive conclusion. In addition, the *acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11* octuple mutant with only functional *ACS8* did not show SA-enhanced ethylene production (Fig. 8), suggesting that *ACS8* is not involved in the SA potentiation of ethylene biosynthesis in response to *Pst-avrRpt2* inoculation either, which is similar to that in response to *Pst* inoculation.

SA-potentiated ethylene production in seedlings inoculated with *Pst-avrRpt2* was dependent on functional *NPR1* (Fig. 9), similar to that inoculated with *Pst* (Fig. 3). It was interesting to see that the mutation of *NPR1* also reduced ethylene induction by *Pst-avrRpt2* in seedlings without SA pretreatment, suggesting that the SA signaling pathway is required for the effector-triggered ethylene production. Examination of *NahG* transgenic and *sid2-2* mutant seedlings gave similar results (Fig. 9, −SA groups). The ethylene production in *npr1*, *NahG*, or *sid2-2* seedlings inoculated with *Pst-avrRpt2* was similar to that in seedlings inoculated with *Pst* (Fig. 3) in kinetics and magnitude. It is likely that the elevated ethylene induction triggered by effector is dependent on endogenously produced SA and signaling. We found that supplementation of SA cannot restore the potentiation of ethylene biosynthesis in *sid2-2* mutant seedlings (Fig. 9; Supplemental Fig. S6), suggesting that SALICYLIC ACID INDUCTION DEFICIENT2 (SID2) may function more than simply as an SA biosynthetic enzyme. It is also possible that one-time application of exogenously supplied SA cannot fulfill the function of endogenously produced SA due to differences in the kinetics and magnitude of cellular SA levels.

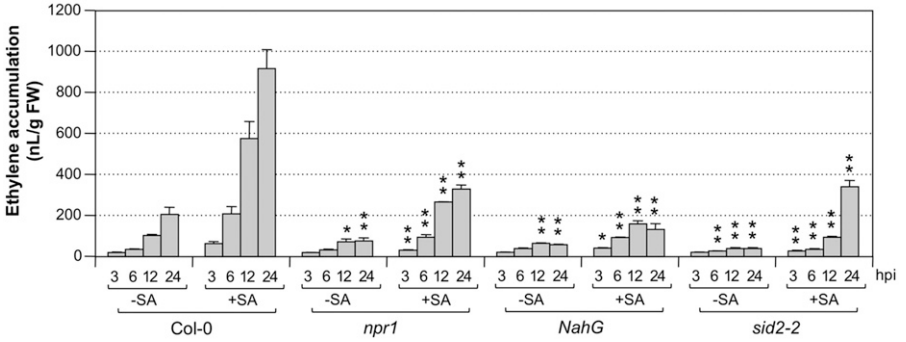

Figure 9. Elevated ethylene biosynthesis in response to *Pst-avrRpt2* inoculation and SA pretreatment is dependent on functional *NPR1* and *SID2*, and the *NahG* transgene abolishes this SA effect. Twelve-day-old wild-type (Col-0), *npr1*, *sid2-2*, or *NahG* Arabidopsis seedlings grown in GC vials were treated with SA (+SA; final concentration of 100 μM) or DMSO (−SA). Two days later, they were inoculated with *Pst-avrRpt2* (final OD_{600} = 0.02). Ethylene accumulations in the headspace were determined at the indicated times. Error bars indicate SD (n = 3). Student's t test was used to compare mutants and the wild type at the same time point after the same treatment (*, $P \leq 0.05$; and **, $P \leq 0.01$). FW, Fresh weight; hpi, hours post inoculation.

