An *Arabidopsis thaliana* gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense

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**Summary**

Emission of methylsalicylate (MeSA), and occasionally of methylbenzoate (MeBA), from *Arabidopsis thaliana* leaves was detected following the application of some forms of both biotic and abiotic stresses to the plant. Maximal emission of MeSA was observed following alamethicin treatment of leaves. A gene (AtBSMT1) encoding a protein with both benzoic acid (BA) and salicylic acid (SA) carboxyl methyltransferase activity was identified using a biochemical genomics approach. Its ortholog (AIBSMT1) in *A. lyrata*, a close relative of *A. thaliana*, was also isolated. The AtBSMT1 protein utilizes SA more efficiently than BA, whereas AIBSMT1 catalyzes the methylation of SA less effectively than that of BA. The AtBSMT1 and AIBSMT1 genes showed expression in leaves under normal growth conditions and were more highly expressed in the flowers. In *A. thaliana* leaves, the expression of AtBSMT1 was induced by alamethicin, *Plutella xylostella* herbivory, uprooting, physical wounding, and methyl jasmonate. SA was not an effective inducer. Using a β-glucuronidase (GUS) reporter approach, the promoter activity of AtBSMT1 was localized to the sepalas of flowers, and also to leaf trichomes and hydathodes. Upon thrip damage to leaves, AtBSMT1 promoter activity was induced specifically around the lesions.

**Keywords: Arabidopsis lyrata**, benzenoids, emission, gene family, methyltransferase, volatile esters.

**Introduction**

Many plants, particularly those pollinated by moths, emit floral scents that contain benzenoid esters such as methylsalicylate (MeSA) and methylbenzoate (MeBA) to attract these insect pollinators (Knudsen et al., 1993). The moth *Hyles lineata* showed strong electroantennogram response to MeSA, indicating that it can detect MeSA at very low concentrations (Raguso et al., 1996). Some flowers, such as *Anthurium majus* (snapdragon) and *Stephanotis floribunda*, show a diurnal rhythm of MeSA and/or MeBA emission, with peak emission coinciding with the peak activity of the respective pollinator, and this rhythm appears to be controlled by a circadian clock (Kolosova et al., 2001; Pott et al., 2002).

Reports of the synthesis and/or emission of benzenoid esters from leaves are fewer, but this may simply reflect the paucity of studies of vegetative volatiles compared with floral scent analyses. Shulaev et al. (1997) reported that tobacco leaves infected with the tobacco mosaic virus (TMV) synthesized and emitted MeSA from both infected and non-infected leaves, and that under very high concentrations of airborne MeSA, neighboring plants could absorb MeSA, convert it to salicylic acid (SA), and thereby induce SA-mediated defense response against TMV (Shulaev et al., 1997). More recently, leaves of *Arabidopsis thaliana* attacked by herbivorous insects were shown to emit MeSA as well as many other volatiles (Van Poecke et al., 2001). Although the specific biological function of MeSA in this case is currently unknown, it was hypothesized that the emission of a blend of volatiles is part of an ecological mechanism to attract predatory insects that prey on the herbivorous insects causing the initial damage to the plant (Van Poecke et al., 2001). MeSA and MeBA also function as
animal and insect toxins that exert their deleterious effects internally after being ingested by the organism (Lindberg et al., 2000). Moreover, MeSA has also been shown to possess microbicidal properties (Demirci et al., 2000).

MeSA and MeBA are synthesized in planta via a reaction catalyzed by methyltransferases whereby a methyl group is transferred from the donor molecule S-adenosine-L-methionine (SAM) to the carboxyl group of SA or benzoic acid (BA), respectively. SA methyltransferase (SMT) activity has been characterized in flowers of three different species – Clarkia breweri, S. floribunda, and snapdragon, and cDNAs encoding SMT from these species have been obtained (Negre et al., 2002; Pott et al., 2002; Ross, 2002; Ross et al., 1999). The purified enzymes can methylate both SA and BA, but all possess Km values for BA that are 3–10-fold higher than their Km values for SA (Negre et al., 2002; Pott et al., 2002; Ross et al., 1999). In contrast, only one protein with predominantly BA methylating activity has been characterized thus far. The snapdragon BA methyltransferase (BAMT) showed activity with BA but not with SA, although the Km value for the physiological substrate BA, 1.1 mM (Dudareva et al., 2000), is considerably higher than the reported Km values for SMTs using SA, which range from 24 to 83 μM. So far, no SMT or BAMT enzymatic activities in these species have been detected in organs other than flowers (a putative SMT, with a poorly characterized substrate specificity, was reported to be induced in hairy root cultures of Atropa belladonna upon SA treatment; Fukami et al. (2002)). Specifically, neither the expression of SMT and BAMT could be detected in leaves under normal conditions, nor was the expression of SMT and BAMT induced in leaves by various treatments, including administration of SA or methyljasmonate (MeJA; Negre et al., 2002; Ross, 2002).

