Biochemical and Structural Characterization of Benzenoid Carboxyl Methyltransferases Involved in Floral Scent Production in *Stephanotis floribunda* and *Nicotiana suaveolens*[1]

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Flower-specific benzenoid carboxyl methyltransferases from *Stephanotis floribunda* and *Nicotiana suaveolens* were biochemically and structurally characterized. The floral scents of both these species contain higher levels of methyl benzoate and lower levels of methyl salicylate. The *S. floribunda* enzyme has a 12-fold lower K_m value for salicylic acid (SA) than for benzoic acid (BA), and results of in silico modeling of the active site of the *S. floribunda* enzyme, based on the crystal structure of *Clarkia breweri* salicylic acid methyltransferase (SAMT), are consistent with this functional observation. The enzyme was therefore designated SAMT. The internal concentration of BA in *S. floribunda* flowers is three orders of magnitude higher than the SA concentration, providing a rationale for the observation that these flowers synthesize and emit more methyl benzoate than methyl salicylate. The *N. suaveolens* enzyme has similar K_m values for BA and SA, and the in silico modeling results are again consistent with this in vitro observation. This enzyme was therefore designated BSMT. However, the internal concentration of BA in *N. suaveolens* petals was also three orders of magnitude higher than the concentration of SA. Both *S. floribunda* SAMT and *N. suaveolens* BSMT are able to methylate a range of other benzenoid-related compounds and, in the case of *S. floribunda* SAMT, also several cinnamic acid derivatives, an observation that is consistent with the larger active site cavity of each of these two enzymes compared to the SAMT from *C. breweri*, as shown by the models. Broad substrate specificity may indicate recent evolution or an adaptation to changing substrate availability.

Methyl salicylate and methyl benzoate are common components of floral scent and are believed to be important attractants of insect pollinators (Dobson, 1994). Enzymes that catalyze the formation of methyl salicylate and methyl benzoate from salicylic acid (SA) and benzoic acid (BA), respectively, have been reported from flowers of *Clarkia breweri*, snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), and *Stephanotis floribunda* (Ross et al., 1999; Murfitt et al., 2000; Negre et al., 2002; Pott et al., 2002). While these enzymes use *S*-adenosyl-L-met (SAM) as the methyl donor as do many previously characterized methyltransferases that act on a variety of substrates (e.g. DNA, protein, phenylpropanoids), these SA and BA carboxyl methyltransferases (SAMT and BAMT, respectively) display primary amino acid sequences that show no significant sequence identity to other methyltransferases. Interestingly, it was subsequently discovered that a group of *N*-methyltransferases involved in the biosynthesis of the alkaloid caffeine, including theobromine synthase, also share sequence similarity with SAMT and BAMT (D'Auria et al., 2003). These enzymes were therefore grouped into a new class of methyltransferases designated the SABATH methyltransferases, and this family now also includes jasmonic acid methyltransferase (Seo et al., 2001) and indole-acetic acid methyltransferase (Zubieta et al., 2003). The recently obtained three-dimensional structure of *C. breweri* SAMT (Zubieta et al., 2003) also clearly indicates that these enzymes have a unique structure distinct from those of unrelated methyltransferases found in plants (Noel et al., 2003).

It is estimated that the total number of different compounds with specialized functions synthesized

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by various plant species exceeds 100,000 but that each species synthesizes only a small fraction of the potential chemical diversity of the plant kingdom (Pichersky and Gang, 2000; Verpoorte et al., 2000). An intriguing question yet to be addressed in detail at the molecular level is how different plant species adapted to unique environmental niches to evolve biosynthetic systems that can provide specialized small metabolites that confer adaptative advantages on the host plant. Various hypotheses have been advanced, including (1) gene duplication and divergence of the genes to encode highly specific enzymes, (2) the presence of enzymes that are less specific so that they can carry out the same chemical reaction with several different substrates, and (3) a combination of both mechanisms (Pichersky and Gang, 2000; Firn and Jones, 2003; Schwab, 2003).

The floral enzymes involved in benzenoid ester production provide an excellent opportunity to examine the issue of enzyme specificity and promiscuity in specialized metabolism and the role of each in the evolution of plant-specialized metabolism. BA and SA are very similar compounds, differing only in the presence of an ortho hydroxyl group on the benzyl ring of SA (Fig. 1), yet some flowers synthesize one or the other of these two methyl ester derivatives and other species synthesize both. For example, *S. floribunda* flowers emit primarily methyl benzoate and little methyl salicylate (Pott et al., 2002), while its relative *Hoya carnosa* emits only methyl salicylate (Altenburger and Matile, 1988). The *Nicotiana* species, *Nicotiana suaveolens*, emits methyl salicylate and methyl benzoate while *Nicotiana sylvestris* and *Nicotiana alata* emit only methyl benzoate, and *Nicotiana rustica*, *Nicotiana tomentosiformis*, *Nicotiana longiflora*, *Nicotiana plumpaginifolia,* and *Nicotiana forgetiana* emit neither (Loughrin et al., 1990; Raguso et al., 2003). In this report, we have characterized the biochemical properties of benzenoid methyltransferases from *S. floribunda* and *N. suaveolens* to determine whether the amount of specific products that their flowers synthesize (methyl salicylate and methyl benzoate) is due to the specificity of the enzyme or whether other factors are involved.

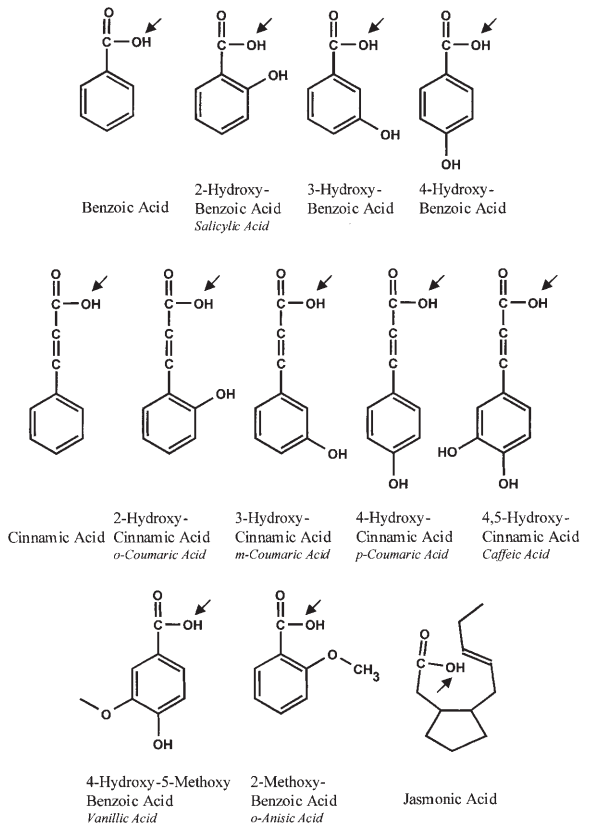

Figure 1. Structures of carboxyl group containing small molecules. Chemical structures of the carboxyl-containing small molecules used in the assays. The possible methylation site is indicated by an arrow. Generic names of the substrates, when available, are indicated in italics.

