

S-Adenosyl-L-Methionine:Salicylic Acid Carboxyl Methyltransferase, an Enzyme Involved in Floral Scent Production and Plant Defense, Represents a New Class of Plant Methyltransferases¹

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S-Adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (SAMT) was partially purified from petals of the annual California plant *Clarkia breweri*. SAMT catalyzes the formation of methylsalicylate, an important floral scent compound in *C. breweri*, from salicylic acid and S-adenosyl-L-methionine (SAM). The native enzyme is a dimer with a subunit molecular weight of 40.3 kDa, and it has a K_m for salicylic acid of 24 μ M and a K_m for SAM of 9 μ M. A cDNA encoding SAMT was isolated from a *C. breweri* cDNA library prepared from floral mRNA. The sequence of the protein encoded by SAMT cDNA shows no significant sequence similarity to any protein in the data bank whose biochemical function is known. It does show significant sequence similarity (20–40% identity) to proteins encoded by at least seven *Arabidopsis thaliana* genes whose sequences have recently been determined in large-scale sequencing projects. The *C. breweri* SAMT cDNA was expressed in *E. coli* and the bacterial cells synthesized a functional SAMT protein with properties nearly identical to those of the plant-purified enzyme. © 1999 Academic Press

Key Words: *Clarkia breweri*; floral scent; flavor; methylsalicylate; O-methyltransferase; salicylic acid.

Methylsalicylate (MSA)³ is a benzenoid ester that is widespread in the plant world. It is a common ingredi-

ent of floral scent, especially of flowers that are moth-pollinated (1). It may also be part of the plant defense responses that are mediated by salicylic acid (SA). Several reports have shown that MSA accumulates in damaged tissue together with SA (2, 3) and may even be an air-borne signal that induces the defense responses in nondamaged organs of the same plant or in adjacent ones, perhaps by being absorbed by the tissue and then converted back into SA (3). An enzymatic activity that catalyzes the formation of MSA from SA, and which uses SAM as the methyl donor (SAM:salicylate carboxyl methyltransferase, or SAMT), has been reported from petals of the scented flowers of *Clarkia breweri*, an annual, noncultivated plant native to California (4). However, the purification and characterization of SAMT have not yet been reported.

Methyl esters in general are widespread plant secondary compounds and are often involved in plant-animal interaction. Many are found in floral scents, such as methyljasmonate and methylbenzoate (1). Some of these, and other methyl esters, are also produced in vegetative tissues. Since they often contribute to the creation of a distinct flavor that humans find attractive, several plant varieties have been selected and bred that are rich in methyl esters, for example basil varieties that are rich in methylcinnamate (5). MSA itself is recognized as the flavor ingredient found naturally in leaves and flowers of wintergreen and is often produced synthetically to flavor many types of food, candy, and medicine (6–8). Yet to date, no plant enzymes catalyzing the formation of such methyl esters have been fully purified and characterized. Here,

polyvinylidene fluoride; SA, salicylic acid; SAM, S-adenosyl-L-methionine; SAMT, SAM:salicylic acid carboxyl methyltransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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³ Abbreviations used: Bis-Tris, bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane; COMT, caffeic acid O-methyltransferase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid; FPLC, fast protein liquid chromatography; HAP, hydroxyapatite; IEMT, SAM:(i-so)eugenol O-methyltransferase; IPTG, isopropyl β -D-thiogalactopyranoside; MSA, methylsalicylate; OMP, O-methyltransferase; PVDF,

we report the purification and characterization of SAMT and of SAMT cDNA. The complete sequence of the protein, deduced from the cDNA, indicates that SAMT is part of a hitherto unrecognized protein family of methyltransferases distinct from all previously identified methyltransferases.

MATERIALS AND METHODS

Plant material and growth conditions. Details of the growing conditions of the *C. breweri* plants were previously described (9).

SAMT enzyme extraction, assays, and product analysis. A crude protein extract was prepared as previously described (10). A ratio of 10:1 (v/w) extraction buffer to tissue fresh weight was used. Enzyme assays were prepared and activity levels calculated as previously described (4) except that assay temperature was 22°C. The identity of the products was verified by radio-TLC and by GC-MS, as previously described (4, 10, 11).

