A novel methyltransferase from the intracellular pathogen *Plasmodiophora brassicae* methylates salicylic acid

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SUMMARY

The obligate biotrophic pathogen *Plasmodiophora brassicae* causes clubroot disease in *Arabidopsis thaliana*, which is characterized by large root galls. Salicylic acid (SA) production is a defence response in plants, and its methyl ester is involved in systemic signalling. *Plasmodiophora brassicae* seems to suppress plant defence reactions, but information on how this is achieved is scarce. Here, we profile the changes in SA metabolism during *Arabidopsis* clubroot disease. The accumulation of SA and the emission of methylated SA (methyl salicylate, MeSA) were observed in *P. brassicae*-infected *Arabidopsis* 28 days after inoculation. There is evidence that MeSA is transported from infected roots to the upper plant. Analysis of the mutant *Atbsmt1*, deficient in the methylation of SA, indicated that the *Arabidopsis* SA methyltransferase was not responsible for alterations in clubroot symptoms. We found that *P. brassicae* possesses a methyltransferase (PbBSMT) with homology to plant methyltransferases. The *PbBSMT* gene is maximally transcribed when SA production is highest. By heterologous expression and enzymatic analyses, we showed that PbBSMT can methylate SA, benzoic and anthranilic acids.

Keywords: *Arabidopsis thaliana*, clubroot disease, methyltransferase, *Plasmodiophora brassicae*, salicylic acid.

INTRODUCTION

In order to access host carbon sources, obligate biotrophic plant pathogens establish an intricate relationship with their host that influences physiology and biochemical processes (Chandran *et al.*, 2010; Hok *et al.*, 2010). The phytomyxid *Plasmodiophora brassicae* is a biotrophic protist that causes clubroot in Brassicaceae—a disease of economic importance worldwide (Dixon, 2009). This

pathogen lives together with the host cells in an intricately balanced manner and, by reprogramming host plant cells, causes the formation of large hypertrophic root galls, which is largely dependent on alterations in plant hormone levels (Ludwig-Müller *et al.*, 2009). Its life cycle is mainly intracellular, and the major stage is an intracellular plasmodium which later develops into multiple resting spores (for a recent review of the life cycle, see Kageyama and Asano, 2009).

The phytomyxids belong to the eukaryotic supergroup Rhizaria (Bass *et al.*, 2005). They are phylogenetically distant from fungal and stramenopile plant pathogens which have also evolved biotrophic infection strategies (e.g. Baxter *et al.*, 2010; Schirawski *et al.*, 2010; Spanu *et al.*, 2010). Consequently, the study of *P. brassicae* may greatly expand the body of knowledge on pathogen infection strategies.

Salicylic acid (SA) is a crucial signal molecule for the activation of plant defence responses (Dempsey *et al.*, 2011; Loake and Grant, 2007). The regulation of SA in the plant cell is achieved, in part, by its maintenance in a range of different forms. SA is rendered largely inactive in localized defence induction by SA glucosyltransferase-mediated conjugation to glucose (forming SA β -glucoside) (Song *et al.*, 2008) or methylation to form methyl salicylate (MeSA) (Seskar *et al.*, 1998). Methylation of SA from the *S*-adenosine-L-methionine (SAM) methyl group donor is achieved by members of the distinct SABATH group of methyltransferases (D'Auria *et al.*, 2003). These enzymes have been shown to have similar and/or overlapping methylation activity against both SA and benzoic acid (BA) (Chen *et al.*, 2003; Dudareva *et al.*, 2000; Ross *et al.*, 1999). In addition to SA and BA, other SABATH enzymes act on diverse secondary metabolites, including jasmonic acid (JA), indole-3-acetic acid (IAA) and gibberellic acid (Qin *et al.*, 2005; Seo *et al.*, 2001; Varbanova *et al.*, 2007; Zhao *et al.*, 2008). The substrate targets for the great majority of the 24 *Arabidopsis* and 41 *Oryza sativa* SABATH enzymes remain unconfirmed.

The role of MeSA in plant defence and its interrelationship with benzoic acid/salicylic acid methyltransferase (BSMT) enzyme activity are not precisely understood. Because of its greater hydrophobicity than SA, MeSA is more volatile and membrane permeable

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and hence can move more easily between cells. MeSA can be converted back to free, active SA via the activity of an MeSA esterase (Forouhar *et al.*, 2005; Vlot *et al.*, 2008). Evidence that MeSA is a long-distance systemic acquired resistance (SAR) signal has been presented in tobacco (Park *et al.*, 2007). MeSA has been shown to be fully or partially required for the development of SAR, depending on the amount of light following infection (Liu *et al.*, 2011). Both biotic and abiotic stresses induced the transcription of Arabidopsis *AtBSMT1* in leaves (Chen *et al.*, 2003). Studies of Arabidopsis containing knockouts of *AtBSMT1* showed a near-complete reduction in MeSA production following pathogen infection (Attaran *et al.*, 2009; Liu *et al.*, 2010). Overexpression of the rice *OsBSMT1* gene in Arabidopsis led to reduced SA accumulation, constitutive MeSA production and greater susceptibility to attack by both *Pseudomonas syringae* and *Golovinomyces orontii* (Koo *et al.*, 2007).

Much research into pathogen infection strategies has focused on protein molecules that are secreted into cells to shut down plant defences, redirect nutrient flows, promote tissue colonization and influence signalling (Giraldo and Valent, 2013; Oliva *et al.*, 2010; Panstruga and Dodds, 2009). Bacterial proteins are the best understood effectors, and roles for a number have been confirmed, including proteases and E3 ligases (Deslandes and Rivas, 2012; Mansfield, 2009). By contrast, the molecular mechanisms of effectors from eukaryotes are less well understood; avirulence proteins from oomycetes and fungi have been shown to translocate into cells, but evidence for specific biochemical roles for these molecules is accumulating slowly (Djamei *et al.*, 2011; Hemetsberger *et al.*, 2012; Lee *et al.*, 2013). It is presumed that *P. brassicae* must secrete effectors into host cells to cause the wholesale cellular and tissue rearrangements seen in clubroot disease. However, the understanding of the molecular biology of *P. brassicae* is rudimentary (Siemens *et al.*, 2009a). Of the 76 genes examined in the principal study investigating full-length *P. brassicae* cDNAs to date, 17 had predicted signal sequences, but few, if any, of these presented a clear role in pathogenesis based on sequence similarity (Bulman *et al.*, 2006). A single *P. brassicae* gene from a subsequent collection (Bulman *et al.*, 2007) has been characterized in some detail at the biochemical level; a putatively secreted protein, Pro1, was shown to have *in vitro* serine protease activity (Feng *et al.*, 2010), although the actual substrate for this protease *in planta* remains unidentified. Furthermore, the addition of Pro1 to canola (*Brassica napus*) root exudates increased the positive effect of these exudates on the germination rate of resting spores, indicating a positive role for Pro1 during spore germination (Feng *et al.*, 2010). The first report of *P. brassicae* transformation has been published recently, but it has not been possible so far to demonstrate the expression of the transgenes in *P. brassicae* (Feng *et al.*, 2013).