SMT and BAMT belong to a structurally related group of methyltransferases termed the SABATH family based on the first three identified enzymes belonging to this group, SMT, BAMT, and theobromine synthase (D’Auria et al., 2003). The SABATH proteins show no significant sequence similarity to other known methyltransferases (D’Auria et al., 2003; Noel et al., 2003). The A. thaliana genome contains 24 intact genes of this type, which are collectively called the SABATH genes (D’Auria et al., 2003). Only one of these has been functionally characterized – jasmonic acid carboxyl methyltransferase (JMT; renamed here AtJMT1), the gene encoding the enzyme that catalyzes the formation of MeJA from jasmonic acid (JA) (Seo et al., 2001). Analysis of sequence similarity indicates that none of the 23 uncharacterized AtSABATH proteins is particularly similar in sequence to known SAMTs (i.e. they are all <45% identical to the C. breweri SMT) (D’Auria et al., 2003).

We have undertaken the task to determine the function of all AtSABATH genes. As it was previously reported that A. thaliana could synthesize and emit the ubiquitous plant compound MeSA (Van Poecke et al., 2001), we asked whether the enzyme responsible for methylation of SA belongs to the SABATH protein family of methyltransferases, and if so, in what tissues and under what conditions is its gene expressed? Here, we report the identification of a SABATH gene from A. thaliana and its ortholog from A. lyrata that encode enzymes embodied with both SMT and BAMT activities. We also present data suggesting that the A. thaliana gene is involved in the plant response to biotic and abiotic stresses.

Results

Detection of MeSA emission from A. thaliana plants

We have recently shown that A. thaliana plants grown under normal conditions do not emit detectable amounts of benzenoid esters from their vegetative or reproductive tissues (Chen et al., 2003). Similar results had been reported by others (Van Poecke et al., 2001). On the other hand, plants under attack by Pieris rapae caterpillars have been reported to emit a volatile cocktail that includes MeSA (Van Poecke et al., 2001).

We tested several treatments under which biosynthesis and emission of benzenoid esters might be induced. The most effective of the methods we used in inducing MeSA emission was the treatment of detached A. thaliana leaves with alamethicin, a voltage-gated ion-channel-forming antibiotic from the fungus Trichoderma viride that had been found to be an efficient inducer of MeSA emission in lima beans (Engelbert et al., 2001). This treatment resulted in MeSA emission levels of 6.4 ± 0.7 ng g⁻¹ FW h⁻¹ for several hours after the first 21 h of exposure to alamethicin (Figure 1a). No MeBA emission was detected following alamethicin treatment, and detached leaves treated with water emitted only trace amounts of MeSA (Figure 1a).

Feeding by the larvae of the diamondback moth (Plutella xylostella) on rosette leaves of intact plants resulted in MeSA emission levels of 0.7 ± 0.3 ng g⁻¹ FW h⁻¹ 21–30 h after the start of herbivory (Figure 1b). Additionally, small levels of MeBA emission, approximately 10-fold less than the MeSA levels and at the limit of detection, were also observed (Figure 1b). Treatments with MeJA, physical wounding, and thrip herbivory did not result in detectable levels of either benzenoid ester emission or intracellular concentrations of these compounds. Serendipitously, it was found that uprooting the plant from the soil (which simulates soil disturbance) and placing it in a beaker with its roots submerged in water resulted in emission of both MeSA and MeBA, but the emission levels were at the threshold of detection and therefore difficult to quantify.
**Table 1 Primers for RT-PCR analysis**

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<th>Gene name</th>
<th>Sequences (5' to 3')</th>
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</table>

F, forward primer; R, reverse primer.

**Figure 1.** Induction of MeSA and MeBA emission. Volatiles emitted from (a) detached rosette leaves treated with alamethicin or water, and from (b) whole rosette plants under attack by Plutella xylostella larvae or control (no *P. xylostella* larvae) were collected during 9 hr of closed-loop stripping (Donath and Boland, 1995) 21–30 hr after the beginning of treatment and identified using GC/MS analysis. Each experiment was repeated three times, and quantitative data were obtained by integration of peaks and comparison with standards. A portion (from 12 to 22 min) of one gas chromatogram from each experiment is shown. IS represents the internal standard (monoyl acetate).