A Positive Role of Ethylene Biosynthesis in Plant Disease Resistance

Enhanced ethylene biosynthesis is associated with both PTI and ETI in plant resistance against bacterial infection. In addition, *Pst* actively suppresses PAMP-triggered ethylene production via the TTSS-mediated process, and SA pretreatment potentiates *Pst*-induced ethylene biosynthesis, suggesting that ethylene might play an important role in plant immunity against bacterial infection. As a result, we examined the resistance of *acs* mutants infiltrated with *Pst* with and without SA pretreatment. As shown in Figure 10, loss of function of *ACS2* and *ACS6* genes, which are targets of the MPK3/MPK6-regulated ethylene production pathway, leads to higher pathogen susceptibility. Mutation of additional *ACS* genes, such as in *acs2 acs6 acs4 acs5 acs9* quintuple, *acs2 acs6 acs4 acs5 acs9 acs7* sextuple, and *acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11* octuple mutants, did not further enhance the susceptibility (Fig. 10). The increase in *Pst* growth in *acs* mutants was small, less than 1 log, but the difference was significant and reproducible (Fig. 10A). In contrast, the growth of *Pst-avrRpt2* was about 1 log higher in *acs* mutants than in the Col-0 control (Fig. 10B), suggesting that ethylene plays a positive role in both PTI and ETI in plant immunity against *Pst* infection.

SA pretreatment resulted in about a 1-log decrease in bacterial counts in *Pst*-inoculated plants (Fig. 10A). However, there was no significant difference in *Pst* counts in the wild-type control (Col-0) and various *acs* mutants after SA pretreatment. The loss of *Pst* susceptibility in *acs* mutants after SA pretreatment is likely due to a strong systemic acquired resistance (SAR) effect on other defense responses, which overshadowed the effect of ethylene on plant resistance against *Pst*. In contrast, SA pretreatment did not significantly affect the growth of *Pst-avrRpt2* in Arabidopsis (Fig. 10B). Furthermore, even with SA pretreatment, *acs* mutants were still more susceptible to *Pst-avrRpt2* (i.e. SA could not mask the requirement of ethylene biosynthesis during Arabidopsis resistance against *Pst-avrRpt2*). This result suggests that ethylene induction is more important to ETI and resistance against *Pst-avrRpt2*, which is consistent with the higher levels of ethylene induction during Arabidopsis-*Pst-avrRpt2* interaction.

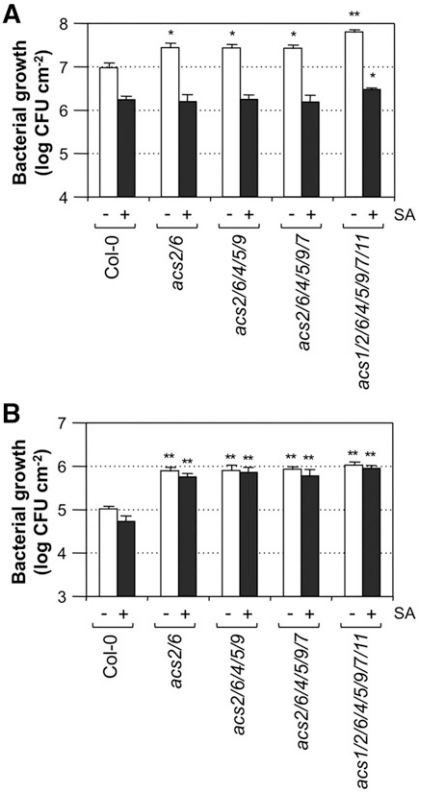

Figure 10. Ethylene is involved in plant resistance against both *Pst* and *Pst-avrRpt2*. Five-week-old wild-type (Col-0) and *acs* mutant Arabidopsis plants grown under a short-day light cycle were used for pathogen assays. Fully expanded leaves (three per plant) were infiltrated with either *Pst* (A) or *Pst-avrRpt2* (B; OD_{600} = 0.001). SA pretreatment was performed by spraying the plants with SA (1 mM) and drenching the soil with SA (1 mM) at the same time for 24 h before pathogen infiltration. Bacterial growth was quantified 3 d post inoculation. Student's *t* test was used to compare mutants and the wild type (*, $P \leq 0.05$; and **, $P \leq 0.01$). CFU, Colony-forming units.

