Altering Expression of *Benzoic Acid/Salicylic Acid Carboxyl Methyltransferase 1* Compromises Systemic Acquired Resistance and PAMP-Triggered Immunity in *Arabidopsis*

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Methyl salicylate (MeSA), which is synthesized in plants from salicylic acid (SA) by methyltransferases, has roles in defense against microbial and insect pests. Most of the MeSA that accumulates after pathogen attack is synthesized by benzoic acid/SA carboxyl methyltransferase 1 (AtBSMT1). To investigate the role of AtBSMT1 in plant defense, transgenic *Arabidopsis* with altered AtBSMT1 function or expression were assessed for their ability to resist pathogen infection. A knockout mutant (*Atbsmt1*) failed to accumulate MeSA following pathogen infection; these plants also failed to accumulate SA or its glucoside in the uninoculated leaves and did not develop systemic acquired resistance (SAR). However, the *Atbsmt1* mutant exhibited normal levels of effector-triggered immunity and pathogen-associated molecular pattern (PAMP)-triggered immunity to *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*. Analyses of transgenic *Arabidopsis* plants overexpressing AtBSMT1 revealed that they accumulate elevated levels of MeSA in pathogen-infected leaves but fail to develop SAR. Since the levels of SA and its glucoside were reduced in uninoculated systemic leaves of these plants whereas MeSA levels were elevated, AtBSMT1-mediated conversion of SA to MeSA probably compromised SAR development by suppressing SA accumulation in uninoculated leaves. PAMP-triggered immunity also was compromised in the AtBSMT1 overexpressing plants, although effector-triggered immunity was not.

Plants utilize multiple levels of inducible defenses for protection against microbial pathogens. A low-level resistance termed pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI, formerly called basal resistance) is activated when cell surface receptors recognize molecules unique to microbes (Chisholm et al. 2006; Jones and Dangl 2006). Some microbes have evolved effectors to suppress PTI; in turn, plants have evolved resistance (*R*) gene-encoded proteins that recognize either a cognate pathogen-derived effector or an effector-mediated alteration to a host target. This recognition results in rapid and robust activation of the plant's defense

responses, conferring a strong resistance to pathogen infection termed effector-triggered immunity (ETI, formerly called *R* gene-mediated resistance). ETI is associated with increased production of reactive oxygen species, nitric oxide, and SA, as well as a hypersensitive response (HR) that is manifested by programmed host cell death at the site or sites of infection (Greenberg and Yao 2004). In addition, a heightened state of resistance and defense is activated in the uninoculated portions of the plant. This resistance, termed systemic acquired resistance (SAR), is SA-dependent, long-lasting, and provides protection against a broad spectrum of pathogens (Vlot et al. 2008a). Although a HR is not strictly required for induction of SAR (Cameron et al. 1994; Mishina and Zeier 2007), SAR signaling generally has been studied in distal systemic tissue of plants undergoing a HR (Vlot et al. 2008a).

Activation of SAR requires production of a mobile signal in the infected tissue and its translocation through the phloem to distal, uninoculated tissue. Multiple mobile SAR signals may be involved. Several studies have implicated one or more lipid-based or -derived signals in *Arabidopsis*. A mutation in the lipid-transfer protein DIR1 compromises *Arabidopsis*' ability to either generate, transmit, or both an SAR signal but does not affect local resistance (Maldonado et al. 2002). Another potential lipid-derived signal is jasmonic acid or a derivative of it, which has been suggested to be an early signal for SAR development (Truman et al. 2007). Other studies argue for the involvement of a glycerolipid-derived SAR signal, based on analyses of mutants with altered plastid lipid and fatty acid metabolism (Chaturvedi et al. 2008; Nandi et al. 2004). Recently, azelaic acid, a nine-carbon dicarboxylic acid, was identified as a phloem-mobile SAR signal that primes plants to accumulate SA upon subsequent infection (Jung et al. 2009).

In tobacco, previous studies have implicated methyl salicylate (MeSA) as a SAR signal (Park et al. 2007). This discovery was based on quantification of MeSA and SA levels in plants resisting infection by *Tobacco mosaic virus* (TMV), along with characterization of grafted tobacco plants in which the genes responsible for MeSA biosynthesis (*SA methyltransferase*, *NtSAMT1*) or MeSA cleavage to SA (*SA-binding protein 2*, *SABP2*) were silenced via RNAi technology. Analysis of these chimeric tobacco plants further indicated that NtSAMT1 is required only in the primary (1°) infected leaves, in which the SAR signal is produced, while SABP2's MeSA esterase activity is needed only in the uninoculated distal leaves, in which the SAR signal is perceived and processed. Additionally, SABP2's esterase activity must be inhibited in the 1° infected

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tissue (by SA binding in the active site) or SAR fails to develop (Forouhar et al. 2005; Park et al. 2007). Subsequent studies in *Arabidopsis* identified an 18-member methyl esterase family. Five members of this family encode proteins exhibiting MeSA esterase activity in vitro; reduced expression of these five family members via knockout (KO) mutation or RNAi-mediated silencing compromised SAR development and correlated with enhanced MeSA accumulation in the uninoculated tissue of SAR-induced plants (Vlot et al. 2008b). Recently, the synthetic SA analog 2,2,2,2'-tetrafluoroacetophenone, which inhibits the MeSA esterase activity of SABP2 and its *Arabidopsis* orthologs, was used to confirm the importance of these enzymes and MeSA for SAR development (Park et al. 2009). In planta analyses using this inhibitor in conjunction with leaf detachment assays and MeSA quantification also revealed the kinetics with which the MeSA/SAR signal is produced in 1° infected tissue, transmitted through the phloem, and processed in the distal healthy tissue in TMV-infected tobacco.