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**Isolation of AtSABATH cDNA from A. thaliana encoding a protein with BAMT and SAMT activities by correlating AtSABATH gene expression with MeSA emission**

We had previously constructed 20 pairs of AtSABATH gene-specific primers and two pairs of primers that are each specific to two AtSABATH genes (Table 1) to measure the expression of all the 24 AtSABATH genes in various organs (D’Auria et al., 2003). Here, we used these primers in RT-PCR experiments (Figure 2) to determine which of the 24 AtSABATH genes were expressed following alamethicin application to detached leaves (Figure 2a, bottom panel).
and *P. xylostella* herbivory on attached leaves (Figure 2b, bottom panel), two treatments that induce MeSA emission, but were not expressed in the respective control treatments, which did not result in MeSA emission. Intact, undamaged leaves showed the fewest number of AtSABATH genes expressed (Figure 2b, top panel). Detached leaves treated with water only (Figure 2a, top panel) showed the expression of several additional AtSABATH genes, some of them also induced by *P. xylostella* herbivory and alamethicin application (e.g. At3g44860 and At3g44870), suggesting that the function of these genes is in a general response to stress. One gene, At5g38020, was induced by alamethicin but not by herbivory, and one or the other member of the closely related pair of genes At1g66690 and At1g66700 (which encode proteins that are 92% identical to each other) was induced by herbivory but not by alamethicin.

However, the expression pattern of only two genes, At1g19640 and At3g11480, correlated perfectly with MeSA emission: their steady-state mRNA levels increased substantially following both alamethicin application and *P. xylostella* herbivory, the two MeSA emission-inducing treatments, in comparison to both control treatments that did not result in MeSA emission (Figures 1 and 2). One of these two induced genes, At1g19640, had previously been identified as *AtMT1* (Seo et al., 2001). The full-length cDNA clone of the remaining induced gene, At3g11480, was obtained by RT-PCR, expressed in *Escherichia coli*, and the crude *E. coli* protein extract was tested for enzymatic activity using several putative substrates, including SA and BA. The lysate of *E. coli* cells expressing gene At3g11480 showed activity with BA and SA (see below), and this gene was therefore named *AtBSMT1*. Proteins obtained in *E. coli* from the expression of full-length cDNA clones of, among others, At1g66700 and At1g15125 did not have methylating activities with SA or BA.

**Additional characterization of *AtBSMT1* expression**

To determine the tissues in which *AtBSMT1* is expressed, total RNA was isolated from flowers, leaves, stems, siliques, and roots of *A. thaliana*. These RNA preparations were used in RNA gel blot analysis. The highest levels of *AtBSMT1* transcripts were observed in flowers, lower levels were seen in leaves and stems, and little or no *AtBSMT1* transcripts were observed in roots or siliques of plants grown under standard conditions (Figure 3a).

Further experiments were performed to identify conditions under which *AtBSMT1* gene expression is induced in *A. thaliana* leaves. Leaf wounding and uprooting caused *AtBSMT1* transcripts to accumulate quickly within 2 h after treatment (with uprooting being more effective), and transcript levels declined rapidly within 24 h (Figure 3b). In contrast, MeJA treatment, in which plants were exposed to MeJA vapors in a closed container, resulted in slower induction, and transcript levels at 24 h after treatment were higher than those at 2 h after the beginning of induction. β-Glucuronidase (GUS) staining of several lines of transgenic plants carrying an *AtBSMT1* promoter-GUS fusion gene indicated that under normal growing conditions, the *AtBSMT1* promoter was active in leaves only at the base of the trichomes, in the hyathodes (Figure 4a,b), and in the
upper part of the sepals of flowers (Figure 4c). In addition, strong AtBSMT1 promoter activity was observed around lesions in the leaves caused by thrips of the genus Frankliniella (Figure 4d). These herbivores damage the plant by sucking the cytoplasm out of the cell with their specialized proboscis, leaving the cell walls intact (Hunter and Ullman, 1992).

Isolation of the A. lyrata ortholog of AtSAMT1 and characterization of its expression

We isolated a cDNA of the orthologous gene to AtSAMT1 from the closely related perennial species A. lyrata by RT-PCR with leaf mRNA and the AtBSMT1 primer pair. The A. lyrata cDNA was expressed in E. coli, and the resulting protein also showed methyltransferase activity with SA and BA (see below). It was therefore named AIBSMT1.

