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Mimicking the Host Regulation of Salicylic Acid: A Virulence Strategy by the Clubroot Pathogen *Plasmodiophora brassicae*

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The plant hormone salicylic acid (SA) plays a critical role in defense against biotrophic pathogens such as *Plasmodiophora brassicae*, which is an obligate pathogen of crucifer species and the causal agent of clubroot disease of canola (*Brassica napus*). *P. brassicae* encodes a protein, predicted to be secreted, with very limited homology to benzoic acid (BA)/SA–methyltransferase, designated PbBSMT. PbBSMT has a SA- and an indole-3-acetic acid–binding domain, which are also present in *Arabidopsis thaliana* BSMT1 (*AtBSMT1*) and, like AtBSMT1, has been shown to methylate BA and SA. In support of the hypothesis that *P. brassicae* uses PbBSMT to overcome SA-mediated defenses by converting SA into inactive methyl salicylate (MeSA), here, we show that PbBSMT suppresses local defense and provide evidence that PbBSMT is much more effective than AtBSMT1 at suppressing the levels of SA and its associated effects. Basal SA levels in *Arabidopsis* plants that constitutively overexpress *PbBSMT* compared with those in *Arabidopsis* wild-type Col-0 (WT) were reduced approximately 80% versus only a 50% reduction in plants overexpressing *AtBSMT1*. *PbBSMT*-overexpressing plants were more susceptible to *P. brassicae* than WT plants; they also were partially compromised in nonhost resistance to *Albugo candida*. In contrast, *AtBSMT1*-overexpressing plants were not more susceptible than WT to either *P. brassicae* or *A. candida*. Furthermore, transgenic *Arabidopsis* and tobacco plants overexpressing *PbBSMT* exhibited increased susceptibility to virulent *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) and virulent *Pseudomonas syringae* pv. *tabaci*, respectively. Gene-mediated resistance to DC3000/AvrRpt2 and tobacco mosaic virus (TMV) was also compromised in *Arabidopsis* and *Nicotiana tabacum* 'Xanthi-nc' plants overexpressing *PbBSMT*, respectively. Transient expression of *PbBSMT* or *AtBSMT1* in lower leaves of *N. tabacum* Xanthi-nc resulted in systemic acquired resistance (SAR)-like enhanced resistance to TMV in the distal systemic leaves. Chimeric grafting experiments revealed that, similar to SAR, the development of a PbBSMT-mediated SAR-like phenotype was also dependent on the MeSA esterase activity of NtSABP2 in the systemic leaves. Collectively, these results strongly suggest that PbBSMT is a novel effector, which is secreted by *P. brassicae* into its host plant to deplete pathogen-induced SA accumulation.

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Clubroot disease of brassica species is caused by the biotrophic protist pathogen *Plasmodiophora brassicae*. Infection of canola (*Brassica napus*) by *P. brassicae* induces tumor and gall formation on the roots, which alters source/sink relationships, impedes water transport, and results in significant yield loss in severely infected crops (Dixon 2009). Plant pathogens including bacteria, true fungi, and protists, like oomycetes, secrete effectors to suppress plant defense responses through secretion of effectors into the host plant. Effectors are defined as small, secreted proteins produced by the pathogen to support pathogen colonization through the suppression of host immunity (Win et al. 2012). Several pathotypes of *P. brassicae* cause disease in their host species. The most prevalent pathotypes in Canada are pathotype 3 (Pb3), which is virulent on canola and pathotype 6 (Pb6), which mainly infects vegetable brassicas (Strelkov and Hwang 2014). Pb3 encodes approximately 590 secreted proteins, of which over 200 are small secreted proteins with plausible roles in pathogenicity (Rolfe et al. 2016).

Formation of galls by plant pathogens such as *P. brassicae* is a consequence of pathogen-triggered hormonal changes in the host. Auxin and cytokinin are two major hormones involved in the infection and development of root galls. Cytokinin levels are believed to rise in early stages of infection, while an increase in auxin levels occurs at later stages of infection. Genes that are potentially involved in cytokinin biosynthesis have been identified in *P. brassicae*, while the pathogen is believed to manipulate and utilize auxin produced by the host (Rolfe et al. 2016).

Based on the type of the pathogen encountered and the battery of specific effectors involved, plants employ a targeted array of deactivation or attenuation tactics against pathogens to restrict further ingress. To successfully defend against invading pathogens, plants have generally evolved two types of immunity responses, including pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). PTI is triggered by recognition of conserved microbial molecular patterns by associated plant pattern recognition receptors; while, in ETI, the recognition of a specific pathogen effector by

its cognate plant resistance (R) protein triggers a strong immune response, typically comprising a hypersensitive response.

Salicylic acid (SA) is the major plant hormone in defense against biotrophic pathogens, including clubroot (Lemarié et al. 2015; Ludwig-Müller et al. 2015; Rolfe et al. 2016; Vlot et al. 2009). Agarwal et al. (2011) showed that application of exogenous SA suppresses clubroot disease in *Arabidopsis thaliana*. To counteract SA-related defenses, it has been hypothesized that *P. brassicae* inactivates the plant-derived SA by converting it into biologically inactive methyl salicylate (MeSA) using a benzoic acid (BA)/SA methyltransferase protein (PbBSMT; National Center for Biotechnology Information accession number AFK13134) (Ludwig-Müller et al. 2015). *PbBSMT* identified in Pb3 (PbPT3Sc00026_A_1.308_1) encodes a predicted secreted protein. Sequence analysis revealed that PbBSMT has little overall homology to plant BSMT. However, PbBSMT shares partial homology to *S*-adenosyl methionine (SAM)-binding motifs of AtBSMT1 and NtSAMT1 (Ludwig-Müller et al. 2015; Rolfe et al. 2016). Moreover, recombinant PbBSMT made in *Eschierchia coli* has been shown to methylate SA and BA as well as anthranilic acid (Ludwig-Müller et al. 2015). Expression of *PbBSMT* was found to be very low in *Brassica napus*–infected roots during the first week after inoculation, then increased in week 2, and peaked in weeks 3 and 4 (Rolfe et al. 2016). Upregulation of *PbBSMT* coincides with increased SA production in *P. brassicae*–infected plants (Ludwig-Müller et al. 2015; Rolfe et al. 2016), suggesting a potential role for this enzyme in suppression of plant SA-dependent defense responses in *Arabidopsis*.

Many different plant species produce MeSA (Effmert et al. 2005). Volatile MeSA can function as fragrance for attraction of beneficial insects, like pollinators, to the plant (Goff and Klee 2006; Widhalm and Dudareva 2015). In addition to attraction, volatile MeSA is known to function as an airborne defense signal for plant-plant communication and tritrophic interactions of plant, herbivory insects, and parasitoids (Baldwin et al. 2006; Dempsey and Klessig 2012; Shulaev et al. 1997). MeSA was the first of several phloem-mobile signals for induction of systemic acquired resistance (SAR) to be identified in tobacco and *Arabidopsis* (Dempsey and Klessig 2012; Park et al. 2007; Vlot et al. 2008). Notably, MeSA has been reported to be transported from *P. brassicae*–infected roots to the aerial tissue (Ludwig-Müller et al. 2015).

A model has been proposed for SAR activation in *Arabidopsis* and tobacco by MeSA (Dempsey and Klessig 2012; Kumar and Klessig 2003; Liu et al. 2010; Park et al. 2007; Vlot et al. 2008). Rising SA in the primary infected leaves is converted to MeSA by NtSAMT1/AtBSMT1. MeSA accumulation is further enhanced in this tissue by SA inhibition of the MeSA esterase activity of tobacco SA-binding protein 2 (NtSABP2) or of the orthologous *Arabidopsis* MeSA esterases (AtMSEs). Following translocation to the distal systemic tissues, MeSA is converted back to SA by NtSABP2 (AtMSEs); the released SA then activates or primes systemic immune responses. A similar signaling pathway appears to operate in potato, since suppressing the activity of StMES1 compromised SAR (Manosalva et al. 2010). The levels of released SA in the systemic tissue are too low to inhibit the MeSA esterases, thereby allowing SA levels to increase.