MeSA is synthesized in plants by enzymes that transfer the methyl group from *S*-adenosyl-L-methionine to the carboxyl group of SA or benzoic acid (BA) or both. These SA carboxyl methyltransferase (SAMT) and BA carboxyl methyltransferases belong to a structurally related group of methyltransferases termed the SABATH family (D'Auria et al. 2003). *Arabidopsis thaliana* contains 24 intact SABATH members, two of which have been implicated in plant defense response. AtJMT1 (Atlg19640) methylates jasmonic acid (Seo et al. 2001). Expression of this gene is induced locally and systemically by wounding or methyl jasmonate treatment, and overexpression of AtJMT1 resulted in enhanced resistance to virulent *Botrytis cinerea*. By contrast, AtBSMT1 (At3g11480) utilizes both SA and BA as methyl acceptors (Chen et al. 2003). AtBSMT1 is constitutively expressed in leaves, stems, and floral tissue, although its expression in leaves can be further induced by wounding, herbivory, methyl jasmonate, or alamethicin, a channel-forming peptide that mimics pathogen attack. Characterization of a T-DNA KO mutant in this gene (*Atbsmt1*; SAIL_766_B10) suggested that AtBSMT1 is responsible for increased biosynthesis of MeSA after attack by insects (M. Dicke, *personal communication*).

To further investigate the role of AtBSMT1 in SAR signaling, we monitored the responses of an *Atbsmt1* KO mutant and *AtBSMT1*-overexpressing plants to infection with virulent and avirulent strains of bacterial and oomycete pathogens. Unexpectedly, SAR was compromised regardless of whether *AtBSMT1* was constitutively overexpressed or its function knocked out. By contrast, PTI and ETI were affected differently by the loss or overexpression of *AtBSMT1*. How these results relate to our model for SAR signaling is discussed.

RESULTS

SAR is compromised in *Atbsmt1*.

To assess the roles of AtBSMT1 and MeSA in plant disease resistance, a T-DNA insertion line, SAIL_766_B10 in the Columbia-0 (Col-0) ecotype (*Atbsmt1*), was characterized. Sequencing the mutated gene and its corresponding mRNA indicated that the insertion caused a deletion of 328 nt spanning 91 nt from the 3' end of intron 2 and 237 nt from the 5' end of exon 3. These nucleotides were replaced with a similar number of nucleotides from the T-DNA (Supplemental Fig. S1A). The insertion also led to use of a novel 3' splice site that removed an additional 36 nt from the 5' end of exon 3. Since the resultant 91-amino acid deletion in the mutant protein spans all residues involved in *S*-adenosyl-L-methionine binding (Zubieta et al. 2003), it is very unlikely that this protein has methyl transferase activity.

To determine whether MeSA levels are altered in *Atbsmt1* plants, inoculated leaves were collected at various times after infection with coronatine-deficient avirulent *Pseudomonas syringae* pv. *maculicola* carrying *AvrRpt2* (*P. syringae* pv. *maculicola* *AvrRpt2 cor*⁻), and gas chromatography-mass spectrometry (GC-MS) analysis was performed. MeSA levels were only slightly, if at all, lower in *Atbsmt1* plants than in wild-type (Wt) Col-0 plants before infection. However, while MeSA levels in the inoculated leaves of Wt plants rose steadily, displaying an approximately threefold increase by 24 h post-primary infection (hplⁱ), MeSA levels in *Atbsmt1* increased only slightly during the timecourse (Fig. 1A).

Whether SAR is altered in *Atbsmt1* plants was then assessed. *Atbsmt1* and Wt plants were induced for SAR by infecting three leaves with *P. syringae* pv. *maculicola* *AvrRpt2 cor*⁻ and, 2 to 3 days later, challenging the uninoculated leaves with virulent *P. syringae* pv. *tomato* DC3000. Wt plants that received this treatment developed SAR, as indicated by the 10- to 100-fold reduction in virulent *P. syringae* pv. *tomato* DC3000 growth, as compared with Wt plants that received a 1° mock inoculation (Fig. 1D). In contrast, SAR was compromised in *Atbsmt1* plants, since virulent *P. syringae* pv. *tomato* DC3000 growth was not significantly lower in SAR-induced plants compared with mock-inoculated plants. Development of SAR was also partially compromised in *med4-1*, in which several of the MeSA esterase-encoding genes were silenced via RNAi technology (Vlot et al. 2008b).

MeSA is hypothesized to signal SAR by moving from the inoculated to the uninoculated tissue, in which it is hydrolyzed by MeSA esterases to generate SA. Thus, we tested whether SA levels are reduced in the uninfected tissue of *P. syringae* pv. *maculicola* *AvrRpt2 cor*⁻-infected *Atbsmt1* as compared with Wt plants. Both SA and SA 0- β -glucoside (SAG) levels were reduced in the uninfected leaves of *Atbsmt1*, as compared with Wt plants; by 60 hplⁱ, SA levels were reduced approximately twofold and SAG levels were depressed approximately fivefold (Fig. 1B and C).