To verify that the A. lyrata gene AIBSMT1 is orthologous to AtBSMT1, we performed PCR on genomic DNA from both A. thaliana and A. lyrata. Two primers were designed based on the genomic sequence of A. thaliana. The forward primer 5’-GGTTCTACCTTACGGA-3’ corresponds to exon 4 of At3g11480 (AtBSMT1), and the reverse primer 5’-CTGCTACTGCTCGATG-3’ corresponds to exon 5 of gene At3g11490, the adjacent downstream gene to At3g11480 (which is transcribed in the direction toward At3g11480) on chromosome III (Figure 5). When A. thaliana genomic DNA was used in the PCR reaction, a fragment of 1282 nucleotides was obtained (Figure 5). When A. lyrata genomic DNA was used, a fragment of 1256 nucleotides was amplified, which showed 82% identity to the A. thaliana fragment (Figure 5). Sequence analysis confirmed that one end of the A. lyrata fragment comprises the sequence of the 3’ end of AIBSMT1, including part of the fourth exon, an intron, and the fifth exon. The sequence identity of this segment to the corresponding A. thaliana fragment is 94% in the exons and 75% in the intron (there are also three deletions/insertions greater than one nucleotide each in one sequence compared with the other in this area). The other end of the fragment, containing the 3’ end of the coding region of the adjacent gene, is 93% identical to the 3’-coding region of At3g11490. The middle of this fragment constitutes the intergenic spacer, which has 84% sequence conservation between the two genomes (but 10 deletions/insertions). As these results indicate that the same gene is found downstream to AtBSMT1 and AIBSMT1, we conclude that the latter two are orthologous.
RNA gel blot analysis showed that AIBSMT1 transcript levels were, similarly to AtBSMT1 transcripts in A. thaliana, higher in flowers and detectable also in leaves (Figure 6).

Analysis of the sequences of the proteins encoded by AtBSMT1 and AIBSMT1

The AtBSMT1 encodes a protein comprising 379 amino acid residues, while the AIBSMT1-encoded protein is one residue longer (Figure 7a). The two proteins are 92% identical, and the critical residues putatively involved in binding the acid substrate are identical in both (Figure 7a). Both AtBSMT1 and AIBSMT1 are less than 50% identical to any of the four currently known SAMTs or the snadragon BAMT (AmBAMT). Compared with most of these other proteins, both AtBSMT1 and AIBSMT1 possess an N-terminal extension of about 20 amino acids. The snadragon SAMT (AmSAMT) also has an N-terminal extension, but it is shorter (Figure 7a). Phylogenetic analysis of the sequences presented in Figure 7(a) indicates that AtBSMT1 and AIBSMT1 are more similar to AmBAMT and also to AtJMT1 than they are to other SAMTs, including C. breweri SAMT (CbSAMT; Figure 7b).

Enzymatic characterization of AtBSMT1 and AIBSMT1

The coding regions of AtBSMT1 and AIBSMT1 were ligated into the vector pCRT7/CT-TOPO TA for functional expression in E. coli as non-fusion proteins. The two proteins were purified to near homogeneity in two steps, first using anion-exchange column diethylaminoethyl cellulose followed by Mono-Q anion-exchange chromatography (Figure 8; Table 2). The molecular mass of both holoenzymes was estimated by size exclusion chromatography to be 91 kDa. The subunit molecular mass of both enzymes, calculated from their sequences, is 43.4 kDa (Figure 8).
Together, these results indicate that the AtBSMT1 and AIBSMT1 enzymes exist as homodimers under physiological conditions, as do previously characterized SAMTs and BAMTs (Murfitt et al., 2000; Ross et al., 1999).

The two enzymes were further evaluated for their ability to catalyze the SAM-dependent carboxyl methylation of a number of potential substrates (Table 3). Of the substrates tested, SA, BA, anthranilic acid, and m-hydroxybenzoic acid all served as in vitro substrates in the reactions catalyzed by the AtBSMT1 protein. At substrate concentrations of 1 mM, AtBSMT1 showed the highest activity with BA and m-hydroxybenzoic acid rather than with SA. AIBSMT1 also exhibited methyltransferase activity with BA, SA, and anthranilic acid, but lacked detectable activity with 3-hydroxybenzoic acid. Notably, AIBSMT1 also exhibited a higher activity with BA than with SA under the conditions tested.