DISCUSSION

Our understanding of ethylene involvement in plant immunity is mostly based on analyses of ethylene sensing/signaling mutants, such as *ethylene response1* and *ethylene insensitive2* (*ein2*). Detailed measurement of ethylene induction in plant immunity and its functional analysis using ethylene biosynthetic mutants are lacking. In this study, we characterized the complex interplay between Arabidopsis and *Pst* in controlling ethylene biosynthesis (Fig. 11). Ethylene is highly induced after plant sensing of pathogen-derived PAMPs or effectors in Arabidopsis. It is interesting that *Pst* actively suppresses PAMP-triggered ethylene induction in Arabidopsis via a TTSS-dependent pathway. The battle between Arabidopsis and *Pst* over ethylene induction would suggest a positive role of ethylene in plant immunity. Consistent with this speculation, high-order *acs* mutants with greatly reduced ethylene induction are more susceptible to both *Pst* and *Pst-avrRpt2*, supporting a positive role of ethylene in plant immunity.

Pretreatment of Arabidopsis with SA, a key hormone in plant SAR, greatly potentiates the ethylene induction in response to *Pst* inoculation. The SA potentiation effect is dependent mostly on MPK6 and MPK3, two pathogen-responsive MAPKs, and their downstream ACS2 and ACS6, two type I ACS isoforms. In addition, the sole member of type III ACS, ACS7, whose expression is induced by *Pst* infection and enhanced by SA pretreatment, also contributes to the pathogen-induced ethylene production. *Pst-avrRpt2* induces a higher level of ethylene production, which is dependent

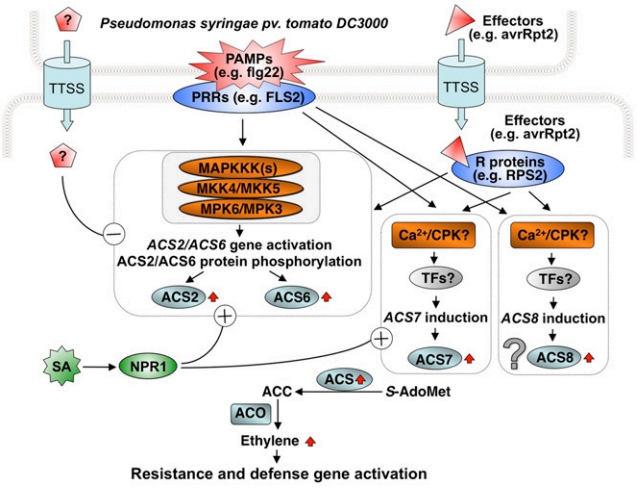

Figure 11. Model depicting the regulation of ethylene biosynthesis in the Arabidopsis-*Pst* interaction, which involves PTI, effector-mediated suppression, ETI, and SA-induced potentiation. Three ACS members contribute to the *Pst*-induced ethylene biosynthesis. Among them, ACS2 and ACS6 are regulated by the stress-responsive MPK3/MPK6 cascade, while ACS7 is regulated by the unidentified signaling pathways. The contribution of ACS8 is uncertain due to the ethylene production by *Pst*. Even ACS8 is involved; its contribution should be minimal based on the low residual ethylene production in the *acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11* mutant inoculated with *Pst*. All four *ACS* genes are activated at the transcriptional level. ACS2 and ACS6 are also regulated at the protein stability level by MPK3/MPK6 phosphorylation. Both the MAPK and unidentified pathways are activated in PTI and ETI. The MPK3/MPK6-regulated ACS2/ACS6 branch is also targeted by the unidentified effectors. This pathway is also the major target of the SA-induced potentiation of ethylene biosynthesis. In addition, induction of *ACS7* expression by *Pst* is potentiated by SA pretreatment, which is similar to *ACS2* and *ACS6*. In contrast, ACS8 is not involved in SA-potentiated ethylene production. ACC, 1-Aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; FLS2, FLAGELLIN SENSING2; PRRs, pattern-recognition receptors; *S*-AdoMet, *S*-adenosylmethionine; TFs, transcription factors.

on RPS2, the R protein corresponding to avrRpt2. Elevated ethylene induction after *Pst-avrRpt2* inoculation is dependent on *NPR1* and *SID2*, two key components in SA signaling and biosynthesis, respectively. This is consistent with a positive role of SA in both local immunity and the SAR response. Previously, it was reported that ethylene production in plants inoculated with *Pst-avrRpt2* is partially compromised in *NahG* plants (Lieberherr et al., 2003). However, ethylene induction by *Pst* without the avrRpt2 effector was not characterized; therefore, it was unclear whether the SA-dependent ethylene induction is part of the PTI or ETI.