To rule out the possibility that the impact of AtBSMT1 on SAR is due to methylation of compounds other than SA, MeSA infiltration experiments were performed. MeSA infiltration into lower leaves resulted in elevated levels of SA and SAG in the noninfiltrated leaves of *Atbsmt1* and induced SAR in the untreated leaves of *Atbsmt1* and Wt plants (Fig. 1E through G). These results support the argument that the role of AtBSMT1 in SAR is to convert SA to MeSA. To assess whether AtBSMT1 was required in the 1° infected leaves, which produce the mobile SAR signal, or in the distal leaves, which perceive and process the signal, or in both infected and distal leaves, phloem or petiole exudates from 1° infected Wt or *Atbsmt1* leaves were infiltrated into Wt or *Atbsmt1* leaves of uninfected plants 60 h before challenge with virulent *P. syringae* pv. *tomato* DC3000. Exudates from Wt, but not from *Atbsmt1*, plants induced enhanced resistance in both Wt and *Atbsmt1* plants, indicating that AtBSMT1 is required only for SAR signal production in the 1° infected tissue (Fig. 1H).

Atbsmt1 is not compromised for PTI or ETI.

To assess whether AtBSMT1 plays a role in other forms of resistance such as PTI or ETI, *Atbsmt1* was challenged with virulent or avirulent isolates of *P. syringae* pv. *tomato* DC3000 or *Hyaloperonospora arabidopsidis*. *Atbsmt1* and Wt plants showed similar levels of pathogen growth and disease-symptom development following infection by virulent strains *P. syringae* pv. *tomato* DC3000 (Fig. 2A and B) or *H. arabidopsidis* Noco-2 (Fig. 2C). Resistance to avirulent strains *P. syringae* pv. *tomato* DC3000 *AvrRpt2* (Fig. 2D) or *H. arabidopsidis* *Emwa-1* (Fig. 2E) also was comparable in *Atbsmt1* and Wt

plants. Therefore, the role or roles of AtBSMT1 in PTI or ETI appears to be minimal; alternatively, its loss may be masked by other functionally redundant methyltransferases.

Overexpression of AtBSMT1 compromises SAR.

In a complementary set of experiments, the effect of AtBSMT1 overexpression on disease resistance was determined using transgenic plants expressing AtBSMT1 under control of the constitutive *Cauliflower mosaic virus* 35S promoter. 35S::AtBSMT1 transgenic plants accumulated elevated, although vari-

able, levels of the transgene mRNA (Fig. 3A) and contained elevated levels of MeSA in their inoculated leaves prior to and following infection with *P. syringae* pv. *maculicola* AvrRpt2 *cor* (Fig. 3B). Analysis of pathogen growth in SAR-induced 35S::AtBSMT1 and Wt plants revealed that SAR was compromised by AtBSMT1 overexpression. Like *med4-1* plants, 35S::AtBSMT1 transgenics displayed similar levels of virulent *P. syringae* pv. *tomato* DC3000 growth in the secondary (2°) inoculated leaves, regardless of whether these plants previously received a SAR-inducing 1° infection with *P. syringae* pv. *macu-*

Fig. 1. Characterization of the *Atbsmt1* knockout (KO) mutant. Kinetics of **A**, methyl salicylate (MeSA), **B**, salicylic acid (SA), and **C**, SA β -glucoside (SAG) accumulation after infection with coronatine-deficient *Pseudomonas syringae* pv. *maculicola* carrying *AvrRpt2* (*Psm AvrRpt2 cor⁻*) in *Atbsmt1* and wild-type (Wt) Col-0. MeSA content was quantified in primary (1°) infected leaves of 3- to 4-week-old plants at the indicated hours post-primary infection (hpi). SA and SAG content was quantified in the uninoculated leaves of 3- to 4-week-old plants at the indicated times. All experiments illustrated in **C** through **E** were done twice with the similar results and mean $\pm$ standard deviation (SD) values are presented. White and black bars represent Wt and *Atbsmt1* plants, respectively. Asterisks indicate statistically significant differences (* = $P < 0.05$, ** = $P < 0.01$, Student *t*-test) between levels in *Atbsmt1* KO to those in Wt Col-0 for each timepoint. **D**, *Atbsmt1* KO is compromised for development of systemic acquired resistance (SAR). At 2 to 3 days post-primary infection with $MgCl_2$ (mock, black bars) or *P. syringae* pv. *maculicola* *AvrRpt2 cor⁻* (SAR induction, striped bars), two or three uninoculated leaves were challenged with virulent *P. syringae* pv. *tomato* DC3000. *P. syringae* pv. *tomato* growth was determined at 3 days post-secondary infection (dpi). The *med4-1* transgenic plants, in which multiple MeSA esterases are silenced, were only partially compromised for SAR in the experiment shown but, in three other experiments, were completely SAR-defective and served as a negative control. The mean $\pm$ SD values are presented on a logarithmic scale. All four independent experiments gave similar results for the *Atbsmt1* KO, showing compromised SAR development with no significant difference in *P. syringae* pv. *tomato* growth on mock- versus *P. syringae* pv. *maculicola* *AvrRpt2 cor⁻*-infected plants. Asterisks indicate statistically significant differences (* = $P < 0.05$, ** = $P < 0.01$, Student *t*-test) between *P. syringae* pv. *tomato* growth in *P. syringae* pv. *maculicola* *AvrRpt2 cor⁻* to that in the control (Mock) for each genotype. **E** through **G**, Infiltration of MeSA at the rate of 1 mg/liter (6.6 μ M) into lower leaves induced elevated SA and SAG levels at 30 h postinfiltration in the untreated leaves of *Atbsmt1* plants and induced enhanced resistance to subsequent challenge by *P. syringae* pv. *tomato* 3 days after treatment. A different letter (in **E** and **F**) indicates statistically significant difference ($P < 0.05$, Student *t*-test) among levels for all treatments and genotypes, while asterisks in **G** indicate statistically significant differences (* = $P < 0.05$, ** = $P < 0.01$, Student *t*-test) between *P. syringae* pv. *tomato* growth in *P. syringae* pv. *maculicola* *AvrRpt2 cor⁻* to that in the control (Mock) or between plants treated with MeSA at 0.5 or 1 mg/liter versus MeSA at 0 mg/liter (control) for each genotype. **H**, Petiole exudates were collected from Wt and *Atbsmt1* leaves 12 to 60 h after mock infection with $MgCl_2$ buffer (PEX-M) or infection with *P. syringae* pv. *maculicola* *AvrRpt2 cor⁻* (PEX-I). The exudates were infiltrated into three leaves of uninfected Wt or *Atbsmt1* plants. After 60 h, two noninfiltrated leaves of these plants were challenged with *P. syringae* pv. *tomato* and were assayed for growth of this virulent pathogen 2 dpi. Asterisks indicate statistically significant differences (* = $P < 0.05$, ** = $P < 0.01$, Student *t*-test) between *P. syringae* pv. *tomato* growth in plants treated with PEX-I versus PEX-M (control) for each donor treatment. Experiments shown in **E** through **H** were done twice with similar results.