RESULTS

Emission of Methyl Salicylate and Methyl Benzoate from Flowers of *N. suaveolens* and *S. floribunda*

While the emission of *S. floribunda* flowers has been characterized (Matile and Altenburger, 1988), quantitative measurements have not been previously reported. We determined the emission levels of methyl salicylate and methyl benzoate from *S. floribunda* flowers (1–3 d after flower opening) to be 1 μg/g fresh weight (FW)/h and 14.73 μg/g FW/h, respectively (Table I). The methyl benzoate emission exceeded methyl salicylate emission by almost 15-fold. The highest levels of volatile emission were found on the 2nd d after flower opening between 6 PM and 10 PM (37.6 μg methyl benzoate/g FW/h). Emission of both compounds is reduced by 60% in the morning (9 AM; Pott et al., 2002). The quantitative emission levels of methyl benzoate and methyl salicylate from *N. suaveolens* flowers had been previously determined by several groups (Loughrin et al., 1990, 1993; Kolosova et al., 2001; Raguso et al., 2003). Coincidently, these groups reported that methyl benzoate emission exceeded methyl salicylate emission. We quantitatively determined the emission of 2-d-old *N. suaveolens* flowers between 8 AM and 10 AM, and 6 PM and 10 PM. At both time points the emission was approximately the same for both compounds, and methyl benzoate emission (0.67 and 0.71 μg/g FW/h) exceeds methyl salicylate emission (0.162 and 0.192 μg/g FW/h) approximately 4-fold (Table I). Maximum emission levels were found between 10 PM and 2 AM (2.6 μg/g FW/h) while lowest levels (0.27 μg/g FW/h) were reached 12 h later.

Carboxyl Methyltransferase Activities in Petals of *N. suaveolens* and *S. floribunda*

To determine whether carboxyl methylation activities are present in these flowers, protein extracts from petals were incubated with BA, SA, and several other

Table I. *Emission of methyl salicylate and methyl benzoate from S. floribunda and N. suaveolens flowers*

	S. floribunda 8–10 AM	*S. floribunda* 6–10 PM	*N. suaveolens* 8–10 AM	*N. suaveolens* 6–10 PM
	μg/g FW/h	*μg/g FW/h*	*μg/g FW/h*	*μg/g FW/h*
Methyl salicylate	0.41[a]	1.03 ± 0.48	0.16 ± 0.11	0.192 ± 0.06
Methyl benzoate	5.89[a]	14.73 ± 6.1	0.67 ± 0.3	0.71 ± 0.36

[a]Forty percent of maximum levels (Pott et al., 2002).

related compounds as well as with jasmonic acid (Fig. 1) together with [^{14}C]SAM. The extract of *S. floribunda* showed the highest activity with SA as well as relatively high levels of activity with several related hydroxylated BA derivatives (Table II). The *N. suaveolens* extract possessed the highest activity with 2-methoxy BA (*o*-anisic acid) and 4-hydroxy BA (Table II). Little methylation activity was observed with jasmonic acid. *S. floribunda* flowers emit about 15-fold less methyl salicylate than methyl benzoate (Table I), which is a contradictory reflection of the 9-fold higher SA-methylating activitiy in the flower extract of *S. floribunda*. In *N. suaveolens*, which also emits more methyl benzoate than methyl salicylate (4-fold), BA-methylating activity was 4-fold higher than SA-methylating activity. Moreover, the overall methylating activity levels in *S. floribunda* per g FW were much higher than the methylating activity levels found in the floral extract of *N. suaveolens* (Table II).

Isolation of cDNAs Encoding Floral Benzenoid Carboxyl Methyltransferases from *S. floribunda* and *N. suaveolens*, and Biochemical Characterization of the Encoded Enzymes

The isolation of a cDNA from *S. floribunda* that encodes an enzyme capable of methylating SA was previously reported (Pott et al., 2002). For this study, a cDNA of a homologous gene was isolated from *N. suaveolens* by reverse transcription (RT)-PCR based on sequence information of the previously characterized *S. floribunda* benzenoid carboxyl methyltransferase (Pott et al., 2002). The coding region of this *N. suaveolens* cDNA encodes a protein of 355 amino acids (Fig. 2). Comparison of the predicted amino acid sequence of the *S. floribunda* SAMT with homologous proteins from several other species, including the *N. suaveolens* protein, showed a maximum sequence identity of 56% to 63% with the proteins from *C. breweri*, *N. suaveolens*, and petunia and a minimum of

Table II. *Relative carboxyl methyltransferase activities with various substrates (each at 1 mM concentration) of crude petal extracts of S. floribunda and N. suaveolens, and of the purified enzymes from the two species*

	S. floribunda Petal Extract	*S. floribunda* Purified SAMT	*N. suaveolens* Petal Extract	*N. suaveolens* Purified BSMT
SA	100 (235)[a]	100 (13.1)[b]	12.8	22.5
BA	12.5	31.9	49.6	100 (2.3)[b]
3-Hydroxy BA	7.2	75.6	18.8	15.3
4-Hydroxy BA	2.8	34	99.3	76.2
(E/Z) Jasmonic acid	2.6	4.8	6.0	nd[c]
(E) Cinnamic acid (CA)	2.4	34.5	2.0	16.5
(E) 2-Hydroxy CA	2.7	25.0	6.0	21.0
(E) 3-Hydroxy CA	2.6	23.8	0	4.9
(E) 4-Hydroxy CA	2.9	4.4	1.5	2.55
Vanillic acid	nd	17	nd	nd
Caffeic acid	nd	32.5	nd	nd
2,3-Hydroxy BA	68.9	99.5	13.5	14.9
2,4-Hydroxy BA	9.0	98.5	20.3	18.2
2,5-Hydroxy BA	17.9	1.5	0	2.4
2,6-Hydroxy BA	1.1	1.0	0	1.6
3,4-Hydroxy BA	3.4	27.9	26.3	5.7
3,5-Hydroxy BA	3.3	2.5	2.6	1.8
2-Methoxy BA (o-anisic acid)	3.4	nd	100 (4.4)[a]	83.9

[a]Numbers in parentheses represent activities in pkat/g FW. [b]Numbers in parentheses represent specific activities in pkat/mg protein. [c]nd, Not determined.