Steady-state kinetic parameters for each enzyme were also determined (Table 2). AtBSMT1 and AIBSMT1 showed similar apparent $K_m$ values for SAM, 67 $\mu$M and 87 $\mu$M, respectively, and a twofold difference in apparent $K_m$ values for BA, 65 and 131 $\mu$M, respectively. However, the two enzymes had very different $K_m$ values for SA:

Figure 6. RNA gel blot analysis of the relative abundance of AIBSMT1 mRNA transcripts.

Figure 7. Sequence analyses of AtBSMT1, AIBSMT1, and other known SABATH proteins.

(a) Amino acid sequence alignment of all proteins using the CLUSTALX program (Thompson et al., 1997). Amino acid residues conserved in five or more sequences are shown in white letters on black background. The positions of the residues involved in the active site binding site, identified from the three-dimensional structure of CsSAMT (Noel et al., 2003; Zubieta et al., 2003) are indicated with the symbol $\&$.

(b) Maximum parsimony phylogenetic tree constructed from the alignment in (a) using the program PAUP*.

CsSAMT: C. brevis SANT (Accession # AF133353); AmSAMT, Antirrhinum majus (snapdragon) SAMT (Accession # AF15294); SfSAMT, S. floribunda SAMT (Accession # AF385970); AmBAMT: A. majus SAMT (Accession # AF196492); AtJMT1, A. thaliana JMT (Accession # AY009843); AbSAMT, A. belladonna SAMT (Accession # ABO49752). The Accession number of AtBSSMT1 is AY224596, and the Accession number of AIBSMT1 is AY224596.
$K_m$ value of AtBSMT1 for SA was 16 µM, whereas the $K_m$ value of AIBSMT1 for SA was 127 µM (Table 2). Thus, while the A. thaliana enzyme had similar catalytic efficiency with both SA and BA, the A. lyrate enzyme was about threefold more efficient with BA than AtBSMT1, but about 11-fold less efficient SA than with the A. thaliana enzyme. Put another way, the catalytic efficiency of AtBSMT1 with SA was 50% higher than with BA, whereas the catalytic efficiency of AIBSMT1 with SA was 24-fold lower than with BA.

**Discussion**

**Identification of AtBSMT1**

All previously characterized carboxyl methyltransferases involved in the formation of various plant-specialized metabolites belong to the SABATH family (D’Auria et al., 2003), of which CbSAMT was the first recognized member (Ross et al., 1999). A. thaliana contains 24 SABATH family members (D’Auria et al., 2003). However, the identification of A. thaliana genes that encode proteins with SAMT or BAMT activity has not been straightforward. The A. thaliana SABATH protein with the highest sequence identity to known SAMTs from other species is AtUMT1 (Figure 7b), and this enzyme has been shown to methylate JA but not SA or BA (Seo et al., 2001). In addition, a group of seven AtSABATH proteins cluster together with AmBAMT (D’Auria et al., 2003), but these seven AtSABATH proteins are all equally divergent in primary sequence from AmBAMT (with approximately 40% identity to AmBAMT). Thus, an identification and functional annotation of A. thaliana genes encoding SAMT or BAMT activities could not be straightforwardly attempted based simply on overall sequence similarity.

Instead, we employed a biochemical genomics approach that involved metabolic profiling and the compilation of gene expression data of the entire family, together with enzymatic assays with potential substrates. By correlating the expression of AtSABATH genes under two types of treatments that induce MeSA emission (alamethicin treatment of cut leaves and P. xyllostella herbivory on rosette plants) and two types of treatments that do not result in MeSA emission (water treatment of cut leaves and normal growth conditions), two strong candidate genes for SAMT-encoding capacity were identified. One of these genes, AtUMT1, was ruled out based on previously published work.

**Table 2 Properties of AtBSMT1 and AIBSMT1**

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<th>Enzyme</th>
<th>Holoenzyme mass (Da) by size exclusion chromatography</th>
<th>Subunit mass (Da) by SDS-PAGE</th>
<th>Calculated subunit mass (Da)</th>
<th>pH optimum</th>
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<th>Apparent $K_m$ (µM) for acid</th>
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**Table 3 Relative activity of AtBSMT1 and AIBSMT1 with benzoic acid, salicylic acid, and related substrates**

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<td>p-Coumaric acid</td>
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<td>0</td>
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<tr>
<td>Cinnamic acid</td>
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<tr>
<td>(±)Jasmonic acid</td>
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<td>0</td>
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<tr>
<td>p-Aminobenzoic acid</td>
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*Values are averages of three independent measurements. All substrates were tested at 1 mM concentration. The activity of the two enzymes with benzoic acid was set arbitrarily at 100%.
by Seo et al. (2001), and the second one, At3g11480, was expressed in E. coli and shown by biochemical assays to indeed encode an enzyme, AtBSMT1, with SA- as well as BA-methylating activities.