Fig. 2. Pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) in response to virulent and avirulent pathogens in wild-type (Wt), *Atbsmt1*, and 35S::AtBSMT1 plants. **A**, PTI was analyzed in *Pseudomonas syringae* pv. *tomato* (*Pst*)-inoculated leaves of 4- to 5-week-old plants at 0, 2, and 3 days postinoculation (dpi) with virulent *P. syringae* pv. *tomato*. **B**, Leaves of representative *P. syringae* pv. *tomato*-inoculated plants showing disease symptoms at 5 dpi. **D**, ETI triggered by *P. syringae* pv. *tomato* *AvrRpt2* (*Pst AvrRpt2*) was analyzed as in **A**. White, black, and striped bars represent Wt, *Atbsmt1*, and 35S::AtBSMT1 plants, respectively. **C**, Virulent *Hyaloperonospora arabidopsidis* isolate Noco-2 and **E**, avirulent *Emwa-1* isolate (1×10^6 spores/ml) were inoculated on approximately 2-week-old seedlings of Wt, *Atbsmt1*, and 35S::AtBSMT1 plants. The number of sporangio-phores on individual leaves was counted at 10 dpi. Leaves were categorized into one of six groups based on the number of sporangio-phores per leaf (Group 1, 0 sporangio-phores per leaf, Group 2, 1 to 5, Group 3, 6 to 10, Group 4, 11 to 15, Group 5, 16 to 20, or Group 6, >20). The percentage of leaves in each group in a total 100% is presented. **A** through **E**, Two independent experiments were done with similar results. Mean $\pm$ standard deviation values are presented. Asterisks indicate statistically significant differences (* = $P < 0.05$, ** = $P < 0.01$, Student *t*-test) between *P. syringae* pv. *tomato* growth (or *H. arabidopsidis* growth) in *Atbsmt1* KO or in 35S::AtBSMT1 compared with that in Wt Col-0 at each timepoint.

licola AvrRpt2 cor or a 1° inoculation with buffer (Fig. 3C). Consistent with the loss of SAR, SA and SAG levels in the uninoculated leaves of 35S::AtBSMT1 transgenic plants accumulated to lower levels than in comparable Wt plants, both at the time of inoculation and in the hours following *P. syringae* pv. *maculicola AvrRpt2 cor* infection (Fig. 3D and E). By contrast, MeSA levels in the uninoculated leaves of these plants were substantially greater than those in Wt plants (Fig. 3F)

Overexpression of AtBSMT1 compromises PTI but not ETI.

To determine whether overexpression of AtBSMT1 affected PTI or ETI, 35S::AtBSMT1 plants were inoculated with virulent or avirulent *P. syringae* pv. *tomato* DC3000 or *H. arabidopsidis*. 35S::AtBSMT1 showed increased susceptibility to virulent *P. syringae* pv. *tomato* DC3000 (Fig. 2A and B) and virulent *H. arabidopsidis* Noco-2 (Fig. 2C) as compared with

Wt (or *Atbsmt1*) plants. In contrast, resistance to avirulent *P. syringae* pv. *tomato* DC3000 *AvrRpt2* (Fig. 2D) and *H. arabidopsidis* *Emwa-1* (Fig. 2E) was similar in 35S::AtBSMT1 and Wt (or *Atbsmt1*) plants. Thus, overexpression of AtBSMT1 negatively affects PTI but has little or no effect on ETI.