The Battle between *Pst* and Arabidopsis in Controlling Ethylene Biosynthesis during Plant Immunity

Ethylene induction during plant immunity is dependent on pathogen perception via pattern recognition receptors and R proteins, downstream signaling pathways such as the MPK3/MPK6 cascade, transcription factors involved in *ACS* gene activation, and phosphorylation stabilization of ACS proteins by MPK3/MPK6 and, possibly, CPKs (Fig. 11; Chae and Kieber, 2005; Xu and Zhang, 2014, 2015a). In addition, ethylene biosynthesis is targeted by *Pst* effectors, either directly or indirectly. Within the first 3 h of inoculation, the rates of ethylene production induced by *Pst*, *Pst-hrcC*$^-$, and *Pst-avrRpt2* were similar (Fig. 1B), which is a part of the PTI in response to all three *Pst* strains. After that, *Pst*-induced ethylene biosynthesis decreases in comparison with that induced by *Pst-hrcC*$^-$, suggesting that PAMP-induced ethylene biosynthesis is suppressed by effectors delivered into plant cells. In contrast, *Pst-avrRpt2* induces higher ethylene production as a result of ETI (Figs. 1B and 2). At this stage, the exact mechanism underlying the effector-mediated suppression of ethylene production is unclear. We can exclude a direct suppression of the pathogen-responsive MPK3/MPK6 in this case, because MPK3/MPK6 activation in seedlings inoculated with *Pst* is comparable to that inoculated with *Pst-hrcC*$^-$ (Fig. 4; Supplemental Fig. S3A), suggesting that *Pst* lacks effectors that negatively impact the activation of the MPK3/MPK6 cascade, a key signaling pathway in ethylene induction. We also observed high-level *ACS2* and *ACS6* gene activation in response to *Pst* inoculation in Arabidopsis (Fig. 7, −SA groups), which, together with MPK3/MPK6 activation, should have led to an increase in ACS activity and higher ethylene production. However, there is little/no contribution from *ACS2* and *ACS6* to *Pst*-induced ethylene production (Fig. 6, −SA groups), suggesting that *Pst* effectors might target these two ACS isoforms and impede their function directly or indirectly.

In SA-pretreated seedlings, *Pst*-induced ethylene biosynthesis is much higher than that induced by *Pst-hrcC*$^-$ (Fig. 1, C and D), suggesting that SA not only can reverse the suppression of *Pst* effectors but also can enhance ethylene biosynthesis beyond the maximal level that can be induced by PAMPs. It is likely that SA pretreatment induces additional sensing/signaling pathways that are otherwise absent in plant cells without SA pretreatment. These induced pathways can recognize additional PAMPs, DAMPs, and/or effectors upon bacterial infection. This speculation is consistent with the finding that flg22 treatment of SA-pretreated seedlings resulted in little increase in ethylene biosynthesis (Supplemental Fig. S7), indicating that SA does not elevate PAMP-triggered responses in general but rather may promote the recognition of additional PAMPs, DAMPs, and/or effectors, thus leading to more robust defense responses. Supporting this speculation, it was recently reported that SA affects Arabidopsis microbial pattern receptor kinase levels and signaling (Tateda et al., 2014).