Suppression of clubroot with exogenous SA (Agarwal et al. 2011) and the ability of recombinant PbBSMT to convert biologically active SA into MeSA, which is unable to induce immune responses (Ludwig-Müller et al. 2015), strongly suggests that PbBSMT is an effector. The most direct and rigorous test of this would be to delete or inactivate the PbBSMT and assess the effect on *P. brassicae* virulence. Since *P. brassicae* is not amenable to such genetic manipulation to help address this question, we determined the effects of overexpression of PbBSMT in transgenic *Arabidopsis* and tobacco. To assess why PbBSMT might function as an effector when it and the endogenous AtBSMT1 both can convert SA to MeSA, its effects were compared with those of overexpression of AtBSMT1. Here, we report that PbBSMT was much more effective than AtBSMT1 at both reducing endogenous and exogenous SA levels and at suppressing multiple levels of resistance, including nonhost and basal resistance as well as PTI and ETI.

RESULTS

PbBSMT is conserved among *P. brassicae* pathotypes and has a functional secretory signal peptide (SP).

The *P. brassicae* genome contains *PbBSMT*, with similarity to plant SAM-dependent carboxyl methyltransferase (methyltransferase-7 superfamily) (Rolfe et al. 2016). The well-studied *BSMT* gene in *Arabidopsis* (*AtBSMT1*, At3g11480) also belongs to the methyltransferase-7 superfamily (Liu et al. 2010, 2011). The Pb3 *PbBSMT* open reading frame is 1,134 bp long, which encodes a 377–amino acid protein with very limited homology to *AtBSMT1* (Ludwig-Müller et al. 2015). Importantly, recombinant PbBSMT can methylate SA and BA (Ludwig-Müller et al. 2015). *PbBSMT* is conserved in all the *P. brassicae* pathotypes sequenced to date (Rolfe et al. 2016). Despite differences in their host range (Rolfe et al. 2016), *PbBSMT* alleles from Pb3 and Pb6 are 97% identical at the DNA level, while *PbBSMT* alleles from Pb2, Pb3, Pb5, and Pb8, and the European isolate e3 show 100% identity to Pb3 (not shown). PbBSMT has the signatures of plant pathogen effectors, which are the presence of an N-terminal SP and the lack of transmembrane and other subcellular targeting domains. To validate the function of PbBSMT predicted SP (PbBSMT-SP), we used a genetic assay in yeast, as described by Oh et al. (2009). The PbBSMT-SP was fused to the mature yeast invertase sequence, was cloned in the pSUC2 vector, and was transferred to the invertase-deficient mutant yeast strain YTK12. Transgenic strain YTK12 harboring the PbBSMT-SP fused to invertase was able to grow on a medium in which sucrose was replaced with raffinose, thereby demonstrating that PbBSMT-SP is a functional SP (Supplementary Fig. S1).

Expression of *35S::PbBSMT* in planta reduced SA levels and increased susceptibility to clubroot.

Endogenous *PbBSMT* expression in *P. brassicae* increased soon after infection in canola root (Fig. 1A). Expression rose during formation of primary and secondary plasmodia and was highest during gall formation. To help assess the significance of PbBSMT activity for clubroot development, the transgenic *Arabidopsis* lines ectopically expressing *AtBSMT1* (AtT) and *PbBSMT* (PbT) together with the wild type Col-0 (WT) control were evaluated for the effects of these methyltransferases on the levels of free and conjugated SA in leaves and roots. PbT plants were significantly more efficient than AtT plants at reducing the basal levels of SA and conjugated SA (Fig. 2A). Basal levels of biologically active free SA were reduced 80% in roots and leaves of PbT plants compared with those in WT, while the corresponding reductions in AtT were only approximately 50%. Similarly, basal levels of conjugated SA were much more reduced in PbT than in AtT compared with those in WT, for both roots and leaves. Furthermore, MeSA was only detectable in the roots of PbT plants (32 ng g^{-1} fresh weight). In agreement with stronger metabolism of endogenous SA in PbT leaves than in AtT leaves, the residual SA 18 h after infiltration of 0.25 mM SA in PbT was only 2% of that remaining in infiltrated WT. In contrast, this level was reduced just 50% in AtT compared with WT. Thus, PbBSMT is much more effective than AtBSMT1 at

metabolizing both endogenous and exogenous SA in roots and leaves of *Arabidopsis*.

Next we determined the effects of expressing *35S::PbBSMT* and *35S::AtBSMT1* and the resulting enhanced metabolism of SA on clubroot induction by Pb3. The average number of infected cells containing secondary zoospores were over two times higher in the roots of PbT plants compared with those in AtT and WT plants. Furthermore, larger galls formed on PbT plants than on AtT and WT plants, which exhibited similar levels of infection (Fig. 1B and C). Additionally, an *AtBSMT1* T-DNA knockout (*AtBSMT1* KO, SALK_140496) was transformed by *AtBSMT1* (Ask6) and *PbBSMT* (PsK5) and their susceptibility to clubroot was assessed. The infection rate was similar in the *AtBSMT1* KO and AsK6 and comparable to that in WT. In contrast, PsK5 plants produced significantly larger galls than produced by the *AtBSMT1* KO (Supplementary Fig. S5).

Expression of *35S::PbBSMT* impaired nonhost resistance to *Albugo candida*.

We have previously reported that *Arabidopsis* is generally not a host for *Albugo candida* isolate Ac2V, which was isolated from *Brassica* spp. Interestingly, Ac2V is recognized in *Arabidopsis* Col-0 by the toll interleukin 1 receptor-nucleotide binding -leucine-rich repeat–type resistance gene *WRR4* and the resulting resistance is SA dependent (Borhan et al. 2010). To test the effect of PbBSMT on SA-dependent defense, seedlings of PbT plants were inoculated with Ac2V. Trypan blue staining of infected cotyledons showed that ectopic overexpression of *35S::PbBSMT* enabled hyphae branching and growth in PbT cotyledons compared with the immediate arrest of the pathogen at the site of infection in WT Col-0 (Fig. 2B). Impaired resistance was further evidenced by limited mycelial growth and sporulation on PbT, which also occurred on *Arabidopsis* mutant *eds1*, which is defective in SA signaling, and on transgenic *NahG*, which expresses a bacterial-derived SA hydroxylase that converts SA into inactive catechol (Fig. 2B). These results are consistent with PbBSMT suppressing SA-mediated defenses. In contrast to PbT, resistance in AtT to Ac2V was similar to that in WT. Additionally, Ac2V showed partial mycelial growth in PsK5 that was similar to that in PbT (Fig. 2B), however its growth was strongly stopped in the *ATBSMT1* KO and Ask6 cotyledons resembling nonhost resistance in WT Col-0.

PTI and basal resistance were compromised in plants expressing *35S::PbBSMT*.