To assess whether the different effect AtBSMT1 overexpression exerted on PTI versus ETI was associated with differing SA or SAG levels, their kinetics of accumulation were determined after bacterial infection (Fig. 4). SA and SAG levels were reduced in 35S::AtBSMT1 compared with Wt plants regardless of whether the bacterial pathogen was virulent or avirulent, while MeSA levels were elevated in 35S::AtBSMT1 compared with Wt before and after infection with *P. syringae* pv. *tomato* DC3000. However, 35S::AtBSMT1 inoculated with virulent *P. syringae* pv. *tomato* DC3000 displayed almost no increase in SA or SAG within the first 24 hpi, whereas 35S::AtBSMT1 infected with *P. syringae* pv. *tomato* DC3000 *AvrRpt2* or Wt

Fig. 3. A, Characterization of 35S::AtBSMT1 overexpressing plants. **A**, Overexpression of the AtBSMT1 gene in leaves of transgenic 35S::AtBSMT1. RNA was extracted from untreated leaves of wild-type (Wt) Col-0 and four individual 35S::AtBSMT1 plants and was used for semiquantitative reverse transcriptase-polymerase chain reaction of AtBSMT1. ACT2 (*At3g18780*) was used as a control. AtBSMT1 and ACT2 were amplified using 25 and 20 cycles, respectively. **B**, Kinetics of methyl salicylate (MeSA) accumulation in infected leaves of transgenic 35S::AtBSMT1. MeSA was quantified in the *Pseudomonas syringae* pv. *maculicola AvrRpt2 cor*-inoculated leaves of 3- to 4-week-old plants at the indicated times. Mean $\pm$ standard deviation (SD) values are presented. Two independent experiments were analyzed with similar results. **C**, Systemic acquired resistance (SAR) development is compromised in 35S::AtBSMT1 plants. At 2 to 3 days post-primary infection (dp1°i) with MgCl₂ (mock, black bars) or *P. syringae* pv. *maculicola AvrRpt2 cor* (SAR induction, striped bars), two or three uninoculated leaves were challenged with virulent *Pseudomonas syringae* pv. *tomato* (*Pst*), and its growth was determined at 3 dp2°i. SAR-defective *med4-1* was used as a negative control. The mean $\pm$ SD values are presented on a logarithmic scale. Three independent experiments were analyzed with similar results. **D**, Salicylic acid (SA), **E**, SA β -glucoside, and **F**, MeSA content were quantified in uninoculated leaves of 3- to 4-week-old plants at 0, 12, and 24 h post-primary infection with *P. syringae* pv. *maculicola AvrRpt2 cor*. White and striped bars represent Wt and 35S::AtBSMT1, respectively. The mean $\pm$ SD values are presented. Experiments were done twice with similar results. Asterisks in B, D, E, and F indicate statistically significant differences (*= $P < 0.05$, **= $P < 0.01$, Student *t*-test) between levels in 35S::AtBSMT1 versus Wt Col-0 for each timepoint, while asterisks in C indicate statistically significant differences (*= $P < 0.05$, **= $P < 0.01$, Student *t*-test) between *P. syringae* pv. *tomato* growth in *P. syringae* pv. *maculicola AvrRpt2 cor* versus the control (Mock) for each genotype.

plants inoculated with either *P. syringae* pv. *tomato* DC3000 or *P. syringae* pv. *tomato* DC3000 *AvrRpt2* exhibited increased levels of SA and SAG at this time. This lack of increase in SA and SAG may be responsible for the enhanced susceptibility of *35S::AtBSMT1* to *P. syringae* pv. *tomato* DC3000. In contrast, the early rise in SA (and SAG) levels in *35S::AtBSMT1* plants inoculated with *P. syringae* pv. *tomato* DC3000 *AvrRpt2* appears sufficient to activate normal resistance, even though SA levels are lower than those in Wt plants. Consistent with this result, *PR-1* gene expression was similarly induced in *35S::AtBSMT1* and Wt plants after infection with this avirulent bacteria (Fig. 4F).

DISCUSSION

The results from our previous studies in tobacco suggest the following model for SAR development. After infection with an avirulent pathogen, MeSA is produced in the inoculated leaves by a SAMT; this MeSA travels through the phloem as a SAR signal to the distal tissue. Once in the uninoculated leaves, MeSA is hydrolyzed by MeSA esterases (such as SABP2) to active SA, which then activates or primes defense responses leading to SAR (Park et al. 2007). In this paper, we utilized a KO mutant lacking *AtBSMT1* function and a *35S::AtBSMT1*-overexpressing line to demonstrate that *AtBSMT1* plays an

important role in MeSA synthesis and SAR development in *Arabidopsis*. This finding is consistent with our previous demonstration that tobacco plants silenced for expression of *NtSAMT1* are SAR defective (Park et al. 2007). It should be noted that MeSA levels in the *Atbsmt1* KO mutant, which encodes a nonfunctional *AtBSMT1* enzyme, were only slightly, if at all, reduced in comparison to those in Wt plants prior to infection. This result suggests that there are one or more other methyltransferases responsible for the synthesis of basal MeSA levels. However, since MeSA levels increased very little after infection in *Atbsmt1*, compared with an approximately threefold increase in Wt plants, *AtBSMT1* appears to be responsible for the majority of MeSA production induced by infection with an avirulent pathogen.