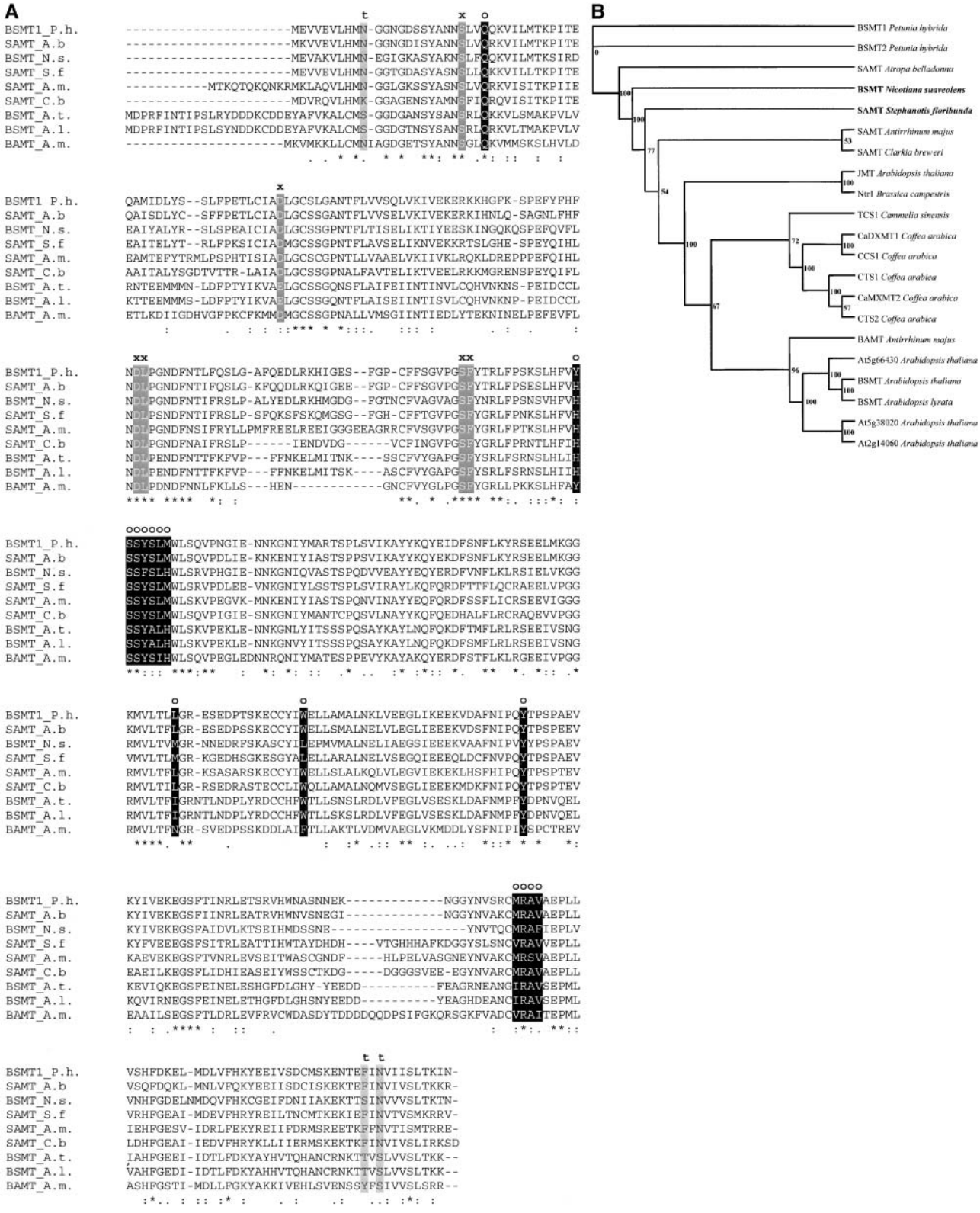

Figure 2. Amino acid sequence comparison of SA- and BA-specific enzymes. A, ClustalX (shaded box) amino acid sequence alignment of SA and BA methyltransferases from *N. suaveolens* (N.s.), *Atropa belladonna* (A.b.), *S. floribunda* (S.f.), snapdragon (A.m.), *C. breweri* (C.b.), petunia (P.h.), *Arabidopsis thaliana* (A.t.), and *Arabidopsis lyrata* (*A.l.*). Conserved amino acids (according to Zubieta et al., 2003) for SAM binding are indicated by ''x'', the amino acids involved in substrate binding are indicated by ''o'', and additionally important amino acids are indicated by ''t.'' *, Identical amino acid; **:**, conserved substitution; **.**, semiconserved substitution. B, Evolutionary relationship of benzenoid methyltransferases and *N*-methyltransferases. The tree was created with PAUP4.0 using the neighbor joining method (Thompson et al., 1997), ClustalW, and Treeview. The two enzymes investigated in this study are presented in bold.

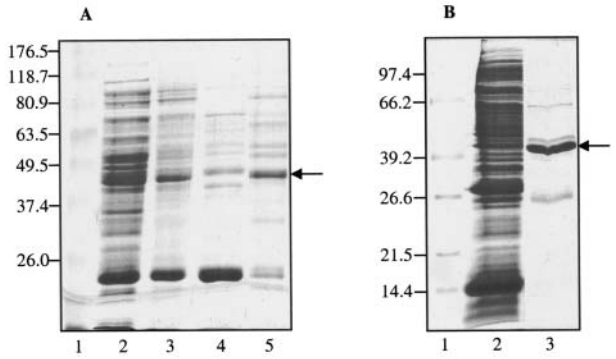

Figure 3. Purification of the recombinant proteins. A, Purification of *S. floribunda* SAMT following overexpression of the cDNA in *E. coli*. Lane 1, protein standard; lane 2, supernatant; lane 3, DE53 fraction mixture of high activity; lane 4, MonoQ fraction, low activity; and lane 5, Mono Q fraction, high activity. The arrow indicates the position of the 40-kD SAMT protein. B, The cloned cDNA from *N. suaveolens* was overexpressed in *E. coli*. Lane 1, protein standard; lane 2, supernatant; and lane 3, purified via nickel NTA affinity chromatography. A protein of 40 kD was enriched (arrow). Protein preparations were separated by 12.5% SDS-PAGE.

approximately 40% identity with the BA methyltransferase from snapdragon. Similarly, the *N. suaveolens* benzenoid carboxyl methyltransferase shares from 52% to 65% identity with homologous sequences from *C. breweri*, *S. floribunda*, and petunia, with the BAMT from snapdragon being the most dissimilar at 40% identity (Fig. 2B).