Protein purification. The purification protocol essentially followed the procedures previously described (9, 10). All manipulations were carried out at 4°C unless stated otherwise. In a typical purification procedure, 250 ml of crude extract (representing 25 g fresh wt of petal tissue) was loaded onto a DEAE-cellulose column (10 ml of DE53; Whatman, Fairfield, NJ) that was preequilibrated with a solution containing 50 mM Bis-Tris, pH 7.0, 10% glycerol, and 10 mM β -mercaptoethanol (buffer A). After washing off unabsorbed material from the column with 15 ml of buffer A containing 100 mM KCl, SAMT was eluted with 15 ml of buffer A containing 250 mM KCl. Fractions (5 ml) were collected and assayed for SAMT activity. Fractions with the highest SAMT activity were pooled (total of 10 ml), diluted threefold with buffer A, and concentrated threefold in an Ultrafree-15 Centrifugal Filter device (Millipore, Bedford, MA). DEAE-cellulose-purified fractions were loaded onto a HAP column (1-cm diameter \times 10 cm, Bio-gel HT; Bio-Rad, Richmond, CA) installed in a Pharmacia FPLC apparatus and preequilibrated with buffer A. Flow rate was 0.5 ml/min. After the enzyme was loaded, the column was washed with 20 ml buffer A and eluted with a linear gradient (100 ml) from 0 to 400 mM Na phosphate in buffer A. Fractions (2 ml) were collected and assayed for SAMT activity.

The fractions containing SAMT activity, eluted from the HAP column with about 100 mM Na phosphate, were pooled (8 ml) and frozen overnight after the addition of KCl to a final concentration of 200 mM and EGTA to a final concentration of 10 mM. Next day the sample was concentrated 10-fold in the Ultrafree-15 filter device, diluted 2-fold in buffer A, and loaded immediately on Mono-Q (0.5-cm diameter \times 6.0 cm, Pharmacia). These intermediate steps between the HAP and Mono-Q columns were performed because dialysis or even storage of SAMT for an extended period in low concentrations of potassium salt solutions always resulted in extensive loss of activity. SAMT was eluted from the Mono-Q column with a 100–400 mM KCl gradient in buffer A. SAMT activity eluted with 180–190 mM KCl. Fractions (2 ml each) were collected and protein content and purity were examined by SDS-PAGE gel electrophoresis followed by Coomassie Brilliant Blue or silver staining of the gel. The fractions which had the largest amount of SAMT protein and the highest degree of purity (4 ml) were concentrated to 100 μ l using the Ultrafree-15 filter device and used for sequencing and for enzyme characterization.

Protein sequence determination. The concentrated proteins present in the Mono-Q fraction with the highest level of SAMT activity were electrophoresed on SDS-PAGE with a 4–12% acrylamide gradient gel. The gel was then blotted onto a polyvinylidene fluoride (PVDF) membrane, the membrane was stained with Coomassie, and individual protein bands were excised and subjected to N-terminal sequencing as previously described (12). Alternatively, the gel was stained with zinc imidazole and zinc sulfate (a negative

stain), and the protein bands were excised and subjected to cyanogen bromide cleavage as previously described (12). The cleaved peptides were extracted and reelectrophoresed on 10% SDS-PAGE, and the gel was blotted onto a PVDF membrane. The separated peptides were identified by staining and excised, and the sequence of individual peptides was determined. Sequence analysis was as previously described (12).