In contrast with most biotrophic eukaryotes, in which feeding structures are bordered by the plant cell membrane, the intracel-

lular growth of *P. brassicae* means that secreted proteins are likely to enter the plant cytoplasm directly. Here, we present the first comprehensive data on SA metabolism and transport during clubroot disease, and propose an SA methyltransferase from *P. brassicae* by which this pathogen may manipulate SA in host cells.

RESULTS

Endogenous SA and MeSA levels in *P. brassicae*-infected plants

In many biotrophic plant–pathogen interactions, the defence hormone SA is induced, but so far this has not been investigated in the interaction between *P. brassicae* and Arabidopsis as host. The level of endogenous SA was therefore measured in Arabidopsis 21 and 28 days after infection (dai) with *P. brassicae*. These time points were chosen because, at 21 dai, galls are well developed and, at 28 dai, plants are completely stunted (culmination of infection; for the time points chosen, see also the respective clubroot symptoms in Fig. 7A). The level of SA in infected roots was less than that in controls at 21 dai, but increased at 28 dai. Leaves from infected and control plants contained similar amounts of SA at 21 dai, whereas, at 28 dai, the leaves of infected plants contained approximately 40 times more SA than the leaves of control plants (Fig. 1A). As high levels of SA were found especially in the leaves of infected plants, we were interested in whether SA would be emitted from leaves as its methyl ester (MeSA).

The increase in endogenous SA level at 28 dai was accompanied by the elicitation of the volatile MeSA, which was emitted from leaves of infected, but not control, plants (Fig. 1B). Although the emission of MeSA from the infected plant was seen in the total ion chromatogram (TIC) modus, this was even more obvious when a diagnostic ion (*m/z* 152) was selected. The complete ion traces are shown in Fig. S1 (see Supporting Information). MeSA emission was not detectable at earlier time points. As anthranilic acid (AA) was also a good substrate for the PbBSMT enzyme (Table 2), we checked for MeAA emission, but none could be found. To determine whether roots are able to produce MeSA, as these organs are in contact with the pathogen, they were incubated with deuterium-labelled SA (SAD₄) and the amount of deuterium-labelled MeSA (MeSAD₄) was analysed. After treatment with exogenous SAD₄, the roots of infected plants contained 17 times more MeSAD₄ at 21 dai, and 22 times more MeSAD₄ at 28 dai, than the roots of control plants (Fig. 1C).

Transport of SA in *P. brassicae*-infected plants

As the level of endogenous SA and the rate of SA methylation were higher in infected than in control plants, we further

Fig. 1 Analysis of salicylic acid (SA) and methyl salicylate (MeSA) transport in *Plasmodiophora brassicae*-infected and control Arabidopsis. For disease progression, see also photographs in Fig. 7A. (A) Gas chromatography-mass spectrometry (GC-MS) measurement of SA, for two time points, in roots and leaves of infected and control plants. The results are means of three experiments. (B) Emission of volatiles determined by GC-MS from leaves of infected and control plants at 28 days after infection (dai). The two right panels show a control sample; the two left panels show a sample from infected roots. The measurement was repeated twice and similar results were obtained. The two top panels show the total ion chromatogram; the two bottom panels represent the selected ion current of m/z 151–152 which is typical for MeSA. MeSA was identified by its mass spectrum; it was found in infected plants; in controls, no MeSA could be determined. The complete spectra are shown in Fig. S2. (C) The amount of deuterium-labelled MeSA (MeSAD₄) in the roots of infected and control plants after incubation (for 5 h) with deuterium-labelled SA (SAD₄). MeSAD₄ = [²H₄]MeSA; SAD₄ = [²H₄]SA. The measurements were performed at 21 and 28 dai. The experiment was repeated three times. (D) The amount of D₄-labelled compounds (SAD₄ and MeSAD₄) in the leaves of infected and control plants at 21 and 28 dai. SAD₄, roots were incubated in [²H₄]SA; MeSAD₄, roots were incubated in [²H₄]MeSA. Data are means $\pm$ SE of three independent experiments. (E) The amount of SAD₄ in leaves of infected Arabidopsis after the incubation of roots with either MeSAD₄ or MeSAD₄ and esterase inhibitor (TFA). The differences were significant between treatments in (E) at $P \leq 0.05$. The experiments in (C)–(E) were repeated three times with independently cultivated plants over different seasons.

investigated the transport of exogenous SAD₄ and MeSAD₄ from roots to leaves of infected and control plants. Plant roots were incubated with SAD₄ or MeSAD₄, and the accumulation of the respective labelled compounds in leaves was determined 5 h later by gas chromatography-mass spectrometry (GC-MS)

(Fig. 1D). MeSAD₄ and SAD₄ could be extracted at different pH values (Fig. S2, see Supporting Information); therefore, within one experiment, it was possible to analyse both metabolites simultaneously. Our experiments showed that both SA and MeSA moved systemically at both time points during clubroot

infection (Fig. 1D). Moreover, we used a synthetic SA analogue, 2,2,2',2'-tetrafluoroacetophenone (TFA), which competitively inhibits the activity of the MeSA esterase SABP2 (Park *et al.*, 2007). If the labelled SA found in the plants comes from endogenous esterase activity, the inhibitor should reduce the level of SAD₄. Indeed, simultaneous incubation of roots with MeSAD₄ and TFA resulted in lower accumulation of SAD₄ in leaves than when incubated with MeSAD₄ alone (Fig. 1E), suggesting that clubroot may affect MeSA movement from roots to leaves in *Arabidopsis*.