Potentiation of *Pst*-Induced Ethylene Biosynthesis by SA Pretreatment in Plant Immunity

Plant immunity involves almost all plant hormones. Besides SA, ethylene, and JA, the three well-recognized

stress/defense hormones, other plant hormones, including abscisic acid, GAs, auxins, cytokinins, and brassinosteroids, can all modulate plant immunity (for review, see Spoel and Dong, 2008; Pieterse et al., 2012; Kazan and Lyons, 2014). They function as secondary signaling molecules downstream of plant sensing of PAMPs, DAMPs, or effectors and can influence plant defense responses either positively or negatively (Glazebrook, 2005; Broekaert et al., 2006; van Loon et al., 2006; Pieterse et al., 2012). An increasing number of pathogen-derived effectors have been found to target/manipulate plant hormone biosynthesis or signaling to facilitate the pathogenesis process, illustrating the importance of plant hormones in plant immunity (Kazan and Lyons, 2014). Cross talk between different hormones, either antagonistically and synergistically, provides another layer of regulation to fine-tune plant immune responses to ward off pathogens.

Pretreatment of Arabidopsis with SA potentiates ethylene biosynthesis in plant immunity, revealing a novel cross talk between SA and ethylene. These enhanced ethylene levels might be important to not only ethylene-regulated defense gene expression and pathogen resistance but also to the maintenance of a balanced SA signaling. It was reported that loss of function of ethylene signaling leads to a higher level of SA biosynthesis and response (Chen et al., 2009). Loss of function of *MPK3/MPK6* compromises the potentiation effect of SA pretreatment (Fig. 5; Supplemental Fig. S4). MPK3/MPK6 involvement in the process is likely a result of their regulation of ACS2 and ACS6, two major ACS isoforms contributing to the SA-potentiated ethylene induction (Figs. 6 and 8). ACS2 and ACS6 are downstream substrates of MPK3/MPK6 (Liu and Zhang, 2004; Han et al., 2010). In addition, *ACS2* and *ACS6* are also regulated at the transcriptional level by the MPK3/MPK6 cascade (Li et al., 2012). In-gel kinase assays revealed that SA pretreatment potentiates MPK3/MPK6 activation after *Pst* and *Pst-avrRpt2* inoculation (Fig. 4; Supplemental Fig. S3). It is likely that enhanced phosphorylation stabilization of ACS2/ACS6 proteins by MPK3/MPK6, together with enhanced *ACS2/ACS6* gene activation (Fig. 7), results in the great enhancement of ethylene biosynthesis in Arabidopsis after SA pretreatment. Consistent with this, SA potentiation of ethylene induction after both *Pst* and *Pst-avrRpt2* inoculation is mostly dependent on *ACS2* and *ACS6*. In the *acs2 acs6* double mutant, more than 80% of the ethylene induction was blocked (Figs. 6 and 8).

Differential Regulation and Contribution of ACS Isoforms to Ethylene Biosynthesis in Plant Immunity

Based on mutant analysis, at least three isoforms of ACS, ACS2, ACS6, and ACS7, contribute to ethylene induction in plant immunity (Figs. 6 and 8). This conclusion is consistent with the activation of their gene expression in the process (Fig. 7). At this stage, whether ACS8 is also involved is hard to determine because of the lack of an *acs8* mutant and the ability of *Pst* to produce low levels of ethylene (Supplemental Fig. S5). Based on their regulation by different signaling pathways, these four ACS isoforms can be classified into three different groups. The first group includes ACS2 and ACS6, two type I ACS that are regulated by the MPK3/MPK6 signaling pathway (Liu and Zhang, 2004; Han et al., 2010; Li et al., 2012). They are the major contributors to SA-potentiated ethylene biosynthesis (Figs. 6 and 8, +SA groups). In addition, they are targeted by *Pst* effectors, which results in their minimal contribution to ethylene induction by *Pst* (Fig. 6, −SA groups), despite (1) the high activation of MPK3/MPK6 (Fig. 4; Supplemental Fig. S3), which should phosphorylate ACS2 and ACS6 and stabilize the protein, and (2) *ACS2/ACS6* gene activation (Fig. 7). Both should have led to their contribution to ethylene biosynthesis if they were not suppressed by *Pst* effectors. The second group includes ACS7, the sole member of type III ACS, which also contributes to the SA-potentiated ethylene induction (Figs. 6 and 8, +SA groups). The third group includes ACS8, a type II ACS. ACS8 might contribute to the low-level induction of ethylene by *Pst*, which is not responsive to SA pretreatment (Figs. 6 and 8, *acs2 acs6 acs4 acs5 acs9 acs7* sextuple and *acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11* octuple mutants with and without SA pretreatment). However, at least part, if not all, of this low-level ethylene is produced by *Pst* (Supplemental Fig. S5), which brings uncertainty to the involvement of *ACS8* (Fig. 11).