However, until all 24 AtSABATH proteins are thoroughly characterized, it is not possible to conclude that all the A. thaliana genes encoding enzymes with BAMT/SAMT activities have been determined. Nor can it be concluded that all possible substrates of AtBSMT1 have been identified.

Origin, structure, and evolution of the AtBSMT1 and AIBSMT1 proteins

The sequences of AtBSMT1 and AIBSMT1 are not particularly closely related to other known SAMTs. Instead, they are somewhat more closely related to AmBAMT (Figure 7). In addition, two critical residues important in the binding of SA in SAMTs, Met 150 and Met 308, are replaced in AtBSMT1, AIBSMT1, and AmBAMT, and all three having His in the equivalent Met 150 position and Ile, Ile, and Val in the equivalent Met 308 position, respectively (Figure 7a). However, active site modeling based on the recently determined three-dimensional structure of CsSAMT (Noel et al., 2003; Zubieta et al., 2003) indicates that the overall topology of the active sites of AtBSMT1 and AIBSMT1, which are identical, are more similar to the topology of the active site of SAMTs, while the topology of the active site of AmBAMT is quite distinct (data not shown). In addition, here we have presented data showing that both AtBSMT1 and AIBSMT1 are equally active with BA, but they differ in their catalytic efficiencies with SA, suggesting that the ancestor enzyme possessed a BAMT activity with low levels of SAMT activity, and that AtBSMT1 evolved its higher catalytic activity with SA only after the A. thaliana and the A. lyrata lineages diverged. These somewhat conflicting data make it difficult to retrace a clear evolutionary path for these two enzymes, emphasizing a common observation regarding enzymes of secondary metabolism whereby one or a few amino acid substitutions may bring about a change in substrate specificity (Frick and Kutchan, 1999; Gang et al., 2002; Wang and Pichersky, 1999). As AtBSMT1 and AIBSMT1 have identical active sites, the difference in their catalytic efficiency with SA must therefore be due to of small changes in the structure of the protein outside the active site.

The presence of an ortholog of the AtBSMT1 in A. lyrata, a close relative of A. thaliana whose lineage diverged 5 million years ago (Koch et al., 2000), presents a unique opportunity to address these questions and to examine the evolution of substrate specificity in the SABATH family. It would also be interesting to examine whether A. lyrata plants emit mostly MeBA or MeSA in response to conditions that result in mostly MeSA emission in A. thaliana.

The involvement of AtBSMT1 in the response to biotic and abiotic stresses

The specific physiological role of AtBSMT1 is not yet clear. Under normal conditions, AtBSMT1 is most highly expressed in flowers, and its expression in leaves is restricted specifically to trichomes and hydathodes. However, headspace analysis of A. thaliana leaves and flowers has failed to detect emission of benzenoid esters from either organs under normal growth conditions (Chen et al., 2003), and internal levels of such esters were also below our detection limits (Chen and Pichersky, unpublished).

Certain biotic and abiotic challenges to leaves led to the induction of AtBSMT1, and in some of these cases, emission of MeSA, and occasionally of MeBA, was also detected. The highest rates of MeSA emission that we have observed occurred when detached leaves were treated with alamethicin, a channel-forming peptide that has been previously used to mimic the effect of pathogen attack on gene expression and cellular functions, as pathogenic fungi are known to secret pore-forming agents (Brewer et al., 1987). Herbivory by P. xylostella larvae for 30 h, which caused extensive damage to the leaves (approximately 40–70% of the leaf material was consumed by the larvae), resulted in lower rates of MeSA emission per gram FW, as well as some low levels of MeBA emission. Both alamethicin application and P. xylostella caused clear induction of AtBSMT1 transcripts.

Physical damage to leaves, and the complex treatment that involved the uprooting of the plant followed by placing the roots in water (a treatment that caused major damage to the roots as well as exposing the roots to a change in osmotic pressure and inorganic ion concentration in the medium and to light), resulted in quick but transient induction of AtBSMT1 (Figure 3b). Additional types of treatments, including flooding the roots of intact plants with distilled water or a saline solution, resulted in only a relatively weak change in AtBSMT1 gene expression (Chen and Pichersky, unpublished). All these abiotic treatments led to only low to undetectable levels of MeSA emissions, a result that is similar to the results of Van Poecke et al. (2001), who failed to detect emission of benzenoid esters from mechanically damaged plants. Strong local induction was observed around areas damaged by piercing-sucking thrips (Figure 4d), but again emission of benzenoid esters, if it occurred, was below detection limits, most likely because of the small area involved. SA, a known global inducer of some defense responses that is also a substrate of AtBSMT1, did not induce AtBSMT1 gene expression, while MeJA, another inducer of systemic defense responses (Seo et al., 2001), did. In both these cases, however, no MeSA emission could be detected.