The two cDNAs from *S. floribunda* and *N. suaveolens* were cloned into the pET101 bacterial expression vector, expressed in *Escherichia coli*, and purified Fig. 3. The purified enzymes were assayed with a range of substrates at a concentration that should saturate or nearly saturate the enzyme (Table II). The *S. floribunda* enzyme was most active with SA, while the *N. suaveolens* protein was most active with BA. However, each protein also methylated a broad range of benzoic and cinnamic acid derivatives with relative activities between 1% and 99% compared to their preferred substrates (Table II). It appears that the spectrum of possible substrates (greater than 20% relative activity) is broader for the *S. floribunda* enzyme than for the enzyme from *N. suaveolens*.

The K_m values of the enzymes for some of these substrates were determined (Table III). The *S. floribunda* enzyme displayed a K_m value for SA that was almost 12-fold lower than its K_m value for BA. 3-Hydroxy BA was preferred over BA; the K_m value for 3-hydroxy BA was 7-fold lower than the K_m value for BA. The turnover number (K_{cat}) of the enzyme with BA was 10-fold higher than with SA; however, the overall catalytic efficiency (K_{cat}/K_m) of the enzyme with SA was only slightly higher than with BA (Table III). The K_m values of the *N. suaveolens* enzyme for SAs and BAs were similar (Table III), but the turnover number with BA was 3-fold higher than with SA (Table III). A 4-fold higher catalytic efficiency with BA over SA was determined for the *N. suaveolens* BSMT.

Determination of Endogenous Concentrations of SA and BA

For scent enzymes that can use more than one substrate, it is conceivable that the amount of synthesized and emitted esters depends at least in part on the availability of specific substrates (Kolosova et al., 2001) and does not simply reflect the K_m values of the enzyme for each of these substrates. The level of total SA and BA were therefore determined for the two plant species investigated here (Table IV). In *S. floribunda* flowers the levels of SA vary 2.5-fold during the day, but the total BA levels are similar. The BA levels are approximately 1,000- to 2,000-fold higher than the SA levels in *S. floribunda* petals. In *N. suaveolens* the levels of total SA were about 1 nmol/g FW in the morning and under the detection level in the evening, while the total BA levels were 2,000 and 2,500 nmol/g FW; therefore, a remarkable 2,000-fold difference between SA and BA levels could be registered in *N. suaveolens* flowers.

Molecular Models of the Benzenoid Carboxyl Methyltransferases

A detailed study of the crystal structure of SAMT from *C. breweri*, including the identification of active site residues, was recently reported (Zubieta et al., 2003). The *C. breweri* protein displays a highly specific SA binding site due to a number of stabilizing SAMT-SA interactions in the active site pocket, which positions the carboxyl acceptor moiety next to the reactive methyl group of SAM. Comparison of the amino acid

Table III. *Comparison of K_m, K_{cat}, and K_{cat}/K_m values of carboxyl methyltransferases from S. floribunda and N. suaveolens*

	S. floribunda SAMT			*N. suaveolens* BSMT		
	K_m	K_{cat}	K_{cat}/K_m	K_m	K_{cat} *N.s.* BSMT	K_{cat}/K_m
	μM	s^{-1}	$M^{-1}\ s^{-1}$	μM	s^{-1}	$M^{-1}\ s^{-1}$
SAM	63	6.9×10^{-3}	109.9	2.25	238×10^{-3}	105.8×10^{3}
SA	250	4.08×10^{-3}	16.3	162.2	26×10^{-3}	160.3
BA	2,900	42.5×10^{-3}	14.6	148.6	93×10^{-3}	625.8
3-Hydroxy BA	440	6.6×10^{-3}	15.0	nd[a]	nd	nd

[a]nd, Not determined.

Table IV. *Substrate concentrations in flower tissues*

	S. floribunda[a] 9 AM	*S. floribunda*[a] 9 PM	*N. suaveolens*[b] 9 AM	*N. suaveolens*[b] 7 PM
	nmol/g FW	*nmol/g FW*	*nmol/g FW*	*nmol/g FW*
Total SA	0.813 ± 0.411	0.353 ± 0.084	0.975 ± 0.06	bdl[c]
Total BA	901.5 ± 97	766.2 ± 88.5	1,928.7 ± 323	2,568.7 ± 874

[a]Average values from 1- and 3-d-old flowers. [b]Average values from 2-d-old flowers. [c]bdl, Below detection limit (1 pmol).

residues of the SAM methyl acceptor binding sites of the enzymes from *S. floribunda* and *N. suaveolens* (Fig. 2), combined with in silico modeling of the active site pocket in each (Fig. 4) show four and six differences, respectively, out of eight potential differences in the acceptor molecule binding site in the *N. suaveolens* and *S. floribunda* enzymes, respectively, compared to the SAMT from *C. breweri* (Table V). Most substitutions are chemically conservative, but others serve to expand or constrict the BA binding pocket.

The substitutions in the *S. floribunda* enzyme allow for the accommodation of bulkier substrates, and so do those in the *N. suaveolens* enzyme, but to a lesser extent. The modeling results are consistent with the empirical observation that the *S. floribunda* enzyme exhibits the broadest substrate tolerance during methylation, accepting 11 of the 17 tested substrates with 20% to 100% relative activity (Table II). The *S. floribunda* enzyme has the lowest K_m value with SA (and is therefore designated SAMT), likely because this acceptor substrate's 2-hydroxyl position forms an intramolecular hydrogen bond with the substrate's own carboxyl group, thus stabilizing the substrate in an orientation favorable for methylation (Zubieta et al., 2003). A substrate with a hydroxyl at the meta position is less favored by *S. floribunda* SAMT, but would permit the formation of a hydrogen bond to Tyr-261 that would provide some stability upon substrate binding. A 4-hydroxyl group does not lie within hydrogen bonding distance of protein residues in the rendered models, so it would appear not to provide additional stabilizing contacts with the active site pocket. However, the fact that the enzyme accepts 3,4-hydroxy BA no better than 4-hydroxy BA suggests that the 4 position substitution may sterically hinder substrate binding perhaps due to clashes with the nearby residues Phe-355, Leu-232, or Val-316. This enzyme disfavors substrates with substitutions at positions 5 and 6, which, again, may prevent proper substrate positioning due to steric conflicts with surrounding residues identified in the models and including Phe-355, Gln-25, Tyr-153, or Val-316. Because