Ethylene Is a Positive Regulator of Plant Immunity against Bacterial Pathogens

Ethylene is involved in diverse biological processes, including plant innate immunity. Based on analyses of ethylene signaling mutants, it is concluded that ethylene plays a positive role in plant resistance against necrotrophic pathogens but negatively influences plant resistance against bacterial pathogens (Glazebrook, 2005; Broekaert et al., 2006; van Loon et al., 2006; Pieterse et al., 2012). For instance, overexpression of Arabidopsis *ETHYLENE RESPONSE FACTOR1*, a transcription factor in the ethylene signaling pathway, results in increased resistance to *B. cinerea* but reduces SA-mediated resistance to *Pst* (Berrocal-Lobo et al., 2002). Analysis of the *ein2* mutant revealed that ethylene signaling is not required for active resistance against bacterial pathogens but is involved in symptom development, and the lack of ethylene signaling makes plants more tolerant to the bacterial pathogens (Bent et al., 1992). In addition, *EIN3* and *EIN3-LIKE1* (*EIL1*) negatively impact the expression of *SID2* and, therefore, SA biosynthesis and plant resistance against bacterial pathogens (Chen et al., 2009). However, recent studies also concluded that ethylene signaling is essential to the expression of genes encoding pattern-recognition receptors or DAMPs, such as *ELICITOR*

PEPTIDE PRECURSORs, and therefore has a positive role in plant immunity against bacterial pathogens (Boutrot et al., 2010; Mersmann et al., 2010; Liu et al., 2013; Tintor et al., 2013; Zipfel, 2013). Furthermore, gene expression profiling analyses revealed that SA, JA, and ethylene all contribute positively to immunity to both biotrophic and necrotrophic pathogens (Glazebrook et al., 2003; Tsuda et al., 2009; Sato et al., 2010).

In this report, we conclude that ethylene plays a positive role in both PTI and ETI during plant immunity against bacterial pathogens based on an analysis of ethylene biosynthetic mutants. How to reconcile these different conclusions about the role of ethylene in plant bacterial resistance? One possible explanation is that key ethylene signaling components such as EIN2 and EIN3/EIL1 are critical to the functions of other plant hormones because of cross talk (Anderson et al., 2004; Chen et al., 2009; Pieterse et al., 2012). Their mutation may interfere with the functions of other plant hormones in plant immunity. For instance, loss of key ethylene signaling components, such as in the *ein3 eil1* double mutant, leads to unchecked SA biosynthesis and/or signaling pathways. The exaggerated SA signaling leads to an enhanced resistance response in ethylene signaling mutants, giving the impression that ethylene has a negative role in plant bacterial resistance (Chen et al., 2009). The use of ethylene biosynthetic mutants in this study should alleviate this problem. Based on our results, ethylene plays a less important role in comparison with SA in plant basal resistance against *Pst*. The compromised resistance of the loss of ethylene biosynthetic mutants against *Pst* can be reversed by SA pretreatment (Fig. 10A), suggesting a dominant role of SA in the process. In contrast, SA pretreatment cannot mask the compromised resistance of ethylene biosynthesis mutants against *Pst-avrRpt2* (Fig. 10B). Previously, ethylene biosynthesis was shown to be critical to plant resistance against *B. cinerea*, a necrotrophic pathogen (Tsuchisaka et al., 2009; Li et al., 2012). Together with the findings in this report, we can conclude that ethylene plays an important role in plant resistance against both fungal and bacterial pathogens.