Van Poecke et al. (2001) suggested that MeSA emission in A. thaliana plays a role in attracting predators of herbivorous insect pests. Our observations of short-lived
AtBSMT1 induction in response to abiotic stress, such as wounding, and the lack of detectable emission of MeSA and MeBA but the strong localized induction of AtBSMT1 expression around the site of feeding by piercing-sucking thrips suggest additional roles for these highly toxic compounds, perhaps in direct defense.

Experimental procedures

Plant materials and treatments

Arabidopsis thaliana (L.) Heynh (ecotype Columbia) plants were grown on soil at 23°C under 16 h light/8 h dark up to 4–6 weeks. For selection of transgenic plants, seeds were sterilized in 75% ethanol for 15 min, rinsed with sterilized water, and germinated on MS medium containing 50 μg ml⁻¹ hygromycin. Hygromycin-resistant seedlings were transferred to soil and later confirmed by PCR. The A. lyrata ssp. lyrata plants used in this report were descended from accessions collected in North Carolina (seeds kindly provided by Dr. Charles Langley, the University of California, Davis, USA). Plants were grown on soil at 23 °C under 20 h light/4 h dark to induce flowering.

All treatments were carried out with 4- to 6-week-old non-bolting plants of A. thaliana. For feeding experiments with P. xyllostella (kindly provided by P. Mitchell-Olds), 6-week-old rosette plants with their root balls wrapped in aluminum foil were placed in 3-l bell jars. Approximately 50 three-fourths instar larvae were applied per plant and allowed to feed for 30 h, leading to an estimated consumption of total leaf area of 40–70%. For alamethicin (Sigma, St Louis, MO, USA) treatment, leaves were cut off from the base of the petiole and stood up in a small glass beaker containing 10 ml of 5 μg ml⁻¹ alamethicin (dissolved 1000-fold in tap water from a 5 mg ml⁻¹ stock solution in 100% methanol). Only the petiole of each leaf was submerged in the solution. The glass beaker was then sealed with Saran wrap and placed in a growth chamber. As a control treatment, leaves were placed in tap water only (including 0.1% methanol). For wounding treatment, two lateral incisions to each side of the midvein of leaves were made with sterile razor blade. Uprooting treatment consisted of removing the entire plant from the soil, rinsing off the soil, and placing the plant in a small beaker with the roots submerged in water. For MeJA treatment, two pots with a total of eight plants were placed in a 5-l glass jar. MeJA (1 μl) was dissolved in 200 μl ethanol and applied on cotton tips placed in the jar, and the jar was sealed. For SA treatment, SA was dissolved in 0.1% Tween (pH 7.0) at a final concentration of 5 mM and used to spray the plants. For treatment with thrips, the plants were placed in a thrip-infested growth chamber for 2 weeks.

Detection of volatile emission from detached leaves treated with alamethicin

Emitted volatiles were collected by a closed-loop stripping method (Chen et al., 2003; Donath and Boland, 1995) in a controlled climate chamber (23°C, 70% humidity, 150 μmol m⁻² sec⁻¹ photosynthetically active radiation (PAR)). After treatment with alamethicin for 21 h (10 h light, 11 h dark) as described in the previous section, the glass beaker with the leaves in alamethicin solution was placed in a 1-l bell jar. For the P. xyllostella treatment, plants were placed as described above in 3-l bell jars and volatiles were collected during 21–30 h of larvae feeding. Volatiles were eluted from the trap with 40 μl CH₂Cl₂ and 120 ng nonyl acetate was added as an internal standard.

Samples from volatile collections were analyzed on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 quadrupole mass selective detector. Separation was performed on (5%-phenyl)-methylpolysiloxane (DB5) or polyethylene glycol (DB-wax) columns (J&W Scientific, Folsom, CA, USA) of 30 m × 0.25 mm i.d. × 0.25 mm thickness. Helium was the carrier gas (flow rate 2 ml min⁻¹), a splitless injection (2 μl) was used, and a temperature gradient of 5°C min⁻¹ from 40°C (3-min hold) to 240°C was applied.