Cloning and structural analysis of *PbBSMT*

During the ongoing isolation of gene transcripts from an enriched cDNA library of *Arabidopsis thaliana* clubroot galls (Bulman *et al.*, 2006), a gene sequence with low primary amino acid sequence identity (maximum of 30%) to other (predicted) methyltransferases was isolated. The highest similarity to plant genes from the SABATH gene family (D'Auria *et al.*, 2003) was $1e^{-09}$. The gene sequence had no significant nucleotide similarity to the *Arabidopsis* genome (if from the plant host, the gene should have had close to 100% nucleotide similarity to *Arabidopsis* sequences), or to gene sequences of other organisms, and was thus judged to be from *P. brassicae*. Specific polymerase chain reaction (PCR) for this gene confirmed that it was present in DNA from infected clubroot galls, but not from uninfected *Arabidopsis* DNA (Fig. S3, see Supporting Information). Based on its putative homology to plant methyltransferases (Fig. 2) and its enzymatic activity (see below), the gene was named *PbBSMT*. PCR amplification showed that *PbBSMT* was also present in the single-spore German *P. brassicae* isolate e3 described by Föhling *et al.* (2003) (Fig. S3). Hybridization of restriction-digested genomic *P. brassicae* DNA provided evidence that *PbBSMT* is a single-copy gene (Fig. S4, see Supporting Information). To rule out contamination from the host plant, genomic DNA from *Brassica rapa* was also hybridized with the *PbBSMT* probe, but no signals were detected.

An apparently complete transcript (JN106050) collected from an oligo-capped library (Bulman *et al.*, 2006) was 1187 bp long with a 21-bp 5' untranslated region (UTR), and encoded a predicted protein of 371 amino acids (Fig. 2). DNA walking was used to obtain a genomic DNA sequence corresponding to the region from 1153 bp upstream of the *PbBSMT* cDNA start site to within 102 bp of the cDNA poly-A tail (JN106049). Alignment of the cDNA sequence with the genomic DNA sequence showed the presence of a single 52-bp intron of consistent structure to that previously seen in *P. brassicae* (Bulman *et al.*, 2007). No obvious evidence for protein coding genes was found in the *PbBSMT* upstream region by tBLASTX/BLASTX versus the GenBank nr (non-redundant protein sequences), env (metagenomic proteins), est (expressed sequence tags) databases. DNA sequencing showed no variation across 1082 bp between the *PbBSMT* genomic DNA

sequence first obtained from the New Zealand *P. brassicae* isolate and that from the German single-spore isolate e3.

BLASTP of the translated *PbBSMT* open reading frame (ORF) gave a hit ($7.37e^{-13}$) against a conserved Methyltransf_7 domain (pfam03492). Although Pfam annotation (Finn *et al.*, 2010) describes the pfam03492 domain as being confined to plant genes, examination of BLAST results indicated that genes from a wide range of organisms contain this domain. For example, BLASTP of the *Nicotiana suaveolens* BSMT (CAF31508) sequence gave a highest non-plant hit of $2e^{-23}$ to a *Mycobacterium vanbaalenii* PYR-1 sequence (YP_954558).

We located pfam03492-containing sequences held in GenBank by a series of iterative BLAST searches against the nr, est_others and env_nt databases. No sequences related to *PbBSMT* were located from the *Spongopora subterranea* 454 transcriptome dataset (Burki *et al.*, 2010). To construct a maximum likelihood phylogenetic tree, 43 full-length pfam03492-containing amino acid sequences were assembled and aligned (Fig. 3). The tree was characterized by long branch lengths, and the relationships between many major groups were unstable. Nevertheless, there were several clear groups of related organisms that indicated vertical evolution of the pfam03492-containing sequences. The plant SABATH sequences formed a single, discrete cluster, as did sequences from fungi. The *P. brassicae* sequence fell at the base of the fungal clade, but the phylogenetic affinity to these sequences was not robust (Fig. 3). The remainder of the sequences were from bacteria, with the exception of a group of aquatic eukaryotes which showed a strong affinity with sequences from marine bacteria/cyanobacteria, suggesting a horizontal gene transfer event. The gene sequence from *Pectobacterium wasabiae* (YP_003260227) was nested within a cluster of sequences from other bacteria, with no evidence of horizontal transfer from a plant genome (Nykyri *et al.*, 2012).

To investigate the structure of *PbBSMT*, the alignment of this protein to known plant SABATH amino acid sequences was examined. A number of motifs and conserved regions involved in substrate interaction and specificity were found in both *PbBSMT* and enzymes from *Arabidopsis*, *Clarkia breweri* and maize (*Zea mays*) (Fig. 2). These included amino acid residues involved in SAM binding, as well as several residues thought to be involved in the binding of SA. From sequences of AAMT (Köllner *et al.*, 2010), AtBSMT (Chen *et al.*, 2003), CbSAMT (Zubieta *et al.*, 2003) and *PbBSMT*, amino acid residues identified as being required for the specific binding of SA, BA or AA in *Nicotiana* species (Hippauf *et al.*, 2010) were examined (Table 1). For example, glutamine-167 (Gln167) found in maize AAMT and leucine-336 (Leu336) found in *Nicotiana* BSMTs were present in *PbBSMT*. Gln25 and valine-311 (Val311), amino acid residues more often found in SAMTs, were also present in *PbBSMT*. Several residues are unique for *PbBSMT*, whereas tryptamine-157 (Trp157) and tyrosine-261 (Tyr261) were found in all the protein sequences compared (Table 1).

Fig. 2 PbsMT has homology to the plant family of SABATH methyltransferases as shown by alignment with several plant SABATH proteins. AAMT, maize anthranilate methyltransferase; AtJMT, Arabidopsis jasmonate methyltransferase; CbSAMT, *Clarkia* salicylate methyltransferase; AtBSMT1, Arabidopsis salicylate/benzoate methyltransferase; AtIAMT, Arabidopsis indole-3-acetic acid (IAA) methyltransferase; PbsMT, *Plasmodiophora brassicae* salicylate/benzoate/anthranilate methyltransferase. The alignment is based on Zubietta *et al.* (2003). Red, S-adenosine-L-methionine (SAM)-binding residues; green, salicylic acid (SA)-binding residues (deduced from AtBSMT); cyan, additional active site residues. Barrels represent α -helices, arrows β -sheets (for an alignment with structural homology, see also Fig. S1).

Fig. 3 PHYML maximum likelihood tree of pfam03492-containing sequences. Figures above the nodes indicate the results of 500 bootstrap pseudoreplicates.