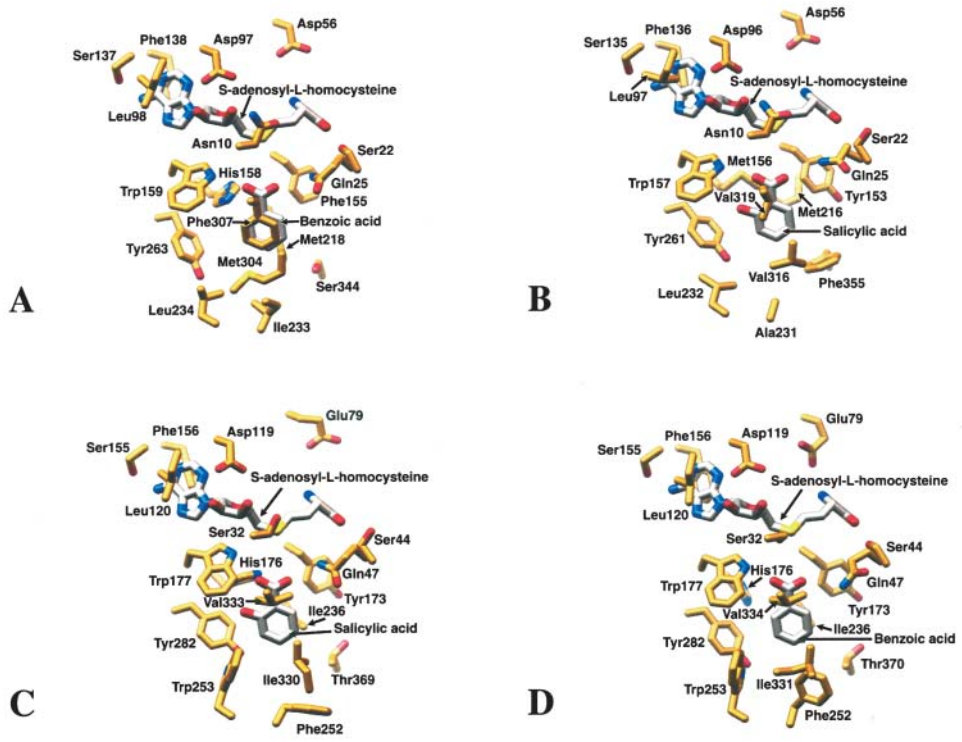

Figure 4. Computer modeling of the active site of four carboxyl methyltransferases. Three-dimensional view of the active sites of the methyltransferases from *S. floribunda* (A), *N. suaveolens* (B), *A. thaliana* (C), and *A. lyrata* (D). The side chains are depicted as half-colored sticks. The modeling was performed as described in Zubieta et al. (2003).

Table V. *Comparison of amino acids at the benzenoid substrate binding sites of the S. floribunda and N. suaveolens benzenoid methyltransferases*

Amino acids in bold are changed compared to the SAMT from *C. breweri*.

S. floribunda	*N. suaveolens*
Val 319	**Phe 307**
Val 316	Met 304
Phe 355	**Ser 344**
Ala 231	Ile 233
Leu 232	**Leu 234**
Met 216	**Met 218**
Met 156	**His 158**
Tyr 153	**Phe 155**

this enzyme possesses a relatively expansive active site, it can also accommodate bulkier substrates such as cinnamic acid and caffeic acid.

The *N. suaveolens* enzyme exhibits higher catalytic rates at saturating substrate concentrations for BA and 4-hydroxy BA, but has similar K_m values for BA and SA (Tables II and III) and is therefore designated BSMT. 4-Hydroxy BA could potentially be stabilized in the enzyme active site by hydrogen-bonding interactions with Ser-344 or Tyr-263, while Phe-307 forms a clamp on the substrate that may prevent optimal substrate positioning whenever there is a substitution at the meta position on the benzoic ring. In the case of 2-methoxy BA, the 2-methoxy group of the substrate likely hydrogen bonds with the ring nitrogen of His-158, thus constraining the substrate in a more favorable position for methylation. This enzyme also displays poor activity with substrates possessing substitutions at positions 5 or 6, likely due to spatial conflicts with nearby residues Phe-24 (not shown), Met-218, Gln-25, or Phe-155 and, unlike *S. floribunda* SAMT, cannot accommodate bulkier substrates.

Expression Analysis of BSMT from *N. suaveolens*

Flower-specific expression of *S. floribunda* SAMT was previously reported (Pott et al., 2002). RNA gel-blot analysis of mRNA from different tissues of *N. suaveolens* revealed selectively high levels in petals and very low or undetectable levels in leaves, roots, shoots, or flower parts such as stigma, styles, and stamens (Fig. 5).

DISCUSSION

Contribution of *S. floribunda* SAMT and *N. suaveolens* BSMT to the Synthesis of Benzenoid Methylesters Emitted from Their Flowers

The *N. suaveolens* emits more methyl benzoate than methyl salicylate, which is in agreement with the flower-specific enzyme we have characterized here that can methylate BA more efficiently than SA. Southern-blot analysis does not indicate the presence of additional floral benzenoid carboxyl homologous sequences (data not shown). It is therefore reasonable to conclude that this *N. suaveolens* BSMT is responsible for the synthesis of both methyl benzoate and methyl salicylate in a physiological setting, and that the amount of each methylated benzenoid produced is largely determined by the internal availability of the respective substrates, with BA levels far exceeding SA levels in plant tissue (Table IV).

A different situation appears to occur in the flowers of *S. floribunda*. We have identified a single flower-specific benzenoid carboxyl methyltransferase enzyme from *S. floribunda* that has similar catalytic efficiency with SA and BA, although the K_m value for SA is 10-fold lower. *S. floribunda* petal extracts also exhibit higher methylation activity with SA than with BA (Table II). Yet, these flowers emit 15-fold more methyl benzoate than methyl salicylate (Table I). This latter observation is most consistent with the additional observation that the concentration of BA in the floral tissue is 1,000- to 2,000-fold higher than the concentration of SA (Table IV).