To evaluate whether PbBSMT alters PTI, PbT, and AtT, plants were inoculated with virulent *Pseudomonas syringae* pv. *tomato* DC3000 (DC3000) (Fig. 3). The SA signaling–defective *NahG* and *eds1* lines were again included as controls. Bacterial growth in PbT was compared with that in WT, AtT, *NahG*, and *eds1* at different times after inoculation. At day 3 postinoculation, DC3000 levels in PbT, AtT, *NahG*, and *eds1* were higher than that in WT (Fig. 3A), with PbT and *NahG* supporting the highest multiplication of DC3000, which was approximately 25-fold more than that in WT. Similarly, increased bacterial growth was observed for Ask6 and PsK5

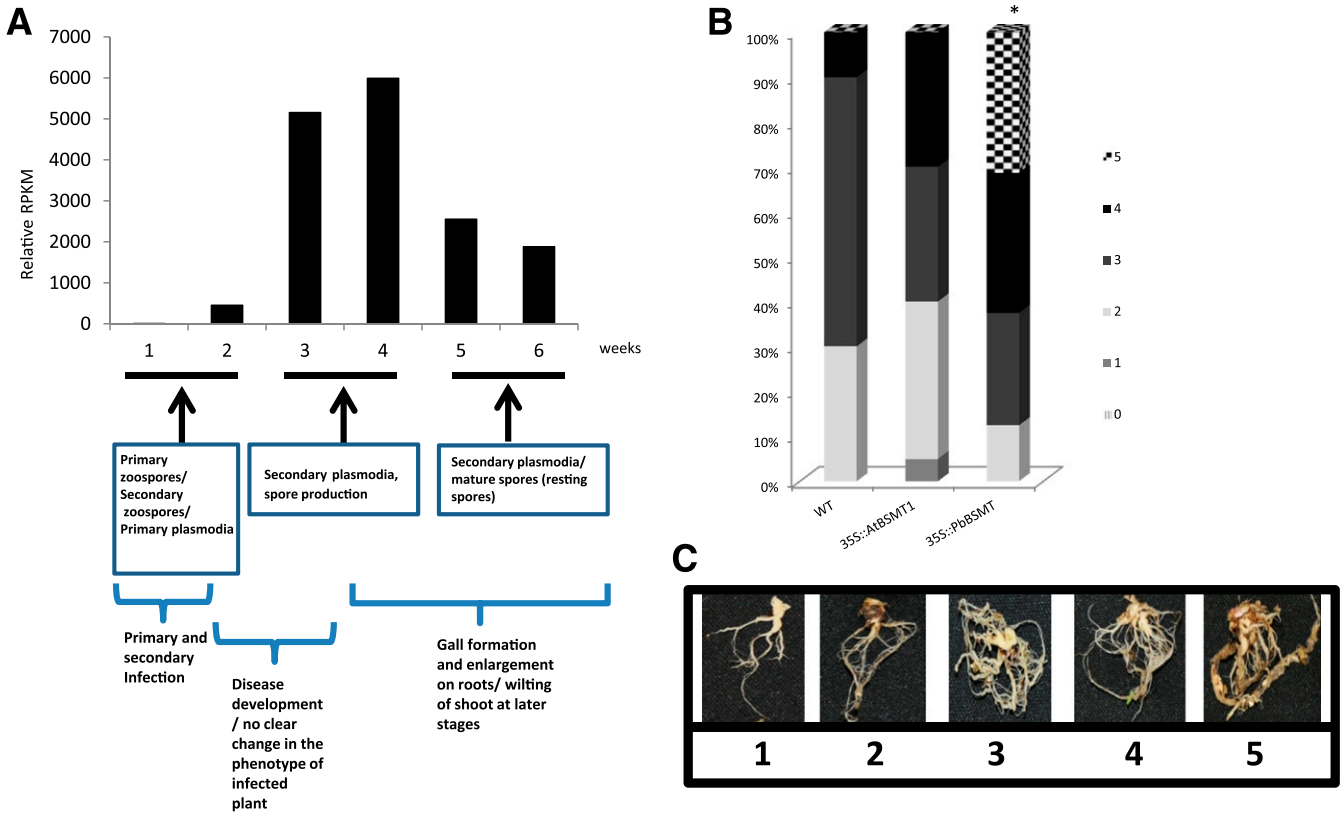


Fig. 1. Rating of clubroot disease on the roots of *Arabidopsis* plants inoculated with 10^7 spores per milliliter of *Plasmodiophora brassicae* pathotype 3 (Pb3). **A,** Expression level of the *P. brassicae* benzoic acid/salicylic acid methyltransferase gene (*PbBSMT*) in canola (*Brassica napus*) roots infected with Pb3, at different growth stages of the pathogen. This panel is reproduced from data reported by Rolfe et al. (2016) to present a visual correlation between *PbBSMT* expression with pathogen growth and disease development in canola. **B,** Disease severity defined on scales of 0 to 5, in which 0 was healthy roots with no galls and 5 was roots with severe gall formation. The percentage of roots in each group from a total of 100% is presented. Asterisks indicate statistically significant different distributions of disease severity classes between *35S::PbBSMT* expressing plants versus *35S::AtBSMT1* overexpressing line and WT (Chi-square test, $\alpha = 0.05$). Three independent experiments were conducted with similar results. **C,** Images of plant roots infected with Pb3 representative of each step (1 to 5) of the scale. RPKM = reads per kilobase million.

plants compared with that in the *AtBSMT1* KO on day 3 after challenge-inoculation by virulent DC3000.

The expression of the SA-responsive marker gene *PR1* was dramatically reduced in the leaves of PbT and *NahG* compared with WT, while *eds1* exhibited marginal reduction (Fig. 3B). Likewise, *PR1* expression was significantly reduced in the infected leaves of PSK5 compared with the *AtBSMT1* KO and Ask6 plants.

Next, the effect of *35S::PbBSMT* overexpression on basal resistance was assessed following *Pseudomonas syringae* pv. *tabaci* 11584 infection of transgenic *Nicotiana benthamiana* (Nb-PbBSMT). Transgenic lines designated benth 7-6 and benth 7-8 supported a greater than sevenfold more growth of *P. syringae* pv. *tabaci* than WT *N. benthamiana* plants (Fig. 3C). Infiltration of *P. syringae* pv. *tabaci* into *N. benthamiana* leaves causes water-soaking and necrosis. Nb-PbBSMT plants infiltrated with *P. syringae* pv. *tabaci* developed necrotic symptoms almost 2 days earlier than WT plants. Moreover, lesion diameter was two to three times larger in transgenic *35S::PbBSMT* plants. These results argue that in planta expression of *35S::PbBSMT* impaired PTI induced by DC3000 in *Arabidopsis* and basal resistance to *P. syringae* pv. *tabaci* in *N. benthamiana*.

ETI was attenuated in *Arabidopsis* and tobacco plants expressing *35S::PbBSMT*.

To evaluate whether PbBSMT is able to alter ETI (also termed resistance [*R*] gene–mediated resistance), PbT plants were inoculated with a strain of DC3000 harboring *AvrRpt2*, which is recognized by the *Arabidopsis* Col-0 *R* gene *RPS2*. PbT plants were less resistant to DC3000/*AvrRpt2* compared with the WT control, with bacterial levels 10-fold higher in PbT (Fig. 3D). In contrast and in agreement with Liu et al. (2010), bacterial levels in AtT were similar to those in WT.