MATERIALS AND METHODS

Plant Growth Conditions and Treatments

Soil-grown Arabidopsis (*Arabidopsis thaliana*) plants were maintained at 22°C in a growth chamber with a 14-h light cycle (100 μE m^{-2} s^{-1}). For experiments, seeds were surface sterilized. After imbibition at 4°C for 3 to 5 d, seeds were sown in petri dishes with liquid one-half-strength MS medium and grown in a growth chamber at 22°C with continuous light (70 μE m^{-2} s^{-1}). Six-day-old seedlings were transferred to 20-mL GC vials with 6 mL of liquid one-half-strength MS medium (seven seedlings per vial) and maintained under the same growth conditions. Twelve-day-old seedlings grown in GC vials were pretreated with SA (100 μM final concentration) by the addition of 100 mM stock solution prepared in DMSO solvent. Two days later, seedlings were inoculated with *Pseudomonas syringae* pv *tomato DC3000* (*Pst*), *Pst-hrcC*$^-$, or *Pst-avrRpt2* or mock inoculated.

Pst strains were maintained as glycerol stocks at −80°C. For inoculum preparation, 20-μL stocks were spread on Difco Pseudomonas Agar F plates with appropriate antibiotics. After incubation at 28°C for approximately 24 h, bacteria were scraped off the plates and suspended in one-half-strength MS medium to OD_{600} of 1. *Pst* inoculation was simply done by adding 120 μL of inoculum to the GC vials with 6 mL of medium to a final OD_{600} of 0.02.

For experiments with multiple time points, at least two independent repetitions were performed. For single time point experiments, at least three independent repetitions were performed. Data from one of the independent repetitions with similar results are shown in the figures. Student's *t* test was used for statistical analysis of most of the data. One and two asterisks above the columns are used to indicate differences that are statistically significant ($P < 0.05$) and very significant ($P < 0.01$), respectively. When different high-order *acs* mutants were compared, one-way ANOVA was performed. Lowercase letters above the columns are used to indicate statistically different groups. When two or more time points are significantly different, we conclude that the two genotypes produce statistically different amounts of ethylene.

Mutant Lines and Generation of Transgenic Plants

Arabidopsis ecotype Col-0 was used as the wild-type control. *NahG*, *npr1*, and *sid2-2* seeds were kindly provided by Drs. Daniel Klessig and Xinnian Dong. DEX-inducible promoter-driven *avrRpt2* transgenic plants (*GVG-avrRpt2*) were obtained from Dr. Brian Staskawicz's laboratory (McNellis et al., 1998). Transfer DNA insertion mutant alleles of *MPK3* (At3g45640) and *MPK6* (At2g43790) were described previously (Liu and Zhang, 2004; Wang et al., 2007). High-order *acs* mutants generated in Dr. Athanasios Theologis's laboratory (Tsuchisaka et al., 2009) were obtained from the Arabidopsis Biological Resource Center. The stock numbers are CS16564 (*acs2*), CS16569 (*acs6*), CS16581 (*acs2 acs6*), CS16644 (*acs2 acs6 acs4 acs5 acs9*), CS16649 (*acs2 acs6 acs4 acs5 acs9 acs7*), and CS16651 (*acs1 acs2 acs6 acs4 acs5 acs9 acs7 acs11*). The chemical genetically rescued *mpk3 mpk6* double mutant (*MPK6SR*) was generated by using an NA-PP1 inhibitor-sensitized MPK6 variant, $MPK6^{YG}$, driven by the *MPK6* native promoter (P_{MPK6}:$MPK6^{YG}$). In the absence of NA-PP1 inhibitor, the functional P_{MPK6}:$MPK6^{YG}$ is able to rescue the *mpk3 mpk6* double mutant, resulting in plants with the *mpk3 mpk6* P_{MPK6}:$MPK6^{YG}$ genotype (named *MPK6SR* plants; Xu et al., 2014). Application of the NA-PP1 inhibitor (final concentration of 5 μM) can effectively inhibit the activity of $MPK6^{YG}$, giving rise to the activity null mutants of both MPK3 and MPK6. Two independent transgenic lines were used, and similar results were obtained.