The Benzenoid Acid Methyltransferases from *S. floribunda* and *N. suaveolens* Exhibit Broader Substrate Promiscuity Than Previously Described SAMTs, BAMTs, and BSMTs

Biochemical characterization and structural elucidation of the first benzenoid carboxyl methyltransferase identified, SAMT from *C. breweri*, indicate that this enzyme is relatively specific for SA, although it does methylate BA and a few other similar BA derivatives but at a much lower efficiency then the characterized physiological substrate, SA (Ross et al., 1999; Zubieta

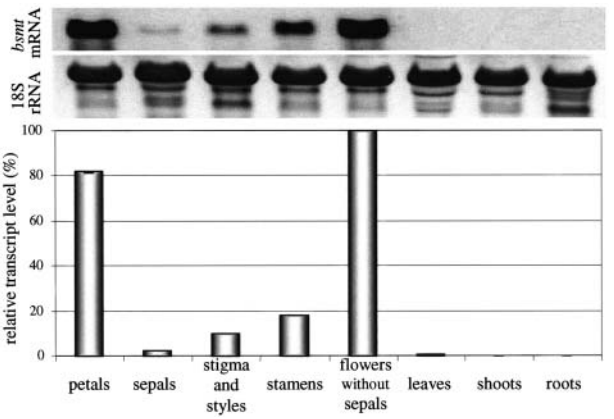

Figure 5. Expression of the *N. suaveolens* BSMT in different organs. Plant material of *N. suaveolens* was harvested at 6 PM. Flower parts were harvested at the first DPA. RNA was extracted and RNA gels were run with 5 μg total RNA. The blots were hybridized with the *N. suaveolens BSMT*-specific probe and rehybridized with an 18S rDNA probe. Relative transcript levels (normalized with the rRNA data) were calculated, and the highest value was set as 100%. Results were obtained from three blots (one representative blot is shown) and error bars indicate SE.

et al., 2003). Similarly, SAMT from snapdragon is also relatively specific for SA, whereas BAMT from snapdragon methylates only BA but not SA (Murfitt et al., 2000; Negre et al., 2002). Another recently characterized enzyme of this type is the *A. thaliana* BA/SA carboxyl methyltransferase (AtBSMT) and its ortholog from *A. thaliana lyrata*, AlBSMT. These two enzymes methylate BA and SA as well as a few BA derivatives, although AtBSMT is more efficient with SA while AlBSMT is more efficient with BA (Chen et al., 2003).

The modeling of the active sites of flower-specific *S. floribunda* SAMT and *N. suaveolens* BSMT shows that these enzymes are able to accommodate several substrate derivatives as well as more bulky substrates, and are therefore less specific than the SAMT of *C. breweri*. However, kinetic measurements show that while the *S. floribunda* enzyme possesses a lower K_m value for SA, its catalytic efficiency is similar with BA and SA, and the *N. suaveolens* enzyme has similar K_m values with SA and BA but is almost 4-fold more efficient with BA (Table III). These observations are explained in part by steric considerations in the active site (Fig. 4, A and B). However, clear differences in efficiency with specific substrates are not, in general, due entirely to factors residing in the active site. Such a situation is demonstrated by comparison of the active sites of AtBSMT and AlBSMT which are identical (Fig. 2A; Fig. 4, C and D), while their efficiencies for BA and SA are reversed, presumably due to differences elsewhere in the protein that affect the structure, dynamics, or both in the enzyme in solution.

Evolution of Specificity in the SABATH Methyltransferase Family

It has been argued that broad substrate promiscuity and concomitant low turnover rates are traits of newly evolved enzymes of secondary metabolism and that over time such enzymes may evolve a restricted substrate range and a faster turnover rate because such changes increase fitness (Firn and Jones, 2000; Pichersky and Gang, 2000). However, in secondary metabolism there may be an advantage for the organism to maintain enzymes with broad substrate specificity because such enzymes may allow the organism to rapidly respond to a changed environment by synthesizing a different set of chemicals appropriate for the situation with its existent set of enzymes (and genes; Schwab, 2003), so the observation that an enzyme has broad-range specificity may not necessarily be an indication that it has recently evolved.

Whether the broad specificity of the two benzenoid carboxyl methyltransferases, whose structural and biochemical characterization is reported here, is due to recent adaptative changes or due to long-term adaptation is still unclear. Addressing this question will require additional investigations into the involvement of these enzymes in the production of a range of methyl esters that *S. floribunda* and *N. suaveolens* flowers synthesize under different environmental conditions and will necessitate a larger scale comparison of these volatile chemical mixtures and the underlying genetic fingerprint across related species existing in unique ecotypes. This comparative study would require a detailed and multifaceted approach using orthologous genes and enzymes in related species but referenced to specific environments wherever possible so as to deconvolute the selective pressure exhibited by the local environment over the natural genetic drift that may occur over time. It is interesting to note that *H. carnosa*, a close relative of *S. floribunda*, which emits methyl salicylate but not methyl benzoate from its flowers (Altenburger and Matile, 1988), has a benzenoid carboxyl methyltransferase that is highly similar overall to *S. floribunda* SAMT (90% identical on the protein level), yet it is highly specific for SA, and its SA binding site is almost identical to that of *C. breweri* SAMT (M.B. Pott, J. Ross, and B. Piechulla, unpublished data). Moreover, the internal concentration of SA in its flowers is similar to that observed in *S. floribunda*, but no BA is detected in the *H. carnosa* flowers (M.B. Pott, A. Slusarenko, and B. Piechulla, unpublished data). While these observations suggest a correlation between the diversity of available intracellular substrates and broad substrate specificity, this pairwise comparison is not sufficient to establish whether broad substrate specificity is the ancestral or derived character for this lineage of benzenoid carboxyl methyltransferase enzymes. A full resolution of this question awaits the characterization of additional orthologous sequences and enzymes from other closely related species.

MATERIALS AND METHODS

Plant Material and Plant Growth Conditions

Stephanotis floribunda (Brongn.) plants were grown in the greenhouse with supplemental light between 6 AM and 10 PM during autumn and winter. *Nicotiana suaveolens* (Lehmann) was grown on vermiculite and watered with Hoagland solution. The plants were kept in growth rooms at 18°C to 22°C with 16 h light (6 AM till 10 PM) of 150 μmol m^{-2} s^{-1}. Day 1 was the day of flower opening.

Plant Extracts, Enzyme Assays, and K_m Value Determination

The crude extracts were prepared as described in Wang et al. (1997), with the following modifications: Petals were cut and immediately submerged in ice-cold extraction buffer (5 μL per mg FW). The plant material was ground on ice in a small mortar, centrifuged for at least 15 min, and the supernatant was transferred to a new tube. Glycerol (1/4 volume) was added and the crude extract was stored at −20°C.