To further assess effect of PbBSMT on ETI, two independent transgenic *N. tabac*um cv. Xanthi-nc (NN genotype) lines harboring *35S::PbBSMT* (Nt-PbBSMT), designated Nt33-3 and Nt33-4, were tested for resistance to tobacco mosaic virus (TMV). *N. tabac*um Xanthi-nc contains the *R* gene *N*, which confers ETI to TMV, including restriction of viral replication and development of necrotic lesions, referred to as a hypersensitive response (Deom et al. 1991). Nt33-3 and Nt33-4 were

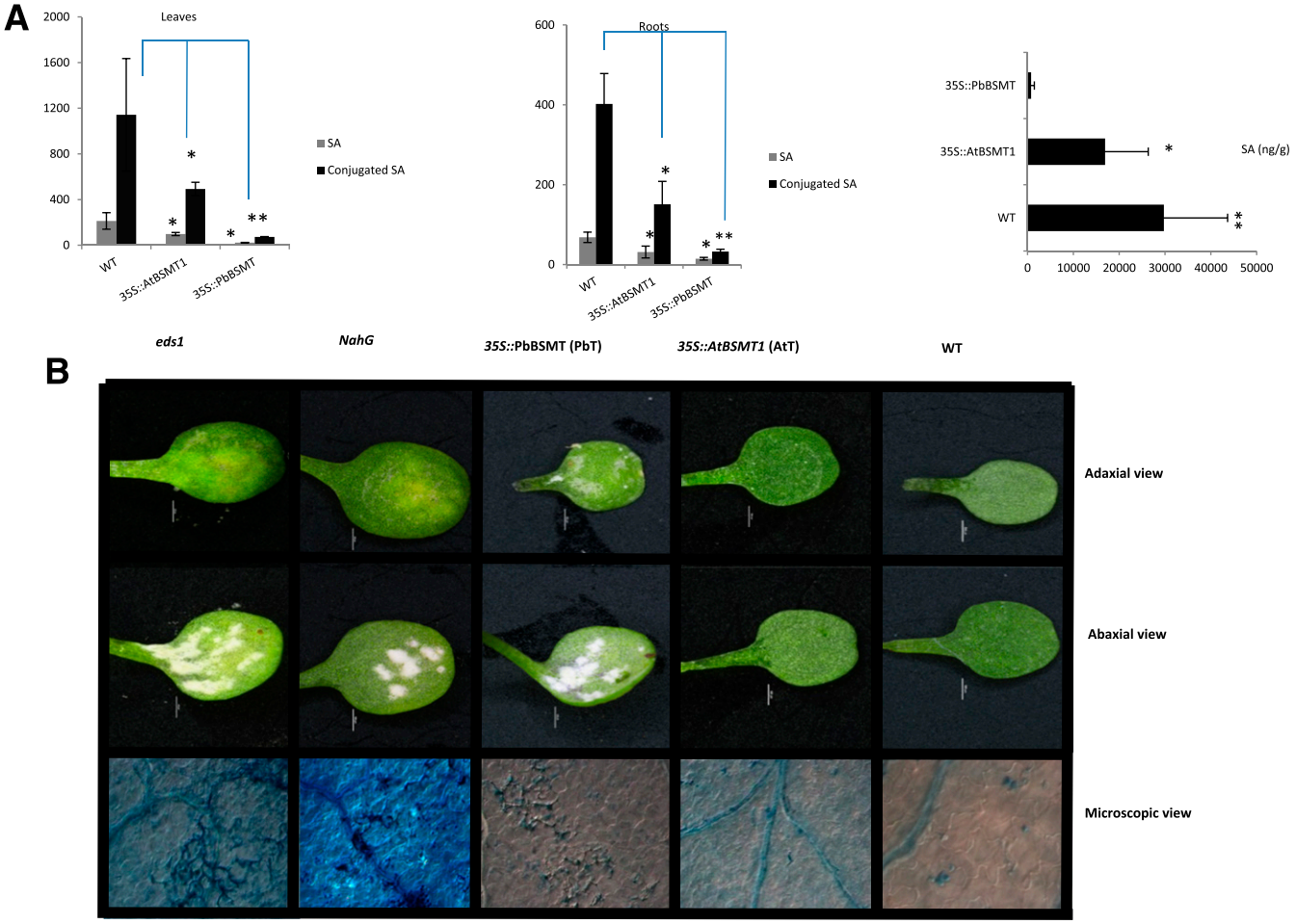


Fig. 2. Levels of salicylic acid (SA) and its conjugated forms in *Arabidopsis thaliana* leaves and roots of noninoculated healthy plants constitutively expressing *35S::AtBSMT1* or *35S::PbBSMT* compared with wild type (WT). **A,** Left and middle graphs show the levels of free and conjugated SA in the leaves and the roots, respectively, of healthy noninoculated plants; the right graph shows free SA in treated leaves 18 h after infiltration with 0.25 mM SA. Values are the mean ± standard deviation fresh weight measured in nanograms per gram. **B,** Nonhost response to *Albugo candida* race Ac2V in cotyledons of WT, transgenic 35S::PbBSMT (PbT), transgenic 35S::AtBSMT (AtT), SA-deficient transgenic NahG, and SA signaling–defective *eds1* mutant SG8-19. While Ac2V is nonvirulent on WT, its growth was facilitated in 35S::PbBSMT plants. In the lower panel, the extent of mycelial growth was visualized by trypan blue staining. The stained infected leaves correspond to the same plants shown in the middle and top panel. White dots on the lower surface of infected cotyledons show formation of pustules due to mycelial growth and sporulation. White scale bars are equal to 500 µm. Asterisks in A indicate statistically significant differences (one asterisk [*] indicates $P \leq 0.05$, two [**] $P \leq 0.01$, Student *t* test) between the levels of SA or its conjugated form in 35S::PbBSMT and 35S::AtBSMT1 versus WT Col-0. For the left and middle graphs, statistical analysis was done between free SA (gray bar) and conjugated SA (black bar) separately. Two independent experiments were done for B with similar results.

less resistant to TMV, as indicated by necrotic lesions that were five to eight times larger than those on the WT control (Fig. 3E). Together, these two sets of data strongly argue that PbBSMT suppresses ETI.

Local *35S::PbBSMT* expression in tobacco-induced SAR to TMV.

PbBSMT methylates SA to form MeSA (Ludwig-Müller et al. 2015), similar to its tobacco (NtSAMT1) and *Arabidopsis* (AtBSMT1) orthologs. NtSAMT1 is involved at the onset of SAR in the primary leaves in which this SAR signal is generated (Liu et al. 2011; Park et al. 2007; Vlot et al. 2008). To examine whether PbBSMT or AtBSMT1 could induce SAR-like phenotype, *35S::PbBSMT* or *35S::AtBSMT1* were transiently expressed in three lower leaves of *N. tabacum* Xanthi-nc and, 1 week later, the upper systemic leaves were challenged with TMV. PbBSMT was detected in the infiltrated leaves of *N. tabacum* Xanthi-nc as early as 4 days post-inoculation (dpi) and persisted up to 16 dpi (data not shown). Average TMV lesion size was reduced by 70 and 50% in the upper systemic leaves of plants transiently expressing *35S::PbBSMT* or *35S::AtBSMT1*, respectively, in the lower leaves compared with that on plants not expressing either, i.e., mock-infiltrated (Fig. 4). These results revealed that expression of either pathogen or plant BSMT was enough to induce systemic resistance to TMV in Xanthi-nc resembling the pathogen-induced SAR.

NtSABP2 was required for enhanced resistance induced by PbBSMT.

Since conversion of inactive MeSA back into active SA by NtSABP2 in the systemic tissues is essential for SAR (Park et al. 2007), we tested whether NtSABP2 is required for PbBSMT-induced SAR-like resistance, using *N. tabacum* Xanthi-nc plants in which SABP2 was silenced (siSABP2). *35S::PbBSMT* was transiently expressed in the lower leaves of siSABP2 and WT plants, which was followed a week later with TMV inoculation of the upper systemic leaves. Again, similar experiments were conducted using transient expression of *35S::AtBSMT1* as a positive control. PbBSMT- as well as