The apparent discrepancy in the amount of increase in MeSA levels in the 1° infected leaves (approximately 125 ng per gram of fresh weight [FW]; Fig. 1A) versus the increase in SA plus SAG levels in the distal leaves (approximately 900 ng per gram of FW; Fig. 1B and C) is likely due to two factors. First, MeSA is a “transient” component that is both translocated and converted to SA and eventually SAG. That is, SA and, particularly, SAG are end products of the translocated MeSA and they can build up and accumulate. Therefore, the level of MeSA in 1° infected leaves at any given time will not necessarily equal the increase in SA plus SAG in the distal

Fig. 4. Kinetics of **A**, salicylic acid, **B**, SA 0-β-glucoside, and **C**, methyl salicylate accumulation after infection with virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) or **D** and **E**, avirulent *P. syringae* pv. *tomato* *AvrRpt2* (*Pst AvrRpt2*) in 4- to 5-week-old wild-type (Wt) and *35S::AtBSMT1* plants. The mean ± standard deviation values are presented. **F**, Induction of *PR-1* gene expression by infection with *P. syringae* pv. *tomato* *AvrRpt2*. RNA was extracted at the times indicated and quantified by reverse transcriptase-polymerase chain reaction. *EIF4a* was used as an internal control. *PR-1* and *EIF4a* were amplified using 20 cycles. Two independent experiments were done with similar results. Asterisks indicate statistically significant differences (* = $P < 0.05$, ** = $P < 0.01$, Student *t*-test) between levels in *35S::AtBSMT1* versus Wt Col-0 for each timepoint.

tissue in which it is accumulating. Second, based on studies in tobacco and cucumber by Raskin and coworkers (Shulaev et al. 1995) and Métraux and coworkers (Mölder et al. 1996), it is likely that some of the SA in the distal tissue is synthesized there de novo. The increase in SA level in the distal tissue resulting from cleavage of translocated MeSA could induce synthesis of SA in this tissue.

If the primary function of AtBSMT1 in SAR is to generate the long-distance signal, knocking out its activity would not be expected to alter PTI or ETI, since they develop in the inoculated leaf. Consistent with this model, neither PTI nor ETI were altered in *Atbsmt1 Arabidopsis* following infection with a bacterial or oomycete pathogen. Similarly, ETI was unaffected in chimeric tobacco containing *NtSAMT1*-silenced scions, since comparably sized lesions formed on the TMV-infected Wt and *NtSAMT1*-silenced scion leaves of uninduced plants (Park et al. 2007).

Another prediction based on our model is that *Arabidopsis* overexpressing *AtBSMT1* would accumulate elevated levels of MeSA in the inoculated leaves, which could enhance SAR development in the uninoculated portions of the plant. The discovery that *35S::AtBSMT1* plants were SAR defective was therefore unanticipated. However, this result can be explained if excess levels of AtBSMT1 out-compete the MeSA esterases in the uninoculated leaves and thereby prevent conversion of the transported MeSA to active SA. Supporting this possibility, the uninoculated leaves of *35S::AtBSMT1* plants contained substantially lower levels of SA and SAG than Wt plants, both at the time of and following pathogen infection. Furthermore, MeSA levels in these leaves were much higher than those in Wt plants. Taken together, these results suggest that overexpression of *AtBSMT1* leads to sequestration of SA as MeSA, thereby preventing the accumulation of enough SA in the uninoculated leaves to activate or prime defense responses for SAR development.

Constitutive overexpression of *AtBSMT1* could similarly sequester SA into MeSA in the inoculated leaves, thereby preventing the activation of PTI and ETI. Indeed, *35S::AtBSMT1* plants displayed enhanced susceptibility to virulent pathogens, and they accumulated only basal levels of SA and SAG following infection. Analysis of *Arabidopsis* overexpressing *OsBSMT1*, the rice homolog of *AtBSMT1*, led to a similar conclusion (Koo et al. 2007); these plants failed to accumulate SA or SAG, accumulated elevated levels of MeSA, and displayed heightened susceptibility to virulent bacterial and fungal pathogens. Unlike PTI, ETI was unaltered in *35S::AtBSMT1* plants. Since infection with an avirulent pathogen induces a greater increase in SA than infection by a virulent pathogen, it is possible that only the stronger or more rapid increase in SA induced by *P. syringae* pv. *tomato* DC3000 *AvrRpt2* is able to exceed the ability of AtBSMT1 to sequester it; this residual SA could then signal ETI. In contrast, *Arabidopsis* plants overexpressing *OsBSMT1* were compromised for ETI triggered by a bacterial or fungal pathogen (Koo et al. 2007). There are several possible explanations for the discrepancy between our results, including differences in one or more of the following: i) the level of overexpression of the two transgenes, ii) the specific activity of the encoded enzymes, iii) plant maturity, or iv) growth conditions. Indeed, recent studies by Zeier and coworkers demonstrated that disease resistance and SAR are strongly dependent on light conditions (Griebel and Zeier 2008; Zeier et al. 2004).

A mounting body of evidence indicates that SAR development is a complex process that involves multiple factors, including one or more defense hormones like SA, the structural integrity of the cuticle in distal leaves (Xia et al. 2009), one or more environmental factors (Zeier et al. 2004), plant maturity (Cameron and Zaton 2004), and several mobile signals (Vlot et

al. 2008a). This complexity likely explains the discrepancy between our results and those obtained by Zeier's group (Attaran et al. 2009). Their claim that MeSA is not a mobile SAR signal in *Arabidopsis* is based on analysis of *Atbsmt1* KO mutants that developed SAR despite their inability to produce MeSA (Attaran et al. 2009). Supporting their conclusion, previous studies from their lab (Zeier et al. 2004) and others (Pieterse et al. 1996) have demonstrated that systemic resistance can be induced in an SA and *PR* gene-independent manner under certain conditions. However, given the complexity of SAR induction, an alternative possibility is that differences in experimental conditions affect whether MeSA is or is not required for SAR development. A major contributing factor may be developmental maturity of the plants at time of infection. Zeier's group used 6-week-old plants; our plants were approximately 3.5 weeks old, which means they were at a much younger developmental stage. While both groups grew the plants under short-day conditions in approximately 70% relative humidity, the light intensity we used was double that of theirs. Another difference is the SAR-inducing pathogen. They used virulent *P. syringae* pv. *maculicola*, while we used avirulent *P. syringae* pv. *maculicola* *AvrRpt2 cor*⁻. Further studies will be required to determine how these variables, either collectively or individually, affect SAR signaling.