A structural model was created for PbBSMT using SWISS MODEL (Arnold *et al.*, 2006; Kiefer *et al.*, 2009; Peitsch, 1995). The sequences producing significant alignments were *Clarkia breweri* CbSMT (1M6E) with an *E* value of $6e^{-10}$ and an *Arabidopsis* methyltransferase specific for IAA, AtIAMT (3B5IA), with $2e^{-07}$. Crystal structures of AtIAMT and CbSMT, found in the PDB database, were employed to further predict homology using T-COFFEE (Notredame *et al.*, 2000). PbBSMT showed 67% structural homology to the *Arabidopsis* protein and 78% homology to the *Clarkia* enzyme (Fig. S6, see Supporting Information), whereas the homologies of the primary protein sequences for these comparisons were between 20% and 25%. The structural comparison showed high homology within the α -helical and β -sheet parts (Fig. S5, see Supporting Information). An overlay of CbSMT and PbBSMT, created using the standard settings in UCSF Chimera (Pettersen *et al.*, 2004), showed good matches between the two proteins (Fig. 4A). The positions of the amino acids important for substrate specificity were modelled into the structure (Fig. 4B). PbBSMT protein sequences with both methionines (Met) as start codons were used as templates in the structural predictions,

but no differences were found in the protein model (data not shown).

Enzymatic activity of PbBSMT

Using TargetP and SignalP (Emanuelsson *et al.*, 2000, 2007), the *P. brassicae* gene was predicted to encode a signal peptide targeting the protein to the secretory pathway. There was one highly probable predicted cleavage site, between amino acids 21 and 22, for the signal peptide (Fig. S7A, B, see Supporting Information). In contrast, the *Arabidopsis* AtBSMT does not contain a secretion signal (Fig. S7C). To test the enzymatic activity of the PbBSMT protein, we cloned the gene coding sequence (Fig. S4) into an *Escherichia coli* expression vector. Because we predicted that the active PbBSMT protein would have the signal peptide cleaved during export, the *E. coli* expressed product was constructed without the first 21 amino acids of the protein. We used Met at amino acid 22 to initiate expression.

PbBSMT was expressed in *E. coli* and purified as an active histidine (His)-tag protein fusion (Fig. 5). As no substrate was

Table 1 Important amino acid residues for PbBSMT substrate specificity (based on Hippauf *et al.*, 2010; Köllner *et al.*, 2010; Zubieta *et al.*, 2003). (A) Residues required for binding of S-adenosine-L-methionine (SAM). (B) Residues required for salicylic acid (SA), benzoic acid (BA) and anthranilic acid (AA) binding. For PbBSMT and AtBSMT, the residues at the respective positions in the CbSMT sequence in an alignment are given. Bold amino acids, similar in all SABATHs; italic amino acids, only present in PbBSMT. The question mark means that the correct position of the Ile residue could only be assumed. AAMT, maize MT; AtBSMT, Arabidopsis MT; CbSMT, *Clarkia breweri* MT; MT, methyltransferase; N.ala, *Nicotiana alata*; N.sua, *Nicotiana suaveolens*; N.syl, *Nicotiana sylvestris*; PbBSMT, *Plasmodiophora brassicae* MT.

PbBSMT	AtBSMT	CbSMT	N.sua SMT	N.ala SMT	N.syl SMT	N.sua BSMT2	N.ala BSMT2	N.syl BSMT2	N.sua BSMT1	AAMT1
(A)										
Ala	Ser	Lys10	Asn10	Asn	Asn	Asn10	Asn	Asn	Asn	Ala
Ser	Ser	Ser22	Ser22	Ser	Ser	Ser22	Ser	Ser	Ser	Ser
Asp	Glu	Asp57	Asp56	Asp	Asp	Asp56	Asp	Asp	Asp	Asp
Asp	Asp	Asp98	Asp96	Asp	Asp	Asp97	Asp	Asp	Asp	Asp
Trp	Leu	Leu99	Leu97	Leu	Leu	Leu98	Leu	Leu	Leu	Leu
Ser	Ser	Ser129	Ser135	Ser	Ser	Ser137	Ser	Ser	Ser	Ser
Phe	Phe	Phe130	Phe136	Phe	Phe	Phe138	Phe	Phe	Phe	Tyr
(B)										
Gln	Gln	Gln25	Gln25	Gln	Gln	Ala25	Ala	Gln	Gln	Gln
Ser	Ser	Tyr147	Tyr153	Tyr	Tyr	Tyr155	Tyr	Tyr	Phe	Phe
Gln	His	Met150	Met156	Met	Met	His158	Gln	His	His	Gln167
Trp	Trp	Trp151	Trp157	Trp	Trp	Trp159	Trp	Trp	Trp	Trp
Pro	Leu	Leu210	Leu216	Leu	Leu	Leu218	Leu	Leu	Met	Leu
Asp	Ile?	Ile225	Ile231	Ile	Ile	Val233	Val	Val	Ile	Leu
Tyr	Trp	Trp226	Trp232	Trp	Trp	Leu234	Leu	Trp	Leu	Tyr
Tyr	Tyr	Tyr255	Tyr261	Tyr	Tyr	Tyr263	Tyr	Tyr	Tyr	Tyr246
Leu	Ile	Met308	Met308	Met	Met	Leu336	Leu	Leu	Met	Leu
Val	Val	Val311	Val311	Val	Val	Val339	Leu	Val	Phe	Val
Trp	Thr	Phe347	Phe347	Phe	Phe	Phe376	Phe	Phe	Ser	His

Ala, alanine; Asn, asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

known for PbBSMT, we used a radioactive assay with [14 C]SAM as methyl donor (Zhao *et al.*, 2008). PbBSMT was able to convert SA, BA and AA to the respective methyl esters (Tables 2 and S1, see Supporting Information) with enzymatic activities between 0.12 and 0.26 nkat/mg protein for the different substrates at 4 μ M SAM (conversion rate of [14 C]SAM with SA as substrate was between 10% and 18%). Increasing [14 C]SAM concentrations also resulted in an increased enzymatic activity at non-limiting concentrations for the acidic substrates SA and BA. The identities of the reaction products MeSA and MeAA were confirmed by GC-MS (Fig. 6). We found some methylation of 4-hydroxybenzoic acid, which is structurally close to SA (2-hydroxybenzoic acid), but the molecule is larger because of the different position of the hydroxyl group. No other activities were detected with IAA, JA, gibberellic or cinnamic acid derivatives as the substrate. Caffeic acid, which is N-methylated by another class of SABATH methyltransferases (Ogawa *et al.*, 2001), was also not a substrate for PbBSMT. Various controls, including empty *E. coli* cells, cells containing the empty vector (vector control), heat-inactivated protein and buffer only, did not show any conversion (Table S1).