The identities of MeSA and MeBA were determined by comparison of retention time and mass spectra to those of authentic standards and to mass spectra in the NIST and Wiley libraries. For quantification, representative single ion peaks of each compound were integrated and compared to the equivalent response of the internal standard (single ion method).

Determination of gene expression by RT-PCR

The method used is essentially the same as that previously reported by Chen et al. (2003). Primers for β-tubulin (F: CTCAA-GAGTTTCTCAGCATGA, R: TCACCTTCTTCAGGGATTG) and actin-8 (F: ATGAGGATTAAGGTGGCG, R: GTTTTTATCCGA-GTTTGAAGGCG) were used to judge equality of the concentration of the cDNA templates in each pair of experiments of a treatment–control combination (for β-tubulin, the amount of cDNA used in the reaction was reduced fivefold, and the number of cycles reduced to 25, to avoid saturation). The β-tubulin and actin-8 RT-PCR experiments showed less than threefold variation among samples, and based on the β-tubulin and actin-8 results, each set of the RT-PCR experiments with the AtSABATH primers was normalized accordingly, loaded, and separated on a 1% agarose gel. Each experiment was repeated at least twice with similar results.

AtBSMT1 cDNA cloning

AtBSMT1 was cloned from alamethicin-treated A. thaliana leaves by RT-PCR. AtBSMT1 was first cloned based on predictions from GenBank using two primers 5'-AATGGGCAACCAAGGCTTGG-3' and 5'-TCTACTTTCTAGTCGAAGGAGC-3'. The protein had no activity with any of the substrates tested. As the protein encoded by this cDNA was relatively shorter at the N-terminal end compared to other known SAMT and SAMLike proteins, 5'-RACE using SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA) was performed with the At3g14800-specific oligonucleotide 5'-GGAGGCTTTGAGGAGGAGCT- GG-3'. The sequence of the 5'-RACE product was compared to the genomic sequence of the gene, and the ATG start was determined. Next, a new forward primer 5'-AATGGATCAAGATTCTCACAGC3'- was made based on the newly identified start of the open-reading frame and used with the original reverse primer for RT-PCR cloning of the full-length AtBSMT1 cDNA.

AIBSMT1 cDNA isolation

The isolation of AIBSMT1 was based on its homology to AtBSMT1. The same two primers that were used to amplify the full-length AtBSMT1 were also used in an RT-PCR experiment to amplify an AIBSMT1 cDNA from leaf RNA of A. lyrata.

RNA gel blot analysis

RNA gel blot analysis was performed as previously described by Chen et al. (2003) and D’Auria et al. (2002).
Construction of the AtBSMT1 promoter fusion to the β-glucuronidase (GUS) reporter gene and histochemical localization of GUS activity

The AtBSMT1 promoter region (1.4 kbp) was isolated via PCR from A. thaliana genomic DNA using the primers 5'-CTCCGATT-TG-GCTTCAAG-GCGGATGTT-3' and 5'-GAAATCTGGAATCATTAGG-CAGATTACGTAC-3', which contain an EcoRI and an Ncol site, respectively. The resulting PCR product was cloned into pCR7/CT-TOPO vector and sequenced. The promoter region of AtBSMT1 was next cut out of the pCR7/CT-TOPO vector by digestion with EcoRI and Ncol, and the fragment was isolated and inserted into the binary vector pCAMBIA1303 (Hajdukiewicz et al., 1994) by replacing the EcoRI–Ncol fragment containing the CaMV 35S promoter. The construct was introduced into Agrobacterium tumefaciens strain GV3101, which was used to transform A. thaliana by floral vacuum infiltration (Bechtold et al., 1993). Transgenic lines transformed with the construct were selected using hygromycin, and confirmed by PCR.

The substrate 5-bromo-4-chloro-3-indolyl β-glucuronide (X-Gluc) was used to determine the localization of the enzyme activity of the GUS gene (Jefferson et al., 1987). Tissue samples were incubated at 37°C in GUS-staining buffer (50 mM sodium phosphate, pH 7.0, 0.1% Triton X-100, and 0.5 μg ml⁻¹ X-Gluc overnight. After detection of the blue color, chlorophyll was extracted with 70% ethanol for 24 h.

Biochemical procedures

Preparation of crude cell-free extracts from E. coli, enzyme assays, protein purification, determination of kinetic properties, and molecular mass estimation were performed as previously described by D’Auria et al. (2002).

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References


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