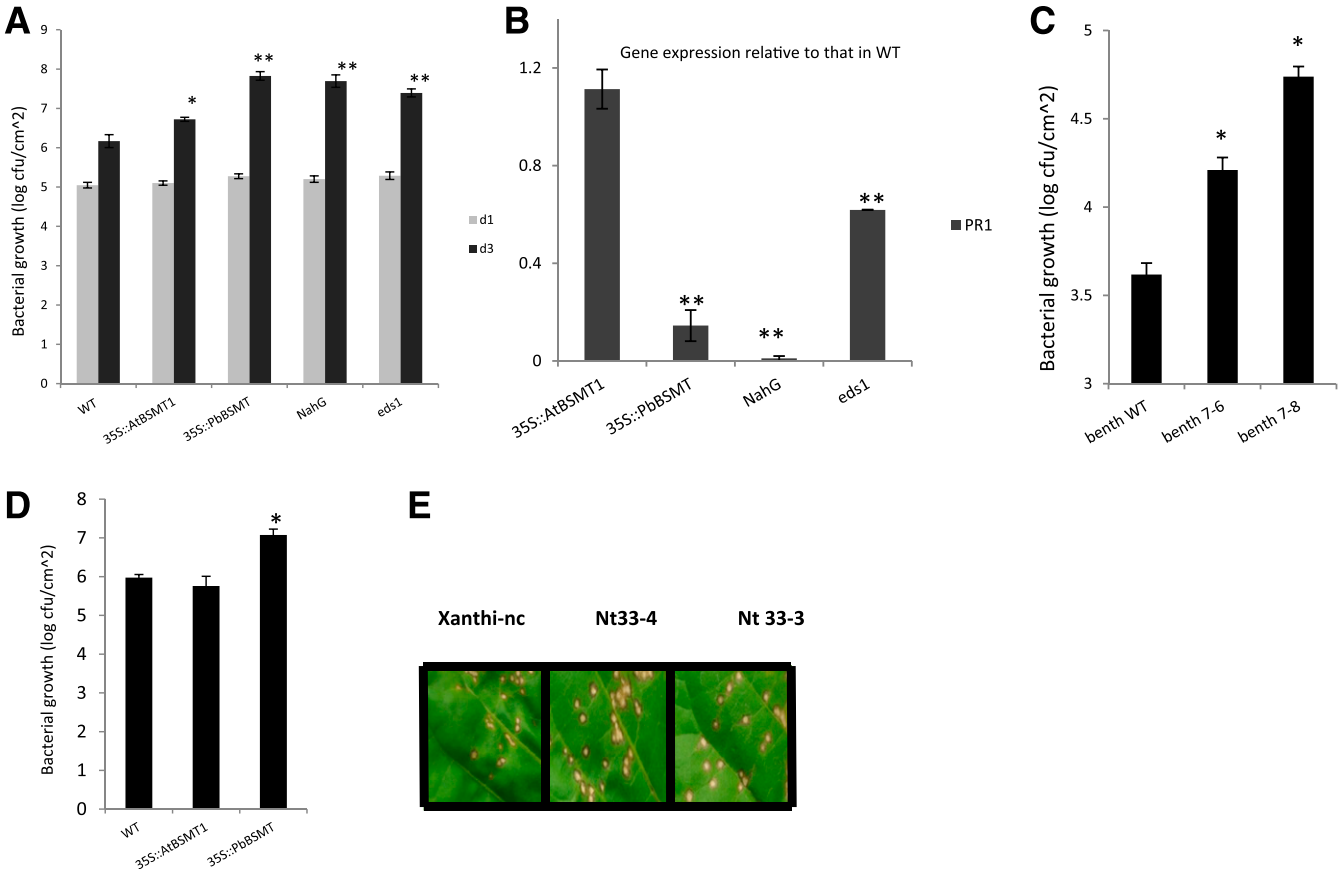


Fig. 3. Pattern- and effector-triggered immunity (PTI and ETI, respectively) in *Arabidopsis* and tobacco plant expressing *35S::PbBSMT* and *35S::AtBSMT1*. For PTI response, three to four leaves of five *Arabidopsis thaliana* and *Nicotiana benthamiana* plants were infiltrated with virulent *Pseudomonas syringae* pv. tomato DC3000 (DC3000) or *Pseudomonas syringae* pv. *tabaci* 11584, respectively. ETI was examined by inoculating three leaves of five individual *A. thaliana* or *N. tabacum* 'Xanthi-nc' with DC3000/AvrRPT2 or tobacco mosaic virus (TMV), respectively. **A,** Wild type (WT), transgenic 35S::PbBSMT (PbT), transgenic 35S::AtBSMT (AtT), salicylic acid (SA)-deficient transgenic NahG, and SA signaling–defective *eds1* mutant SG8-19 were infiltrated with 10^6 CFU ml^{-1} of DC3000; at 1 and 3 days postinoculation, the average number of colony-forming units in the infiltrated leaves was calculated and are presented on a log to the base 10 scale. **B,** mRNA levels of SA-responsive marker gene *PR1* at day 3 postinoculation with DC3000 were quantified with quantitative real-time polymerase chain reaction and are presented relative to those in WT. **C,** The average number of colony-forming units of *Pseudomonas syringae* pv. *tabaci* 11584 3 days postinoculation of WT *N. benthamiana* and of two independent *35S::PbBSMT*-expressing transgenic lines, benth7-6 and benth7-8, are presented on a log scale. **D,** The average number of colony-forming units of DC3000/AvrRPT2 in *35S::PbBSMT*-expressing *Arabidopsis* compared with WT and 35S::AtBSMT1 plants at 3 days postinoculation are presented on a log scale. **E,** TMV lesions on WT *N. tabacum* Xanthi-nc and on two independent *35S::PbBSMT*-expressing *N. tabacum* Xanthi-nc transgenic lines, Nt33-3 and Nt33-4. In A (bacterial growth on day 3) and D, asterisks indicate significant difference (one asterisk [*] indicates $P \leq 0.05$, two [**] $P \leq 0.01$, Student *t* test) between the average values for each plant versus WT Col-0. In B, asterisks indicate significant difference (one asterisk [*] indicates $P \leq 0.05$, two [**] $P \leq 0.01$, Student *t* test) between the average relative values for each plant to that in WT versus WT Col-0 as control. In C, asterisks represent statistical differences between (one asterisk [*] indicates $P \leq 0.05$, Student *t* test) each transformant versus WT *N. benthamiana*. For each experiment, at least two independent experiments were conducted with similar results.

AtBSMT1-induced systemic resistance against TMV was abolished in siSABP2, indicating that SABP2 is required for their induction of SAR-like phenotype (Fig. 4).

The role of NtSABP2 in PbBSMT-mediated SAR-like induction was further investigated with chimeric grafts between WT *N. tabacum* Xanthi-nc and siSABP2. *35S::PbBSMT* was infiltrated into three lower leaves of the rootstock and, 1 week later, TMV was inoculated on the systemic leaves of the scion (Fig. 5A). At 4 to 7 dpi, lesion size was compared with those on control plants in which the rootstock was mock infiltrated before TMV was inoculated on a scion. Again *35S::PbBSMT*-infiltrated rootstocks suppressed TMV lesion size on systemic leaves compared with mock-infiltrated plants (Fig. 5B). Similarly, when siSABP2 was used as rootstock, PbBSMT induced SAR-like resistance in WT *N. tabacum* Xanthi-nc scions. However, the PbBSMT-induced SAR-like phenotype was disrupted when the siSABP2 scion was grafted onto the *35S::PbBSMT*-expressing rootstock (Fig. 5B). These results together confirm that PbBSMT can induce systemic resistance, which is phenotypically similar to pathogen-induced SAR and this is dependent on the activity of NtSABP2 in the systemic tissues.