To test whether AtBSMT1 contributes to the methylation of SA during *P. brassicae* infection, disease development in the Arabidopsis *bsmt1* mutant (Attaran *et al.*, 2009) was investigated. The phytopathological analysis showed no significant difference in clubroot disease severity, as calculated by disease indices of 74 for the wild-type and 65 and 69 for two different alleles of the *Atbsmt1* mutant, respectively (Fig. S8, see Supporting Information).

Transcription of *PbBSMT* during *P. brassicae* infection

The disease progression in Arabidopsis for the gene transcription experiments followed a typical pattern; no gall formation was seen at early time periods (4–11 dai), and root enlargement was first seen at 18 dai. By 21 dai, a major part of the root masses consisted of galls (Fig. 7A).

Amplification of both Arabidopsis reference genes was consistent across the course of the experiment, with *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) showing a higher level of transcription than *Actin8*. The C_t values for *PbBSMT* were near the limits of detection for the 4-dai RNA sample. For the 4-dai sample, the proportion of both *P. brassicae* transcripts relative to those from the plant was low (Fig. 7B). As the infection cycle progressed, the proportion of both the *PbEFL* and *PbBSMT* transcripts increased markedly relative to the plant reference genes. The pattern of increase for the two genes was quite similar during the progression of disease symptoms.

Re-examination of a *B. rapa*—*P. brassicae* callus culture transcriptome dataset (Burki *et al.*, 2010) for reads of the *PbBSMT* sequence showed that *PbBSMT* sequences were present at a surprisingly high frequency. *PbBSMT* was represented by 139 reads, whereas a range of *P. brassicae* housekeeping genes were represented by between two and 25 reads each. Such a high transcript number could indicate the importance of the encoded protein.

Fig. 4 (A) Modelling of PbBSMT on the basis of two plant methyltransferases for which crystal structures are available. An overlay of the protein model of PbBSMT obtained (in grey) with a CbSMT monomer (in magenta) is shown. The model was built in SWISS MODEL and the overlay was created with the standard settings of Chimera. (B) Amino acids, which are believed to be of importance (Table 1) in other SABATH methyltransferase members, are shown for the substrate-binding pocket. The amino acids of the putative salicylic acid-binding site are based on the crystal structure of CbBSMT and models of other methyltransferases (see Table 1); the numbers of the respective amino acids are based on the position in PbBSMT. Green, similar in all methyltransferases of Table 1; magenta, altered amino acid in this position in PbBSMT.

DISCUSSION

Plant infection by phytomyxids requires a sophisticated subversion of host cells. In *P. brassicae*, much attention has been paid to the regulation of the plant hormones auxin and cytokinin during clubroot establishment (Ludwig-Müller and Schuller, 2008). Less is known about SA, ethylene and JA signalling, which are important for plant defence in other plant–pathogen interactions (Grant and Lamb, 2006; Howe, 2004; Koornneef and Pieterse, 2008; Van Loon *et al.*, 2006). SA is a pivotal signal in local and systemic defence (Dempsey *et al.*, 2011; Vlot *et al.*, 2009). However, our understanding of SA signalling during interactions with plant root pathogens lags behind that for foliar pathogens (e.g. Attard *et al.*, 2010).

Although the SA level in control plants decreased with time, in *P. brassicae*-infected plants it increased, and this was particularly obvious in the leaves. At 28 dai, both roots and leaves of infected plants contained more SA than those from control plants; such large-scale SA accumulation occurs in a number of plant–pathogen interactions, although it is unclear whether this is a plant defence signalling process or a secondary effect of cellular stress (Loake and Grant, 2007; Ryals *et al.*, 1996). MeSA was emitted from leaves and moved from roots to the upper plant tissue. Furthermore, it was possible to inhibit MeSAD₄ to SAD₄ conversion in infected plants using the artificial analogue TFA, which competitively inhibits the activity of the MeSA esterase SABP2 (Park *et al.*, 2007). We reasoned that the levels of labelled SA found in infected plants could derive from a plant esterase activity. Indeed, the addition of the esterase inhibitor resulted in lower SAD₄ and higher MeSAD₄ amounts (Fig. 1). This finding suggests that MeSA found in leaves partially originates from the site of *P. brassicae* infection, raising the question of whether this MeSA is produced by Arabidopsis, by *P. brassicae* itself or by the action of both organisms (see model in Fig. 8).

We were therefore intrigued to discover whether a *P. brassicae* gene with similarity to plant SABATH genes was capable of modifying SA. Although SABATH enzymes are reportedly confined to plants (e.g. D'Auria *et al.*, 2003), similarity searches showed that proteins from a wide range of organisms contain the pfam03492 domain. This raises the question as to whether these uncharacterized proteins are functionally related methyltransferases. At a primary sequence level, the *P. brassicae* BSMT protein is only distantly related to the plant SABATH enzymes, and phylogenies constructed from full-length protein sequences gave few clues as to the functions of these genes; the relationships between clusters of sequences were unstable and the plant enzymes formed a discrete cluster from other organisms. The *PbBSMT* sequence did not group robustly with any other sequences, consistent with the phylogenetic separation of Rhizaria from other studied organisms, and a long period of vertical rather than horizontal evolution.

Despite these phylogenetic distances to known methyltransferases, *PbBSMT* heterologously expressed in *E. coli* catalysed the methylation of SA, BA and AA, confirming that a protein from an organism outside of Plantae has SABATH-like methyltransferase activity. The activity of PbBSMT against these particular substrates, but not against a range of other molecules, was consistent with protein alignments and modelling, which showed that PbBSMT had higher structural similarity to the crystal structures of CbSMT than to the closely related AtIAMT (Zhao *et al.*, 2008). Most SA-methylating enzymes have preference for the SA substrate, whereas the conversion of BA and/or AA is less pronounced (Huang *et al.*, 2012). The AtBSMT enzyme differs from PbBSMT in showing only moderate methylation of BA, AA and 4-hydroxybenzoate (Chen *et al.*, 2003).

Fig. 5 Induction and enrichment of PbBSMT for enzymatic assays. (A) Induction with arabinose for different incubation times. (B) Enrichment via metal affinity chromatography (left lane, protein preparation of empty cells; right lane, protein preparation of cells containing the PbBSMT plasmid). The protein concentration was estimated using BSA standards between 0.1 and 1.0 μg protein on the same gel (not shown).

Table 2 Substrate specificity of PbBSMT (nd, not determined). Values are means from at least three different determinations. The percentage conversion is given for 4 μM [^{14}C]SAM. Values given as '0' activity were not above any control. The controls are shown in Table S1.