The enzyme assay was performed according to Wang et al. (1997). The assay solution contained 12.5 μL of crude extract or 1 to 2 μL purified enzyme, 10 μL of assay buffer (250 mM Tris-HCl, pH 7.5, 25 mM KCl), 1 μL of 50 mM BA dissolved in ethanol (or 1 μL of pure ethanol as control), 1 μL [methyl-^{14}C]*S*-adenosyl-L-Met (58 mCi/mmol, in 9:1 [v/v] mixture of sulfuric acid [pH 2.0] and ethanol; Hartmann, Braunschweig, Germany), and H_2O to a final volume of 50 μL. The samples were incubated at 24°C for 50 min or 20°C for 40 min, *S. floribunda* and *N. suaveolens*, respectively, before 100 μL ethyl acetate and then 3 μL concentrated HCl were added to stop the reaction. The tubes were vortexed, briefly centrifuged, and 30 μL of the organic phase (on top) was transferred to a scintillation vial, mixed with 2 mL scintillation fluid (emulsifier-safe; Canberra Packard, Dreieich, Germany) and counted in

a scintillation counter (Tri-Carb 2100 TR; Canberra Packard). For investigation of the substrate spectrum, BA was replaced with the following substrates each at a concentration of 50 mM: 2-hydroxy BA (SA); 3-hydroxy BA; 4-hydroxy BA; 2,3-dihydroxy BA; 2,4-dihydroxy BA; 2,5-dihydroxy BA; 2,6-dihydroxy BA; 3,4-dihydroxy BA; 3,5-dihydroxy BA; cinnamic acid; 2-hydroxy cinnamic acid; 3-hydroxy cinnamic acid; 4-hydroxy cinnamic acid (p-coumaric acid); 2-methoxy BA (o-anisic acid); vanillic acid; caffeic acid; or jasmonic acid.

For product verification the same assay was scaled up to 1 mL with nonradioactive SAM. The products were extracted with 1 mL of hexane and analyzed via gas chromatography-mass spectrometry on a DB-5 column (60 m × 0.25 mm (injector 200°C, interface 235°C, oven program starting at 50°C, hold 2 min, heating to 275°C at a rate of 15°C/min, hold 10 min). Products were identified via mass spectrometry profile and methyl salicylate, methyl benzoate, and methyl jasmonate could also be identified via retention time and standard chemicals.

In all kinetics studies (e.g. K_m value), appropriate enzyme concentration and incubation times were chosen so that the reaction velocity was linear during the incubation time period; at least three replicates were performed. To measure the K_m for each substrate, one substrate concentration was fixed at a saturated level (usually 1 mM; 10 mM BA for K_m determination of *S. floribunda* SAMT) and the concentration of the other substrate to be measured was varied. Lineweaver-Burk, Hanes, and Eadie-Hofstee plots were performed to obtain the K_m and K_{cat} values.

Isolation of *N. suaveolens* BSMT cDNA

RT-PCR

The cloning and sequencing of the *S. floribunda* methyltransferase is described in Pott et al. (2002). The petals of several flowers of *N. suaveolens* (total of 500–1,000 mg) were harvested at 6 PM on the 1st and 2nd d after flower opening. RNA was extracted with lithium chloride purification according to Piechulla et al. (1986) or with guanidinium thiocyanate over a CsCl cushion (Sambrook et al., 1989) or according to the manual of the Qiagen RNeasy Plant Mini kit. RT-PCR reactions were performed with 500 ng to 2.5 μg total RNA and 200 units Moloney murine leukemia virus reverse transcriptase H^- (Access RT-PCR kit; Promega, Mannheim, Germany) according to the manufacturer's instructions. The annealing temperature was 37°C. Five microliters of the 25 μL RT-reaction was used for the PCR with 5 units *Taq* DNA polymerase (48°C). Primers for RT and PCR were STSAMT forward and STSAMT reverse (5′-AATGGAAGTTGTTGAAGTTCTTC-3′ and 5′-TAATTAAACCCTTCTCTTCAT-3′, respectively), specific for the *SAMT* gene of *S. floribunda* as described in Pott et al. (2002). The PCR product was analyzed by gel electrophoresis, and the fragment of about 750 bp was recovered from the agarose gel using a gel extraction kit (Qiagen, Hilden, Germany). The purified fragment was cloned using the pGEM-T Cloning kit (Promega). Both strands were sequenced using the SequiTherm Excel II DNA Sequencing kit with IRD-800 labeled, vector-specific primers and a LI-COR automated sequencer (MWG, Ebersberg, Germany). The amino acid sequences encoded by this fragment were compared with the amino acid sequences of known proteins of the databases using the BLAST Search system (National Center for Biotechnology Information [NCBI], Altschul et al., 1990).

5′-RACE and 3′-RACE

The PCR fragments from *N. suaveolens* were similar to the *SAMT* genes of *Clarkia breweri* and *S. floribunda*, but were incomplete. 5′-RACE was performed with the same RNA sample after digestion with DnaseI (Sigma-Aldrich, St. Louis). For the RT reaction, 2 μg of total RNA was added together with 200 units of Moloney murine leukemia virus reverse transcriptase H^- (Promega). After 62 min of incubation at 47°C the reaction was purified with the PCR purification kit (Qiagen) to remove all nucleotides. Twenty units of terminal desoxynucleotidyl transferase (TdT; Promega) and 0.25 nmol dATP were added together with the corresponding buffer to 2 μL of the eluate and incubated for 2 h at 37°C. An additional 10 units of TdT were added and the reaction incubated overnight at 37°C. After heating to 70°C for 10 min to stop the reaction, 5 μL were used for amplification by PCR with *Taq* DNA polymerase with the following primers: an oligo(dT) primer (5′-GACTGGACTTCAATCAGTTAC$(T)_{16}$-3′) and STSAMT 18 reverse (5′-ATCTGAAAATGGTGTTGAAATCAT-3′). The resulting fragment was analyzed, purified, cloned, and sequenced as described in the previous section.

For the 3′-RACE, total RNA from flowers harvested at midnight on the day of anthesis was used. To eliminate contaminating DNA, a digest with 1 unit DNaseI (Sigma-Aldrich) was performed according to the manufacturer's recommendations. Ten microliters (1.3 μg) RNA from this reaction was used for RT-PCR with the eAMV kit for RT-PCR (Sigma-Aldrich). Three different primers were used in separate reactions: oligo-d$(T)_{15}$A, oligo-d$(T)_{15}$C, and oligo-d$(T)_{15}$G, linked to an adaptor sequence for the RT reaction (5′-GACTGGACTTCAATCAGTTAC$(TTT)_5$A/C/G-3′, respectively). For the RT reaction the following temperature program was adjusted: 15 min 45°C, temperature increment of 2°C every 3 min up to 55°C, hold 15 min, temperature increment of 2°C every 3 min up to 65°C, hold 15 min. Five microliters were then used for the following PCR with a gene-specific and an adaptor primer (5′-GAAGATCGCTTTAGCAAAGCT-3′ and 5′-GACTGGACTTCAATCAGTTAC-3′, respectively), according to the manufacturer's manual. Gel electrophoresis revealed a fragment of about 450 bp, which was recovered from the agarose gel using a Gel Extraction kit (Qiagen). Cloning and sequencing was performed as described in the previous paragraph. The accession number of the *N. suaveolens BSMT* is AJ628349.