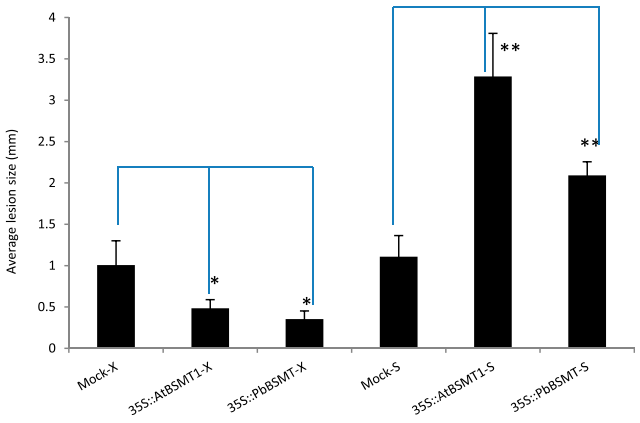


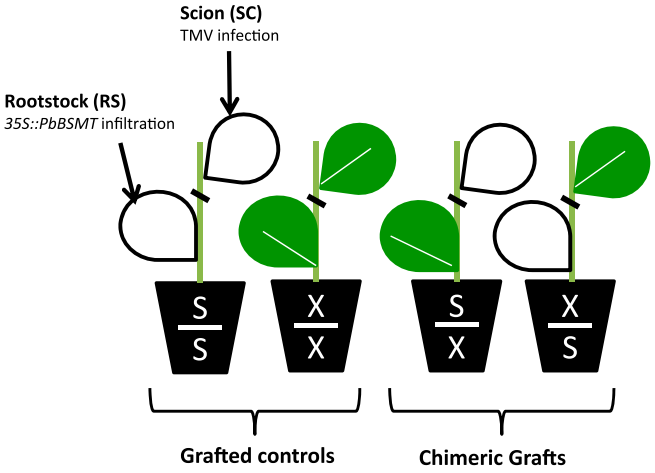
Fig. 4. *PbBSMT* and *AtBSMT1* induced a systemic acquired resistance (SAR)-like phenotype in *Nicotiana tabacum* 'Xanthi-nc' against tobacco mosaic virus (TMV). Average TMV lesion size on the upper leaves of Xanthi-nc (X) or its salicylic acid (SA)-binding protein 2–silenced mutant NtSiSABP2 (S) when *35S::AtBSMT1* or *35S::PbBSMT* were transiently expressed in the three lower leaves compared with average lesion size on plants whose lower leaves were not expressing *35S::AtBSMT1* or *35S::PbBSMT*. Mean ± standard deviation values are presented. Statistical analysis on average induction levels in *N. tabacum* Xanthi-nc (X) and NtSiSABP2 (S) plants was done separately. Asterisks indicate statistically significant differences (one asterisk [*] indicates $P \leq 0.05$, two [**] $P \leq 0.01$, Student *t* test). Two independent experiments were done with similar results.

PbBSMT suppresses SA-mediated retardation of root growth in *Arabidopsis*.

To effectively defend themselves, plants infected by *P. brassicae* accumulate large quantities of SA in their roots (Lemarié et al. 2015). While SA accumulation in the plants serves to protect them from the invading pathogen, high levels of SA can impose fitness costs (van Hulten et al. 2006). To prevent the negative impact of high SA levels, plants convert this highly active phytohormone into inactive metabolites, including MeSA (Dempsey et al. 2011). To assess whether PbBSMT or AtBSMT1 could suppress the toxic effect of high SA levels on root growth WT, AtT, and PbT, plants were grown on Murashige Skoog (MS) agar without or with different concentrations of SA added to the media (Fig. 6A). While root growth was similarly retarded in WT and AtT by SA, PbT exhibited increase tolerance to SA. In addition, when 4-day-old seedlings growing on MS media without SA were transferred onto MS media containing SA, roots of PbT plants grew longer than those of either WT or AtT (Fig. 6B).

Altered hormonal levels in PbT and AtT plants with reduced levels of SA.

Clubroot infection results in changes in the content of plant hormones, including SA, auxin, cytokinin, jasmonic acid (JA),



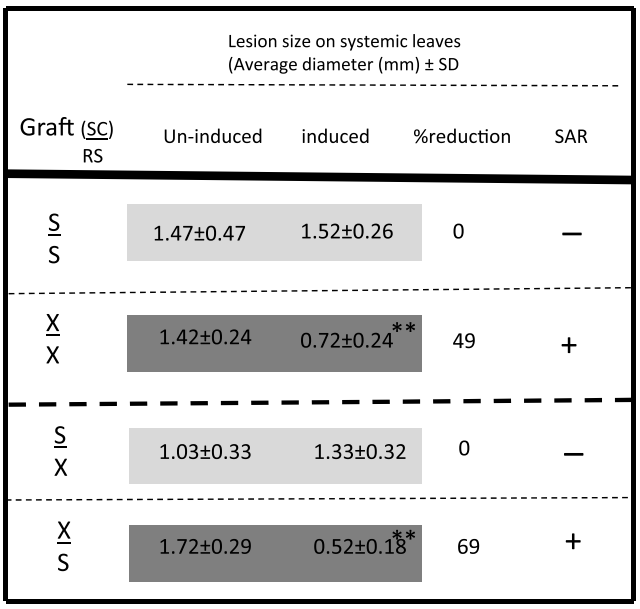


Fig. 5. PbBSMT and AtBSMT1 induced a systemic acquired resistance (SAR)-like phenotype in *Nicotiana tabacum* 'Xanthi-nc' against tobacco mosaic virus (TMV). Average TMV lesion size on the upper leaves of *N. tabacum* Xanthi-nc (X) or its salicylic acid (SA)-binding protein 2–silenced mutant NtSiSABP2 (S) when *35S::AtBSMT1* or *35S::PbBSMT* were transiently expressed in the three lower leaves compared with average lesion size on plants whose lower leaves were not expressing *35S::AtBSMT1* or *35S::PbBSMT*. The mean ± standard deviation values are presented. Statistical analysis on average induction levels in *N. tabacum* Xanthi-nc (X) and NtSiSABP2 (S) plants was done separately. Asterisks indicate statistically significant differences (one asterisk [*] indicates $P \leq 0.05$, two [**] $P \leq 0.01$, Student *t* test). Two independent experiments were done with similar results.

and abscisic acid (ABA) in infected roots (Jülke and Ludwig-Müller 2016; Lemarié et al. 2015). Moreover, the interplay among SA and other plant hormones, including JA, ethylene (ET), ABA, and indole-3-acetic acid (IAA), have been documented in plants responding to various environmental cues. In addition to reduced SA levels (Fig. 2A), PbT and AtT also had changes in other hormones. For example, levels of the ET precursor 1-aminocyclopropane-1-carboxylate (ACC) also was depressed in both leaves and roots of PbT and AtT (Supplementary Fig. S2), while levels of cytokinin and gibberellins were reduced only in the roots of PbT and AtT compared with those in WT (Supplementary Figs. S3 and S4). Levels of ABA and its metabolites were lower only in the leaves and roots of AtT. Of particular interest were the levels of the auxins, since AtBSMT1 and PbBSMT have an apparent indole-3-acetate (IAA) carboxyl methyltransferase domain. The levels of the biologically active IAA and its conjugates *N*-(Indole-3-yl-acetyl)-aspartic acid (IAA-Asp) and *N*-(Indole-3-yl-acetyl)-glutamic acid (IAA-Glu) were not significantly different in PbT, AtT, and WT.