Substrate	Enzymatic activity (nkat/mg protein) [^{14}C]SAM concentration (μM)			Conversion of [^{14}C]SAM (%)
	4	8	12	
Anthranilic acid	0.17 $\pm$ 0.008	nd	nd	15
Benzoic acid	0.12 $\pm$ 0.005	0.18 $\pm$ 0.001	nd	12
Salicylic acid	0.15 $\pm$ 0.025	0.22 $\pm$ 0.085	0.26 $\pm$ 0.027	14
4-Hydroxybenzoic acid	0.04 $\pm$ 0.005	nd	nd	4
Indole-3-acetic acid	0	0	nd	0
Jasmonic acid	0	nd	nd	0
Gibberellic acid	0	nd	nd	0
<i>p</i> -Coumaric acid	0	nd	nd	0
Vanillic acid	0	nd	nd	0
Caffeic acid	0	nd	nd	0

SAM, *S*-adenosine-L-methionine.

Quantitative PCR assays, as well as evidence from an expressed sequence tag (EST) study (Bulman *et al.*, 2006) and callus culture transcriptome work (Bulman *et al.*, 2011; Burki *et al.*, 2010), indicated that *PbBSMT* transcripts were at low levels during early infection and highest when galls were present. At 4 dai, the *PbBSMT* levels were near the detection limit of the quantitative PCR method. By 7 dai, we observed an increase in *PbBSMT* transcription, which is not inconsistent with a role in the modification of SA, as initial *P. brassicae* penetration of the root cortex, signalling the onset of secondary infection, can be detected as early as 5 dai (Siemens *et al.*, 2009b). Although it can be argued that the expression of a *P. brassicae* gene throughout the infection process is consistent with a role in the modification of plant defences (Feng *et al.*, 2010), increased expression of *PbBSMT* later in infection is congruent with its postulated role; if *PbBSMT* hinders

SA-mediated plant defence, it would indeed be most required during secondary infection when that response is strongest. A variety of studies have suggested that initial primary infection by plasmodiophorids meets a limited response from the plant (Siemens *et al.*, 2009a).

That *PbBSMT* methylation of SA would be a plausible mechanism for rendering SA inactive in triggering defence mechanisms (Seskar *et al.*, 1998) is supported by a number of other studies. Following the overexpression of *OsBSMT* in Arabidopsis, MeSA levels were elevated, SA levels were reduced and susceptibility to infection by a biotrophic pathogen was increased (Koo *et al.*, 2007). Similarly, overexpression of *AtBSMT1* in Arabidopsis resulted in an increase in MeSA and compromised SAR (Liu *et al.*, 2010). Knockdown of SAMT in tomato plants led to reduced MeSA production and increased resistance to the root pathogen

Fig. 6 Gas chromatography-mass spectrometry (GC-MS) confirmation of methyl salicylate (MeSA) (A) and methyl anthranilate (MeAA) (B) formation by PbBSMT. The top panels show the respective standards; the bottom panels show the enzymatic product.

Fusarium oxysporum (Ament *et al.*, 2010). In accordance with a function for SA in defence against *P. brassicae*, treatment of Arabidopsis roots with 0.5 mM SA before inoculation completely reduced clubroot symptoms (Agarwal *et al.*, 2011). The possible role for PbBSMT in the methylation of SA is complemented by the additional observation that the *Atbsmt1* mutant did not show altered disease symptoms, suggesting that AtBSMT is not responsible for the SA to MeSA conversion seen during clubroot disease. Measurement of SA metabolism in the *Atbsmt* mutant awaits confirmation, as does the possible role in SA methylation of other Arabidopsis proteins, which must also be considered. Our proposed model for the function of PbBSMT in the light of MeSA transport and emission from Arabidopsis is shown in Fig. 8. It summarizes the idea that the plant's defence route via SA can be altered by an SA methyltransferase from the protist, so that MeSA is the major transport form and finally emitted from leaves. However, future experiments, including more mutants and complementation of plants with the *PbBSMT* gene, could lead to an understanding of this mode of action.

With PbBSMT, the second protein with possible function in pathogenicity from *P. brassicae* was characterized. Although the protease Pro1 has been proposed to play a role in a completely

different process, i.e. germination of resting spores via its proteolytic activity (Feng *et al.*, 2010), PbBSMT is active during later stages of the life cycle, as presumed from the expression analysis (Fig. 7). Although methylation of SA via PbBSMT would be a novel pathogenicity approach, studies of other microorganisms have pointed to related infection strategies. The *Pseudomonas syringae* virulence factor coronatine functions in part to reduce SA-mediated plant defences; it has been shown to repress isochorismate synthase gene 1 and to activate AtBSMT1, thus increasing the production of the volatile MeSA and decreasing SA at infection sites (Attaran *et al.*, 2009; Zheng *et al.*, 2012). The smut fungus *Ustilago maydis* secretes an effector encoding a chorismate mutase into maize (Djamei *et al.*, 2011). This effector converts chorismate to prephenate, making chorismate less available as a precursor for plant SA synthesis. Hence, although *U. maydis* has been proposed to indirectly modulate SA levels, *P. brassicae* may directly metabolize SA, as the protein has a putative secretion signal (Fig. S7A, B) and could therefore act in the host cell. A pfam03492 motif was recently found in a gene from the phytopathogenic bacterium *Pectobacterium wasabiae*, and Nykyri *et al.* (2012) postulated that this may be a horizontally acquired plant gene involved in pathogenesis. Although this represents an

Fig. 7 Transcript analysis of *PbBSMT* during infection in roots of *Arabidopsis* plants at 4 to 24 days after infection (dai). (A) Time course of disease development. Examples of roots of infected plants at 7, 13, 21 and 25 dai are shown because of their distinctive stages in clubroot symptom progression. (B) Samples were analysed for *PbBSMT* and *PbEFL* expression by quantitative polymerase chain reaction (PCR) at different time points after inoculation (dai). Fold difference in expression compared with day 4 normalized to *Arabidopsis glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) is shown. Normalization of data on *Arabidopsis Actin8* (data not shown) gave similar results.

Fig. 8 A model showing the possible role of *PbBSMT* in salicylic acid (SA)-dependent plant defence based on our findings. *Plasmodiophora brassicae* secretes *PbBSMT* into the host cell, where it methylates the defence signal SA. The resulting methyl salicylate (MeSA) fails to up-regulate plant defence reactions and is transmitted to leaves, where it is emitted or converted back to SA.

excellent candidate for further characterization, our phylogenies indicated that this gene is not horizontally acquired from plants.