In summary, our results, presented here and previously (Park et al. 2007, 2009; Vlot et al. 2008b), combined with those of Zeier and coworkers (Attaran et al. 2009) argue for multiple mobile signals for SAR. These signals may be redundant or they may act synergistically or, perhaps, antagonistically, depending on various factors, including the plant-pathogen system, developmental stage of the host, and environmental conditions. For example, there is ample evidence for a lipid-derived mobile signal for SAR (Chaturvedi et al. 2008; Maldonado et al. 2002; Nandi et al. 2004; Truman et al. 2007; Vlot et al. 2008a). Recently, Jung and colleagues (2009) reported that azelaic acid is one such signal. A major challenge will be to decipher how these multiple signals and other factors interact to form an integrated signaling network that effectively controls SAR development under varying abiotic and biotic conditions.

MATERIALS AND METHODS

Plant materials and growth.

All *Arabidopsis thaliana* plants are in the Colombia (Col-0) ecotype background, including the SAIL_776_B10 line (named *Atbsmt1 KO*) obtained from Torrey Mesa Institute and a transgenic line overexpressing *AtBSMT1* under the 35S promoter (named *35S::AtBSMT1*). Seeds for soil-grown plants were sown on autoclaved soil. After 3 days of prechilling at 4°C, the seeds were germinated and grown under i) a 16-h-light and 8-h-dark photoperiod (140 $\mu\text{E m}^{-2}\text{s}^{-1}$) at 22°C and 70% relative humidity (RH) for analyses of PTI and ETI to *P. syringae* pv. *tomato* DC3000 and ii) a 9-h-light and 15-h-dark photoperiod (140 $\mu\text{E m}^{-2}\text{s}^{-1}$) at 22°C and 70% RH for SAR induction by *P. syringae* pv. *maculicola* *AvrRpt2 cor*⁻ and PTI and ETI to *H. arabidopsidis*.

Pathogen experiments.

All bacterial pathogens used in this study were infected by infiltrating the abaxial leaf surface with a 1-ml syringe at the plant stage indicated below. For SAR assays, coronatine-deficient *Pseudomonas syringae* pv. *maculicola* carrying *AvrRpt2* at a concentration of 1×10^6 CFU/ml in 10 mM MgCl_2 was infiltrated into three leaves per plant to induce SAR in 3- to 4-week-old plants grown under a 9-h-light and 15-h-dark photoperiod (140 $\mu\text{E m}^{-2}\text{s}^{-1}$) at 22°C and 70% RH. Plants infiltrated with 10 mM MgCl_2 served as mock controls. At 2 to 3 days post-pri-

mary infection (dpi), two or three untreated and uninoculated distal leaves were challenged with virulent *Pseudomonas syringae* pv. *tomato* DC3000 at a density of 1×10^5 CFU/ml in 10 mM MgCl₂. *P. syringae* pv. *tomato* DC3000 growth was determined by shaking 4-mm leaf disks taken at 3 dpi in 10 mM MgCl₂ supplemented with 0.1 M sucrose at room temperature for 4 h. The resulting bacterial suspensions were serially diluted, and spots of 20 µl per dilution were grown on Kings' B medium with rifampicin at 20 µg/ml, kanamycin at 50 µg/ml, and 1.7% agar and were incubated at 28°C for 48 h, after which time, the number of colonies formed were counted.

For PTI and ETI to *P. syringae* pv. *tomato* DC3000, the avirulent (*P. syringae* pv. *tomato* DC3000 *AvrRpt2*) or virulent (*P. syringae* pv. *tomato* DC3000) strains were inoculated at a density of 1×10^5 CFU/ml in 10 mM MgCl₂ on 4- to 5-week-old plants grown under a 16-h-light and 8-h-dark photoperiod ($140 \mu\text{E m}^{-2}\text{s}^{-1}$) at 22°C and 70% RH. Three 6-mm leaf disks were randomly taken per sample from four to five individual plants; four samples were taken for each genotype at 0, 2, and 3 dpi. Bacterial growth was determined as described in the SAR assays above.

For PTI and ETI to the oomycete pathogen, the *Hyaloperonospora arabidopsidis* isolates were maintained as previously described (Cooley et al. 2000). *H. arabidopsidis* *Noco-2* and *Emwa-1* isolates (1×10^6 spores/ml) were spray-inoculated on approximately 2-week-old seedlings growing under a 9-h-light and 15-h-dark photoperiod ($140 \mu\text{E m}^{-2}\text{s}^{-1}$) at 22°C and 80% RH. After spraying with *H. arabidopsidis* isolates, all seedlings were moved to a growth chamber with a 9-h-light and 15-h-dark photoperiod ($140 \mu\text{E m}^{-2}\text{s}^{-1}$) at 16°C and 99% RH. The numbers of sporangioophores formed by 10 dpi were counted on individual leaves, using a dissecting microscope. Leaves were categorized into one of six groups based on the number of sporangioophores per individual leaf. For each genotype, sporangioophores were counted on three leaves for each of the 12 plants.

MeSA quantification.