Ethylene Measurement

GC vials with Arabidopsis seedlings were flushed and capped immediately after treatment. At the indicated times, ethylene levels in the headspace of the GC vials were measured by GC as described previously (Liu and Zhang, 2004; Han et al., 2010). Ethylene production rates were calculated as the average rates of ethylene production in the intervals of the two adjacent time points. Seedlings were then collected, weighed, frozen in liquid nitrogen, and stored at −80°C for future analysis.

Protein Extraction and In-Gel Kinase Assay

Total proteins were extracted from treated seedlings as described previously (Liu and Zhang, 2004). The concentration of protein was determined by using the Bio-Rad protein assay kit with bovine serum albumin as the standard. MBP was used as the substrate for the in gel-kinase assay (Zhang and Klessig, 1997; Liu and Zhang, 2004).

RNA Extraction and Real-Time PCR Analysis

Total RNA was extracted using the Trizol reagent (Invitrogen). After DNase treatment, RNA (1 μg) was used for reverse transcription. Real-time PCR analysis was performed using an Opticon 2 real-time PCR machine as described previously (Ren et al., 2008). The transcript of the *EF1α* gene was used to normalize the samples. Gene expression was calculated as relative levels to that of the *EF1α* gene in the same sample. The primers used for real-time PCR were *PR1* (At2g14610; 5′-CATACACTCTGGTGGGCCTTA-3′ and 5′-AGTGACCACAAACTCCATTGC-3′), *ACS2* (At1g01480; 5′-GGATGGTTTAGGATTTGCTTTG-3′ and 5′-GCACTCTTGTTCTGGATTACCTG-3′), *ACS6* (At4g11280; 5′-GTTCCAACCCCTTATTATCC-3′ and 5′-CCGTAATCTTGAACCCATTA-3′), *ACS7* (At4g26200; 5′-ACGGTACGATACCATTGTGGA-3′ and 5′-GCTCGCCGTCTTTAGTTTTCT-3′), *ACS8* (At4g37770; 5′-CCTTCCTTCCTTCAAGAATGC-3′ and 5′-GAGAGTCTCGTTAGCCGGAGT-3′), and *EF1α* (At5g60390;

5′-TGAGCACGCTCTTCTTGCTTTCA-3′ and 5′-GGTGGTGGCATCCATCTTGTTACA-3′).

Pathogen Resistance Assay

Five-week-old Col-0 and *acs* mutant plants grown under a short-day light cycle (10 h of light and 14 h of dark) were infiltrated with *Pst* or *Pst-avrRpt2* (OD_{600} = 0.001). Eight leaves were collected 3 d post inoculation for quantification of bacterial growth. SA pretreatment was done by spraying the plants with SA (1 mM) and drenching soil with SA (1 mM) at the same time (Lawton et al., 1994; DeFraia et al., 2010). The flats were then covered with a dome for 24 h before bacterial infiltration. SA solution was prepared in water for drenching and in 0.01% (v/v) Silwet L-77 for spraying. The pH of the SA solutions was adjusted to 6.5 with KOH.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *MPK3* (At3g45640), *MPK6* (At2g43790), *EF1α* (At5g60390), *ACS1* (At3g61510), *ACS2* (At1g01480), *ACS4* (At2g22810), *ACS5* (At5g65800), *ACS6* (At4g11280), *ACS7* (At4g26200), *ACS8* (At4g37770), *ACS9* (At3g49700), and *ACS11* (At4g08040).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Validation of SA responsiveness in liquid-cultured Arabidopsis seedlings.

Supplemental Figure S2. SA treatment weakly induces ethylene biosynthesis in Arabidopsis.

Supplemental Figure S3. Enhanced activation of MPK3 and MPK6 by *Pst* in SA-pretreated Arabidopsis.

Supplemental Figure S4. MPK3 and MPK6 are required for SA-potentiated ethylene biosynthesis in Arabidopsis inoculated with *Pst*.

Supplemental Figure S5. Low-level ethylene production by *Pst*.

Supplemental Figure S6. Potentiation of *Pst*-induced ethylene biosynthesis by SA pretreatment is dependent on the function *sid2-2*.

Supplemental Figure S7. Weak potentiation of flg22-induced ethylene biosynthesis by SA pretreatment in Arabidopsis.

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