As PbBSMT is active against more than one substrate, we cannot rule out that it has another target *in planta*. Nevertheless, we presume that AA is not this target as MeAA was not emitted from infected plants. Similarly, it is possible that the signal sequence does not target the PbBSMT enzyme to the plant cytoplasm, but instead to a location within the *P. brassicae* plasmodia. As plasmodia are in close contact with the host cytoplasm, a role for detoxification of SA can still be made.

Unlike many other candidate effectors, the primary DNA sequence provides a tantalizing clue as to the function of the PbBSMT protein. Here, we have confirmed that PbBSMT has *in vitro* SA methylation capability, and have provided supporting evidence that such activity by PbBSMT may be important during infection. Future studies to confirm the role of this protein during clubroot progression will include the localization of PbBSMT in infected plant cells (e.g. Catanzariti *et al.*, 2006; Kemen *et al.*, 2005) and the transformation and analysis of Arabidopsis mutants (e.g. *Atbsmt1*) with the *PbBSMT* gene for a more in-depth characterization of the *in planta* function of this protein.

EXPERIMENTAL PROCEDURES

Plant and pathogen material

Arabidopsis thaliana ecotype Columbia (Col-0) was used. The Arabidopsis *bsmt1-1* and *bsmt1-2* mutants were those described in Attaran *et al.* (2009). The single-spore *P. brassicae* isolate e3 (Fähling *et al.*, 2003) was propagated in *B. rapa* ssp. *pekinensis*. Resting spores were extracted by homogenization of clubroot galls, filtration through gauze (pore width, 25 µm) and two centrifugation steps (2500 g, 10 min).

Fourteen-day-old Arabidopsis plants were inoculated by injecting the soil around each plant with 2 mL of isolate e3 resting spore suspension (10^6 spores/mL) according to Siemens *et al.* (2002). Control plants were treated with buffer only.

Phytopathological analysis

The disease index (DI) was calculated by categorizing the individual roots at 28 dai into five classes (0, no symptoms; 1 + 2, roots with light symptoms; 3 + 4, roots with severe symptoms), according to Siemens *et al.* (2002), and using the following equation: $DI = (1n_1 + 2n_2 + 3n_3 + 4n_4)100/4N_t$, where n_1 – n_4 are the numbers of plants in the indicated classes and N_t is the total number of plants tested. For each biological experiment, c. 40 Arabidopsis plants were analysed.

Volatile collection

Plant volatile organic compounds (VOCs) were collected for 48 h using the closed-loop stripping method according to Donath and Boland (1995). Here, 10 plants were enclosed in an exsiccator which was connected to a

circulation pump containing a charcoal trap. By providing air circulation, emitted VOCs were continuously collected on charcoal filters. Desorption was performed using methylene chloride (2×20 µL) containing 40 µg/mL *n*-bromodecane as internal standard, and the volatiles were analysed using GC-MS (TRACE 2000 series, Finnigan, Ipswich, Suffolk, UK).

Determination of endogenous SA

SA was determined according to Leitner *et al.* (2008) using GC-MS (TRACE 2000 series, Finnigan) in the selective ion mode. The fragment ion was monitored at m/z 120. The endogenous concentrations of SA were calculated from the peak areas of the respective substance and its standard using calibration curves. The experiment was performed three times and the standard errors are indicated.

Transport of SA and MeSA

The experiment was performed as three independent biological replicates with plants cultivated at different seasons. Plants were harvested at 14, 21 and 28 dai and their roots were incubated in 100 mM, pH 6.5, methanesulfonic acid (MES) buffer containing either 1 mM deuterated SA ($[^2H_4]$ SA) or 1 mM deuterated methyl salicylate ($[^2H_4]$ MeSA). $[^2H_4]$ SA was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). $[^2H_4]$ MeSA was chemically obtained from $[^2H_4]$ SA by methylation with diazomethane (Cohen, 1984). To inhibit esterase activity, 1 mM TFA (Rieke Metals, Lincoln, NE, USA) was added simultaneously with 1 mM $[^2H_4]$ MeSA. The solvent concentration never exceeded 1% methanol. Incubation lasted for 5 h, and then the plants were separated into roots, leaves and inflorescences. The contents of $[^2H_4]$ SA and $[^2H_4]$ MeSA were determined according to Chen *et al.* (1988), with appropriate modifications for SA/MeSA, at pH 3.0 and pH 7.0, respectively. One microlitre of each sample was injected into a GC-MS (Varian Saturn Series, Agilent, Walnut Creek, CA, USA) containing a Phenomenex (Aschaffenburg, Germany) ZB-5 column (30 m $\times$ 0.25 mm $\times$ 0.25 µm). The temperature programme was 60 °C for 1 min, going to 220 °C at a rate of 25 °C/min, and then to 280 °C at 20 °C/min, with a hold for 5 min at 280 °C. $[^2H_4]$ MeSA eluted at 5.5 min. Diagnostic ions were m/z 124 and m/z 156 for $[^2H_4]$ MeSA; the ion at m/z 124 was used for quantification.

To determine the efficiency of extraction of $[^2H_4]$ SA vs. $[^2H_4]$ MeSA, both stock solutions were dissolved in buffer and then extracted at their respective pH values, as described previously (Fig. S1).

Southern blot

Extraction of genomic DNA from plants was performed according to Fähling *et al.* (2003). *Plasmodiophora brassicae* DNA was isolated from resting spores according to Grsic-Rausch *et al.* (2000).

Genomic DNA (20 µg) was cut with *Apal* (1), *Haelll* (2) and *HindIII* (3), and separated on a 1% agarose gel. Southern blotting was performed with a PCR probe amplified with the primers PbBSMTF and PbBSMTR (Table S2, see Supporting Information), generating a c. 580-bp product (Fig. S3). Probe labelling was performed with the Amersham Gene Images AlkPhos Direct Labelling kit (GE Healthcare, Braunschweig, Germany). Detection was carried out with the Chemiluminescence Detection System (GE Healthcare).

Hybridization of the membrane (Serva Nylon bind B; Serva Electrophoresis GmbH, Heidelberg, Germany) was performed overnight at 55 °C. Incubation was carried out for 5 min. X-Ray film was developed for 30 min.