MeSA contents in approximately 100 mg of tissues per sample were measured using GC-MS (CP-3800/Quadrupole-1200L system; Varian, Walnut Creek, CA, U.S.A.), as previously described (Park et al. 2007). MeSA, SA, and SAG quantification was done on plants grown under the same conditions and length of time as those used for the corresponding assessment of disease resistance. For each genotype and timepoint, three to four leaves per plant from approximately 20 plants grown under short-day conditions were used for MeSA quantification. For older (4- to 6-week-old) plants grown under long-day conditions, one to two leaves per plant from approximately four plants were used per timepoint for MeSA quantification, while three to four leaves per plant from approximately eight plants were used per timepoint for SA and SAG quantification.

SA and SAG quantification.

SA and SAG were extracted and quantified from approximately 350 mg of tissue per sample using high-performance liquid chromatography analysis on an ARH-601 organic acids column (100 mm $\times$ 6.5 mm; Transgenomic Inc., Omaha, NE, U.S.A.) run at 55°C in 0.01 N H₂SO₄ with a flow rate of 0.6 ml/min, as previously described (Park et al. 2007). For each genotype and timepoint, three to four leaves per plant from approximately 70 plants grown under short-day conditions were used.

Collection of petiole exudates.

Three- to four-week-old Wt Col-0 and *Atbsmt1* KO plants grown under a 9-h-light and 15-h-dark photoperiod ($140 \mu\text{E m}^{-2}\text{s}^{-1}$) at 22°C and 70% RH were infected with *P. syringae*

pv. *maculicola* *AvrRpt2 cor*⁻ at a concentration of 1×10^6 CFU/ml in 10 mM MgCl₂ to induce SAR. The collection of petiole exudates has been previously described by Maldonado and associates (2002) and Jung and associates (2009). Briefly, 10 infected leaves at 12 hpi with 10 mM MgCl₂ (mock) or *P. syringae* pv. *maculicola* *AvrRpt2 cor*⁻ were excised, were surface-sterilized with 50% ethanol, 0.0006% bleach for 10 s, and then, briefly raised in 1 mM EDTA (pH 8.0). The sterilized petioles were submerged in 500 µl of 1 mM EDTA containing carbenicillin and streptomycin at 50 µg/ml (Sigma, St. Louis) for 48 h. For each genotype and inoculation, two leaves per plant from 20 plants were used for the collection of petiole exudates. After the exudates were clarified by three rounds of centrifugation at 12,000 rpm for 15 min, 50 µl of clarified exudates were placed on the King's B medium with 1.7% agar without antibiotics at 28°C for 3 days, to verify that the collected petiole exudates were bacteria free. The bacteria-free exudates, which were stored at -80°C, were diluted fourfold and were then infiltrated into three leaves of the 3- to 4-week-old plants. After 60 h, two noninfiltrated distal leaves were challenged with the virulent *P. syringae* pv. *tomato* DC3000 for 2 days. Virulent bacterial growth was determined as described in the SAR assays above.

In planta MeSA assay.

MeSA was obtained from Sigma. Three leaves of 3- to 4-week-old Wt Col-0 and *Atbsmt1* KO plants grown under a 9-h-light and 15-h-dark photoperiod ($140 \mu\text{E m}^{-2}\text{s}^{-1}$) at 22°C and 70% RH were infiltrated, using a 1 ml syringe with concentrations of 0 mg/liter, 0.5 mg/liter (3.3 µM), and 1 mg/liter (6.6 µM) of MeSA, which was kept at 4°C. At 3 dpi, two untreated distal leaves were challenged with the virulent *P. syringae* pv. *tomato* DC3000 for 2 days. The growth of virulent bacteria was determined as described in the SAR assays above.

Reverse transcriptase-polymerase chain reaction (RT-PCR).

Total RNA was extracted from leaves, using Trizol reagent (Invitrogen Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. DNase-treated total RNA (2 µg) was used for reverse transcription with a Superscript II kit (Invitrogen). The RT product was subjected to semiquantitative PCR using *AtBSMT1* forward (5'-TATGGAGCTCCAGGTTCTT-3') and *AtBSMT1* reverse (5'-ACGACAAGACTGACAGTCGT-3') primers or *AtPR-1* forward (5'-GTAGGTGCTCTTGTTCTTC-3') and *AtPR-1* reverse (5'-CACATAATTCCACGAGGATC-3') primers. The conditions for semiquantitative PCR to amplify *AtBSMT1* were: one cycle at 94°C (4 min), one cycle at 80°C (2 min), touchdown cycles (94°C for 15 s, 67°C $\rightarrow$ 61°C for 15 s, and 72°C for 30 s, one cycle for each temperature between 67 and 61°C), and 25 cycles at 94°C (15 s), 60°C (15 s), and 72°C (30 s), followed by extension at 72°C (7 min). An *ACTIN* gene, *ACT2*, with specific primers (5'-GCCATCCAAGCTGTTCTCTC-3' and 5'-GAACCAACCGATCCAGACACT-3') as previously described (Liu et al. 2007), or a *EUKARYOTIC TRANSLATION INITIATION FACTOR 4a* (*EIF4a*) with specific primers (5'-AAGAGAGCTGGCTACACAAA-3' and 5'-ATGCATTGATGAGACAGTGA-3') were used as a control in the semiquantitative PCR. The conditions for PCR to amplify *ACT2*, *EIF4a*, and *PR-1* were essentially the same as above, except for the 20-cycle amplification.

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