Cloning Into the Expression Vector

The full-length *N. suaveolens BSMT* cDNA obtained by RT-PCR and the additional fragment obtained by the 3′-RACE were linked via a *Sac*I restriction site and the resulting fragment was amplified with *Pfu* DNA polymerase (Promega) and cloned into the expression vector using the pET101 Directional TOPO Expression kit according to the manufacturer's instructions. Overexpression of the poly-His (6x his) tagged gene was performed in the *Escherichia coli* strain HMS174 (*F*$^-$, *recA*, [*r*$^-$ *K12*, *m*$^+$ *K12*], *Rif*^{r}) *BL21* (*DE3*) *pLys*. The DNA of the complete *S. floribunda SAMT* gene (including start-to-stop codons) was amplified by PCR (primers STSAMT forward and reverse) and cloned without a poly-His tag using the pCR T7/CT TOPO TA Cloning kit (Invitrogen, Karlsruhe, Germany) and transformed into *E. coli* BL21 (codon+) cells. Overexpressed protein was obtained after 2 h of preincubation at 37°C, induction with 1 mM isopropylthio-β-galactoside and continued incubation for 20 h at 20°C. The cells were harvested by centrifugation at 6,000*g* for 15 min, resuspended in 5 mL of lysis buffer (10 mM NaCl; 50 mM Tris/HCl, pH 8.0; 1 mM EDTA; 10% [w/v] glycerol; 14.3 mM mercaptoethanol), sonicated three times for 30 s, and the soluble extract centrifuged at 21,000*g*. Extract activity assays with 10 μL of protein fractions were performed as described above and proteins were separated on 12.5% SDS polyacrylamide gels.

Isolation of RNA and Northern-Blot Hybridizations

At indicated time points, flower petals (300–1,000 mg) were harvested, frozen in liquid nitrogen, and RNA was extracted according to Cheng and Seemann (1998). Lithium chloride precipitation allowed separation of RNA from DNA. Five micrograms RNA aliquots were used for northern blots. The *N. suaveolens BSMT* and 18S rDNA probes were labeled by PCR using digdUTP (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for the *N. suaveolens BSMT* probe used for the PCR reaction were forward, 5′-AATGGAAGTTGTTGAAGTTCTTC-3′; and reverse, 5′-TAATTAAACCCTTCTCTTCAT-3′. Primers for the PCR reaction to obtain the 18S rDNA probe from *N. suaveolens* genomic DNA were forward, 5′-GGTCGCAAGGCTGAAACTT-3′; and reverse, 5′-TTATTGCCTCAAACTTCC-3′. The resulting probe was approximately 300 bp long.

Hybridization was performed with 5× SSC buffer (7% SDS, 50% formamide, 0.1% lauryl sarcosine, 2% blocking solution, 50 mM Na_2HPO_4) at 50°C overnight. Membranes were washed twice for 5 min with 2× SSC, 0.1% SDS at room temperature and for 15 min with 0.1× SSC and 0.1% SDS at 50°C. After hybridization, the membranes were incubated with anti-Dig alkaline phosphate and CSPD (disodium 3-(4-methoxyspiro[lsqb]1,2-dioxetane-3,2[prime]-{5[prime]-chloro}tricyclo{3.3.1.1^{3,7}}decan[rsqb]4-yl; Roche Diagnostics) was used as a substrate. The chemiluminescence signal detected and quantitated with the LAS-1000 (Fuji-Raytest; Straubenhardt, Germany; software Image Gauge, Fujifilm, Tokyo) for 10 to 60 min. Transcript levels were normalized to rRNA levels, which were determined by repeated hybridizations, and the *BSMT* mRNA/rRNA ratios were calculated. The highest ratio of each individual blot was set to 100% and SE was calculated.

Determination of SA and BA Levels in Plant Tissue

The extraction protocol was as described by Meuwly and Metraux (1993). Plant tissue (50–200 mg) was ground in liquid nitrogen and extracted with 700 μL of 90% methanol to which 500 pmol *o*-anisic acid had been added as

a recovery- and internal standard. After addition of 1.4 mL 100% methanol and mixing, the sample was centrifuged (10 min at 14,000 rpm in a microfuge). The methanol was removed from the supernatant by vacuum centrifugation at 43°C. The residue was brought up to 1 mL with 5% (v/v) trichloroacetic acid on ice and separated into two equal aliquots. One aliquot was used as a source to measure free SA and BA, and acidic hydrolysis was performed with the other aliquot by addition of 70 μL 12 M HCl and heating for 1 h at 96°C to release free SA and BA from glycosidic conjugates. To determine SA and BA, each aliquot was extracted twice with 1 mL ethylacetate/cyclohexane/isopropanol (50+50+0.5) while kept on ice, the organic phases were combined, vaccum dried, and resuspended in 200 μL methanol for HPLC analysis. Samples (20 μL) were chromatographed under isocratic conditions with water:methanol:acetic acid (45:50:5) at 0.8 mL/min on a C18 column (Bio-Sil C18 HI 90-5S, 150 $\times$ 4.6 mm). SA was measured via a fluorescence detector set at excitation and detection wavelengths of 313 and 405 nm, respectively (FP920 detector; Jasco, Gross-Umstadt, Germany) and BA via a UV detector (Milton Roy CM4000; Ivyland, PA) at 242 nm.

Computer Modeling

Modeling of the active sites of the *N. suaveolens* BSMT, the *S. floribunda* SAMT, and the Arabidopsis BSMT enzymes was accomplished by fitting the amino acid sequences of each of the three proteins to the previously published three-dimensional structure of the *C. breweri* SAMT (Zubieta et al., 2003) using the program modeler (Sali and Blundell, 1993). The resulting pdb files were visualized, active site residues of the three model proteins aligned with those of the *C. breweri* SAMT, and graphical output images obtained using the University of California, San Francisco, Chimera package, supported by National Institutes of Health P41 RR-01081 (Huang et al., 1996) and the program O (Jones et al. 1993). Three-dimensional images were drawn using POV-Ray (Amundsen, 1997).

Sequence data (for the *N. suaveolens* BSMT) from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ628349 (for the *N. suaveolens* BSMT).

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