Amplification and cloning of full-length *PbBSMT*

The *PbBSMT* coding sequence, starting from the second predicted Met (Fig. S7), was amplified with the *PbBSMTNde1F* and *PbBSMTNot1R* PCR primers (Table S2) using Accuprime Taq (Invitrogen, Life Technologies, Darmstadt, Germany). This fragment did not contain the predicted signal peptide. Fragments were cloned into the pCR4-TOPO vector (Invitrogen). Plasmids containing the correct insert were digested, gel purified and then cloned into the AY2-4 expression vector (Guzman *et al.*, 1995).

Expression and purification of *PbBSMT* in *E. coli*

Escherichia coli BL21(DE3) codon plus cells (Stratagene, Agilent Technologies, Waldbronn, Germany) were grown overnight at 37 °C in Luria–Bertani medium containing 100 µg/mL ampicillin, 50 µg/mL chloramphenicol, 10 µg/mL tetracycline and 0.2% (w/v) glucose. Expression of *PbBSMT* was induced with 0.05% (w/v) arabinose for 4 h at 28 °C. Cells were harvested by centrifugation (5000 g, 10 min), resuspended in lysis buffer [50 mM NaH₂PO₄, 300 mM NaCl, 10 mg/mL lysozyme, 10 mM MgCl₂, 0.25% (v/v) Tween, 10 mM imidazole, pH 8] and subjected to three freeze–thaw cycles (30 s in liquid N₂, 10 min at 37 °C). After centrifugation (12 000 g, 20 min, 4 °C), the supernatant was incubated with Ni-NTA His Bind Superflow (Novagen, Merck, Darmstadt, Germany) for 30 min at room temperature. After four washes with 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8, the protein was eluted using 50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8. Protein purity was analysed by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantified using the BCA Protein Assay Kit with bovine serum albumin as standard (Thermo Fisher Scientific, Waltham, MA, USA).

Enzymatic assay for methyltransferase activity

The enzymatic assay with different substrates (see Table 1) was performed according to Zhao *et al.* (2008). At least three independent assays were performed for each substrate. The non-radioactive assay was performed with SA and AA, and the radioactive assay with SA and BA.

Phylogram of SABATH family members

pfam03492-containing sequences were located by iterative BLAST searches against the GenBank nr, est_others and env_nt databases, and the *Spongopora subterranea*-infected *Solanum tuberosum* transcriptome dataset (SRP003604.2) (Burki *et al.*, 2010). The transcriptome dataset was prescreened to remove *Solanum* sequences (Burki *et al.*, 2010). ORFs were predicted in metagenomic and EST sequences using Geneious Pro 5.0.3 (Drummond *et al.*, 2010). Protein sequences were aligned using MUSCLE (Edgar, 2004). Maximum likelihood phylogenetic trees were constructed using PHYML (Guindon and Gascuel, 2003) with the WAG model. As an

estimate of tree robustness, 500 bootstraps were completed. All phylogenetic manipulations were completed in Geneious.

Structural analysis of *PbBSMT*

Putative signal sequences in *PbBSMT* were predicted using SignalP 3.0 and TargetP (Emanuelsson *et al.*, 2000, 2007).

To compare the amino acids possibly involved in enzymatic activity, an alignment of selected plant SABATH methyltransferase family members with *PbBSMT* was created with CLUSTALW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The *PbBSMT* protein sequence was then manually adjusted based on the alignments of AtBSMT and CbSMT (Zubieta *et al.*, 2003). A model was created for *PbBSMT* using SWISS MODEL (Arnold *et al.*, 2006; Kiefer *et al.*, 2009; Peitsch, 1995), based on the two crystal structures in the PDB database with the highest structural similarity to *PbBSMT*: CbSMT (1M6E) and AtIAMT (3B5IA). Further homology prediction was performed using T-COFFEE (Notredame *et al.*, 2000). The alignments and modeling steps were performed with standard settings. The overlay of CbSMT and *PbBSMT* was created using the standard settings of UCSF Chimera (Pettersen *et al.*, 2004).

Quantitative detection of *PbBSMT* transcription

Arabidopsis growth and infections were carried out essentially as in Bulman *et al.* (2006). Plants were harvested at 4, 7, 11, 18, 21 and 24 dai. Roots were subjected to bead-beating for 2 min. RNA extraction was performed with Concert Plant RNA Reagent (Invitrogen). DNA removal from RNA preparations was performed with a Turbo DNA-free kit (Invitrogen). cDNA synthesis was completed with the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Quantitative reverse transcription-PCR was performed using the SYBR Green Supermix Plus ROX assay (Bio-Rad Laboratories, München, Germany). Amplification was carried out for 40 cycles of 95 °C (15 s), annealing (20 s), 72 °C (20 s), with an initial activation step of 95 °C (2 min 30 s), followed by post-run melt curve analyses (StepOnePlus Real-Time PCR System, Applied Biosystems, Life Technologies, Darmstadt, Germany). Primer annealing temperatures are shown in Table S2. For Arabidopsis, the *GAPDH* and *Actin8* (At1g49240) reference genes were used. In addition to the *PbBSMT* gene (JN106050), quantitative PCR was carried out for the elongation factor-like (*PbEFL*) gene (SRX027224) (Burki *et al.*, 2010). ΔC_T values for the *P. brassicae* genes were calibrated against the Arabidopsis reference genes (Livak and Schmittgen, 2001). Relative quantification was calculated using the $\Delta\Delta C_T$ method with reference to the transcription levels at day 4.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Complete gas chromatography-mass spectrometry (GC-MS) chromatograms from Fig. 1B.

Fig. S2 Chromatograms to distinguish between methyl salicylate (MeSA) and salicylic acid (SA) after extraction of the aqueous phase with ethyl acetate at neutral and acidic pH, respectively.

Fig. S3 Amplification of the *PbBSMT* sequence from genomic DNA and cDNA from the single-spore *Plasmodiophora brassicae* isolate e3.

Fig. S4 *PbBSMT* nucleotide sequence, prediction of patterns for Southern blot analysis and Southern blot.

Fig. S5 Structural homology between *PbBSMT* (Target) and *CbSAMT* (1m6eX) from *Clarkia breweri*, whose crystal structure is available.

Fig. S6 The structural homology for *PbBSMT* with two plant homologues *AtIAMT* and *CbSAMT*.

Fig. S7 Signal peptide analysis for *PbBSMT* and *AtBSMT* reveals that *PbBSMT* has a predicted secretion signal.

Fig. S8 Analysis of the *Arabidopsis bsmt1* mutant for infection with *Plasmodiophora brassicae*.

Table S1 Enzymatic assay of *PbBSMT* with [¹⁴C]-adenosine-L-methionine ([¹⁴C]SAM) as methyl group donor and various substrates.

Table S2 Primers used in this study.