DISCUSSION

Ludwig-Müller and coworkers (2015) proposed that *P. brassicae* overcomes SA-mediated defenses by secreting PbBSMT, which can convert SA into inactive MeSA. Together,

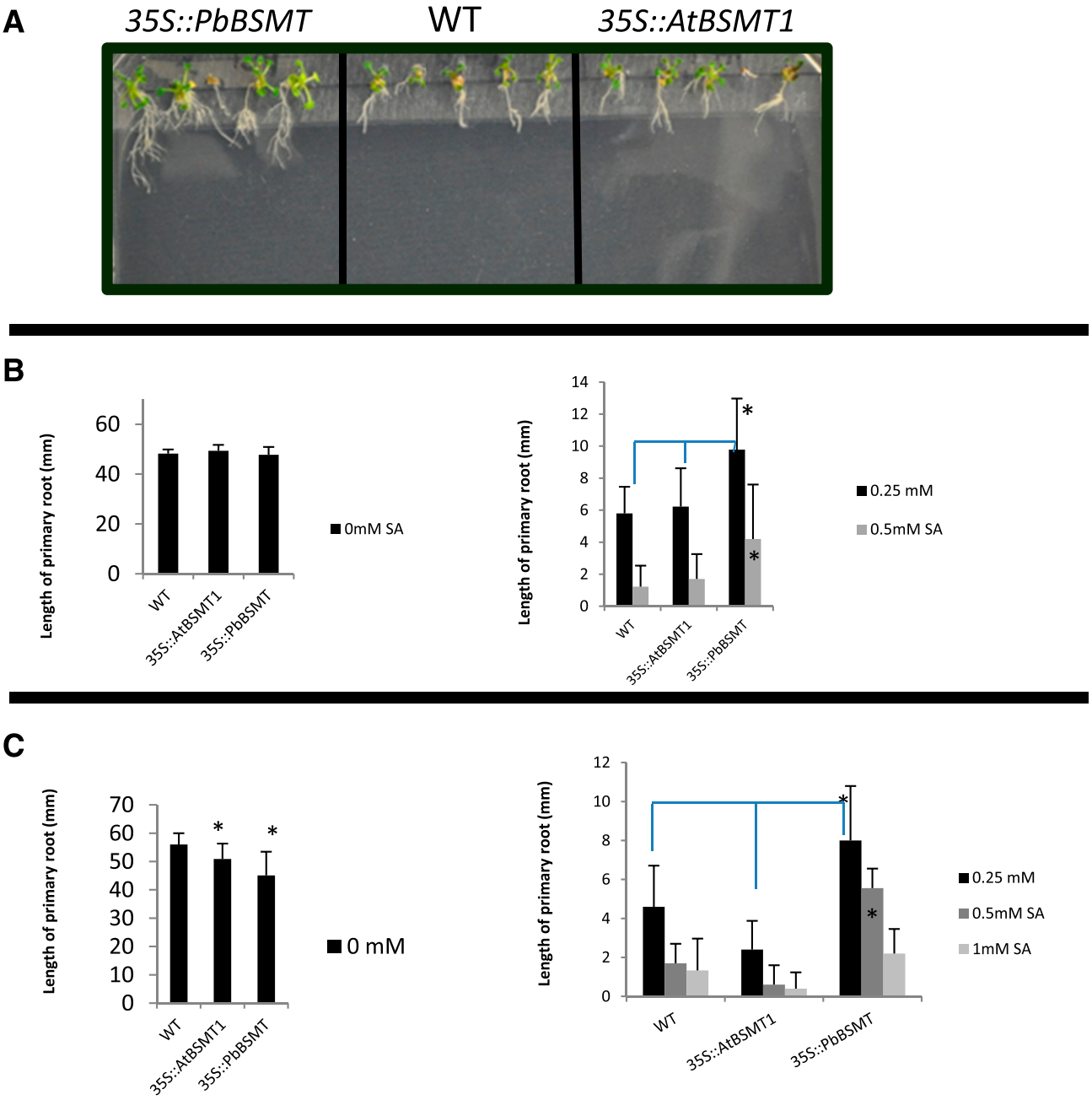


Fig. 6. Effect of salicylic acid (SA) on root growth of wild type (WT) and *35S::AtBSMT1*- or *35S::PbBSMT*-overexpressing *Arabidopsis*. **A,** Growth on Murashige Skoog (MS) agar containing 0.25 mM SA for 21 days. **B,** Average length of primary root at 21 days after planting when seeds were germinated directly on MS media containing various concentrations of SA. **C,** Average length of primary root when seeds were first grown for 4 days on MS agar without SA (left panel) before transfer of the seedlings to MS media containing various concentrations of SA; root length on day 21 minus the root length on day 4, when the seedling were transferred, was plotted (right panel). Asterisks indicate significant difference between each of the plants versus WT Col-0 (one asterisk [*] indicates $P \leq 0.05$, Student *t* test). Two independent experiments were done with similar results.

the results presented here strongly support that proposition. Moreover, they provide an explanation for why PbBSMT could suppress SA-mediated defenses while the endogenous BSMT does not. Ectopic overexpression of *PbBSMT* more effectively reduced levels of endogenous and exogenous SA and its conjugate form than overexpression of *AtBSMT1*. It also suppressed multiple levels of resistance from nonhost to ETI in *Arabidopsis* and tobacco, including resistance to *P. brassicae*. In contrast, overexpression of *AtBSMT1* generally failed to reduce resistance including to *P. brassicae*. Our finding that overexpression of *BSMT* reduced resistance to virulent bacteria is consistent with the results of Liu et al. (2010), who reported that overexpression of *AtBSMT1* in *Arabidopsis* resulted in elevated MeSA levels, whereas levels of SA, its conjugate, and resistance to virulent *Pseudomonas syringae* were substantially reduced. The differential effects of *P. brassicae*–secreted BSMT versus endogenous BSMT likely reflects the higher level of the *P. brassicae* enzyme in the infected tissue than that of the endogenous enzyme, whose expression is not induced by *P. brassicae* infection (S. Rolfe, *personal communication*). It may also be due to possibly higher specific activity of the pathogen enzyme than the plant enzyme, which is suggested by the higher level of conversion of SA to MeSA in PbT plants than in AtT plants.

Similarly, the finding that PbT plants were more resistant to SA-mediated retardation of seed germination and root growth while AtT plants were as sensitive as WT to SA again suggests that the pathogen enzyme is more potent. Importantly, rising levels of SA in infected roots could suppress root growth. The ability of PbBSMT to reduce SA levels would facilitate the growth of the very tissue this biotroph needs to multiply. Thus, PbBSMT inactivation of SA not only suppresses SA-mediated defenses but also suppresses its toxic effect on root growth. It should be noted that, in our experiments, PbBMST protein was expressed ubiquitously while, in *P. brassicae*–infected plants, mature PbBSMT protein is likely concentrated within infected tissue. Effects of root-localized PbBSMT on the defense in distal tissues need to be studied.

Comparison of the effects of overexpression of *PbBSMT* to those caused by a mutation that alters SA signaling (*eds1*) or by a transgene affecting SA accumulation (*NahG*) provides additional support that PbBSMT functions to suppress SA-mediated defenses. Interestingly, although PbT and *NahG* both exhibited reduced resistance to *Albugo candida* and DC3000 while resistance to Pb3 was compromised in PbT, it was not in *NahG*. This difference suggests that perhaps PbBSMT has an additional virulence activity besides inactivating SA. In agreement, Lovelock et al. (2016) showed reduced susceptibility in Col-NahG plants infected by two isolates of *P. brassicae*.

Several plant hormones including IAA, cytokinins, JA, ET, brassinosteroids (BR), and ABA have been shown to have roles in clubroot disease development in crucifers, with SA, BR, and IAA having the most prominent roles (Ludwig-Müller 2014; Rolfe et al. 2016). Interconnection between many hormone pathways has been described as hormone cross-talk. This phenomenon is defined to act as a general plant strategy in various stress responses to environmental and internal cues (Vos et al. 2015). In addition to SA depletion (in the shoots and roots), *PbBSMT* overexpression resulted in reduction in basal levels of ACC (in the shoots and roots) and cytokinins in transgenic plants. While elevated cytokinin is thought to be important for gall development, PbT plants supported growth of larger galls than WT plants despite having lower basal cytokinin levels. Perhaps, in infected roots, cytokinin production is manipulated by the pathogen, since annotated genes of Pb3 include those with a potential role in cytokinin regulation (Rolfe et al. 2016).

In summary, our data argue that PbBSMT functions as a virulent factor by suppressing SA-mediated defenses against biotrophic pathogens through its conversion of biologically active SA into inactive MeSA. In addition, inactivation of SA suppresses its toxic effect on root growth, which also would facilitate *P. brassicae* colonization leading to clubroot disease.

MATERIALS AND METHODS

Plant materials.

Arabidopsis and tobacco plants were grown on a soilless potting mix and were kept in growth cabinets with 8 h of light (100 µmol/m^2/s) at 22 (light) and 20°C (dark) and 70% relative humidity. *A. thaliana* plants overexpressing *Arabidopsis* BSMT (AtBSMT1, AtT), Col-*NahG*, and Col-*eds1* have been described elsewhere (Borhan et al. 2008; Liu et al. 2010; Van Wees and Glazebrook 2003). *Nicotiana tabacum* Xanthi-nc silenced for NtSABP2 was described by Park et al. (2007).

To compare the efficacy of AtBSMT1 and PbBSMT in detoxification of a high concentration of SA, seeds were surface-sterilized and were sown on MS agar medium supplemented with 0.5% sucrose. SA was added to MS agar plates at a concentration of 0.25, 0.5, and 1 mM. After 4 days of stratification at 4°C, the petri dishes were transferred and were positioned vertically under a short-day photoperiod (8 h of light at 22°C). Additionally, seeds were grown on MS media for 4 days, and then, seedlings were transferred onto new MS plates containing various concentrations of SA. The average growth rate of plant roots was calculated.

Systemic expression of NtSABP2 was shown to be necessary for pathogen-induced SAR (Park et al. 2007). To test whether *NtSABP2* expression in distal leaves is also important for induction of resistance against TMV by PbBSMT, grafting was done using *N. tabacum* Xanthi-nc. and *N. tabacum siSABP2*. After successful grafting, three lower leaves of rootstock were infiltrated by either *35S::PbBSMT* or *35S::GFP* as mock. Later, three leaves on the scion were infected by TMV and disease was scored 4 to 7 days later.

Pathogens and bioassays.

Clubroot pathology on *Arabidopsis* plants was carried out as soil drench of an inoculum of the single spore (ss) isolate of *Plasmodiophora brassicae* pathotype 3 (provided by S. E. Strelkov, University of Alberta) prepared at a concentration of 2×10^7 ss ml^{-1}. Four weeks after inoculation, 20 to 25 roots were analyzed for the appearance of root galls. Clubroot symptoms were scored from 0 to 5 for no gall to massive galls on the crown and entire root system. Disease severity was calculated as the average percentage of disease for each rating scale. *Pseudomonas syringae* pv. *tomato* DC3000 (starting inoculum 1×10^6 CFU ml^{-1}) and DC3000 harboring *AvrRpt2* (starting inoculum 1×10^5 CFU ml^{-1}) were used to study PTI (also called PAMP-triggered immunity) and ETI in 5-week-old *Arabidopsis* plants, respectively (Liu et al. 2010). Three leaves of five individual plants were infiltrated with a suspension of each bacterial strain and average numbers of bacteria were calculated on various days. Quantitative real-time PCR (PCR) analysis of *AtPR1* transcript was done on *Arabidopsis* leaves collected at 3 days after inoculation by DC3000 (Sanchez-Vallet et al. 2010).

Pseudomonas syringae pv. *tabaci* 11584 (ATCC11528 provided by D. S. Guttman, University of Toronto) (*P. syringae* pv. *tabaci*) was used to infiltrate the leaves of 4-week-old *Nicotiana benthamiana* plants (10^5 CFU ml^{-1}). Average lesion diameter as well as average bacterial numbers on day 7 after inoculation were calculated. TMV (provided by ?? Kovalchuk, University of Lethbridge) and *Albugo candida* race Ac2V that

were maintained on the host were used to inoculate *N. tabacum* Xanthi-nc and *Arabidopsis* as described before (Borhan et al. 2008; Park et al. 2007). *Albugo* mycelial growth was observed under the light microscope upon staining with trypan blue (van Damme et al. 2008). Average TMV lesion diameter was measured at 4 to 7 days after inoculation.

Cloning, transformation, confocal laser scanning microscopy, and the yeast trap system.

Gateway cloning vectors pDONR/zeo and pEarleyGate 100 were used to clone cDNA of *AtBSMT1* (*At3g11480*) or *PbBSMT* (PbPT3Sc00026_A_1.308_1) and to transform *Arabidopsis* Col-0. Additionally, *N. benthamiana* and *N. tabacum* Xanthi-nc were also transformed with cDNA of PbBSMT. Droplet digital PCR (Whale et al. 2012) was used to determine the transgene copy number and select transformants with a single insertion. In *Arabidopsis*, the *phosphinothricin* (*bar*) selectable marker gene was amplified using the primer set 5′-GGGGCCCGCGTAG GCGATGC-3′ (forward) and 5′-CGTACCGAGCCGCAGGAA CC-3′ (reverse) and was detected with the 6-carboxyfluorescein-labeled probe 5′-ATCCCTGGCTCGTCGCCGAGGTGG-3′. The *Arabidopsis alcohol dehydrogenase* (*AtADH*) reference gene was amplified using the primer set 5′-CTTCCACGTTCAGT GAGTACA-3′ (forward) and 5′-CTCCTAACCCAGTAGACA AACC-3′ (reverse) and was detected with the hexachloro-fluorescein (HEX)-labeled probe 5′-AGCAACCTGACCAG AGTGAACCAC-3′. Individual single copy lines were allowed to self-pollinate and independent homozygous lines were selected for further investigation. The expressions of *PbBSMT* in *N. benthamiana* and *N. tabacum* Xanthi-nc transgenic lines were also determined, using droplet digital PCR. The *phosphinothricin* (*bar*) selectable marker gene was amplified using the same primer set as described above. The *NbADH* reference gene was amplified using the primer set 5′-TCAAGTATCGATTCGTG CTC-3′ (forward) and 5′-AGAGCTAAGCAAAGACAAA GAT-3′ (reverse) and was detected with the HEX-labeled probe 5′-TTGGCAACCATTCCAACTCTGCTT-3′. The *NtAllylADH* reference gene was amplified using the primer set 5′-CACTG CTCTAGTTGGTCTCTTC-3′ (forward) and 5′-GCGTAAACA CAAACAGCTCTAC-3′ (reverse) and was detected with the HEX-labeled probe 5′-AAAGCAAGTCGTGATGGTTTCG CG-3′. Insert copy numbers were calculated in *N. benthamiana* and *N. tabacum* relative to the *NbADH* or *NtAllylADH* reference genes, respectively, which are present as one copy in the *N. benthamiana* and two in the *N. tabacum* genomes.

For the yeast signal sequence trap experiment (Oh et al. 2009), nucleotide sequences corresponding to positions 1 to 21 amino acids (SP fragment) of *PbBMST* was PCR-amplified. Yeast transformation was performed according to the protocol listed in the Yeastmaker Yeast Transformation System 2 (Clontech). The invertase-negative yeast strain YTK12 was transformed with a *pSUC2::SP*(*PbBSMT*) construct or empty vector *pSUC2* (negative control). Transformed colonies were transferred to CMD-W (without Trp) plates and were incubated at 30°C for 3 days. For the invertase secretion assay, transformed colonies were replica-plated on CMD-W plates and YPRAA (1% yeast extract, 2% peptone, 2% raffinose, and 2 µg/ml antimicyn A), containing raffinose and lacking glucose. After 3 days incubation at 30°C, the plates were checked for growth and were photographed.

Hormonal assays.

Plant hormones and their derivatives, including SA, auxin, cytokinin, gibberellic acid, ABA, and the precursor of ET (ACC), were quantified in the leaves and roots of *Arabidopsis* plants using Ultra-performance liquid chromatographic–electrospray ionization-tandem mass spectrometry in the Aquatic and Crop Resource Development lab at the National Research Council Canada, Saskatoon and the Max Planck Institute for Chemical Ecology, Jena, Germany (Chao et al. 2016; Chauvaux et al. 1997; Kodja et al. 2015; Lulai et al. 2016; Lulsdorf et al. 2013; Murmu et al. 2014; Vadassery et al. 2012).

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