cDNA isolation and characterization. To isolate a SAMT cDNA, we prepared a probe in the following way. Based on the peptide sequence MGENSPEYQIFLNDLPGNDFNAIFRSLP, we synthesized the 26-mer oligonucleotide 5'-CC(ACGT)GA(AG)TA(CT)CA(AG)AT(ACT)TT(CT)(CT)T(ACGT)AA(CT)GA-3' which corresponds to the amino acids PEYQIFLND in the sense direction, and the 23-mer oligonucleotide, 5'-AA(AG)TC(AG)TT(ACGT)CC(ACGT)GG(ACGT)A(AG)(AG)TC(AG)TT-3', which corresponds to the peptide sequence NDLPGNDF in the antisense direction. The two oligonucleotides have a common, complimentary region of five nucleotides. Since these oligonucleotide can prime each other, we mixed the two oligonucleotides together (without the addition of random primers) and performed a labeling reaction with [α -³²P]dATP (13). This reaction resulted in the formation of a 44-mer radioactive probe, which was used to screen a *C. breweri* floral cDNA library as previously described (12). Several clones were isolated and characterized by restriction enzyme digests and by sequencing. All proved to encode the same protein. The sequence of the longest clone was completely determined on both strands.

Expression of SAMT in Escherichia coli and protein purification. The coding region of SAMT was amplified with the forward 30-mer oligonucleotide 5'-TTCTAGACATATGGATGTACGGCAAGTTCT-3' (for the first methionine codon) or the 29-mer oligonucleotide 5'-TTCTAGACATATGAAGGGTGGCGCCGGAG-3' (for the second methionine codon) and the backward 28-mer oligonucleotide 5'-TGG-ATCCGATGAATTAATCTGATTTTCG-3'. The forward oligonucleotides introduced an *Nde*I site at the initiating methionine ATG codon. The backward oligonucleotide introduced a *Bam*HI site downstream of the stop codon. The PCR-amplified fragments were cloned into the *Nde*I-*Bam*HI site of the expression vector pET-11a or pET-28 (14). The resulting plasmid was transferred into *E. coli* BL21 cells, and the cells were grown in 2X YT medium (16 g trypton, 10 g yeast extract, and 5 g NaCl in 1 liter of medium) with 25 μ g/ml chloroamphenicol and 50 μ g/ml ampicillin (pET-11a constructs) or 50 μ g/ml kanamycin (pET-28 constructs) at 37°C. When the culture density reached OD₆₀₀ of 0.4–0.5, IPTG was added to make a final concentration of 0.4 mM and the culture was incubated further at 37°C and shaken at 150 RPM for 3 h.

For the purification of nontagged SAMT, the cells were harvested and sonicated in lysis buffer (10 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 10% glycerol, and 10 mM β -mercaptoethanol, pH 8.0) on ice with a microtip probe for four time intervals of 30 s each. The lysate was spun at 12,000g for 10 min to pellet cell debris, and soluble SAMT was collected in the supernatant fraction. Next, the proteins in this supernatant that precipitated in an ammonium sulfate concentration of 75% but not 50% were collected by centrifugation and resuspended in buffer A. The nontagged SAMT protein was further purified by DE53 anion exchange, HAP, and Mono-Q chromatography. The *E. coli*-expressed SAMT, tagged at its N-terminus with a short peptide (20 amino acids) containing six histidine residues (the product of the pET-28 construct), was purified by nickel-based affinity chromatography (1 ml bed volume) according to the manufacturer's protocol (Novagen, Inc.), with the following modifications: After binding the protein to the column, the column was washed in binding buffer without imidazole, and SAMT was next eluted with 6 ml of stripping buffer (100 mM EDTA, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). After elution, the fraction containing SAMT activity was concentrated by centrifugation in the Ultrafree-15 Centrifugal Filter device and resuspended in buffer A.

TABLE I
SAMT Purification from *C. breweri* Petals

Purification step	Total protein mg	Total activity pkat	Specific activity (pkat/mg protein)	Purification (-fold)	Recovery (%)
Crude extract	139.3 ^a	525	3.8	1.0	100
DE-53	14.8	170	11.5	3.0	32.4
HAP	6.4	92.5	14.5	3.8	17.6
Mono-Q	0.049	17.3	353	93	3.3

^a Representing 25 g of fresh weight of *C. breweri* petals.

Extraction of methylsalicylate from the spent medium of E. coli cells and GC-MS analysis. BL21 cells expressing SAMT and controls were grown under conditions described above, except that the growing medium was supplemented with 5 μ g/ml SA. After removing the cells by centrifugation, the spent medium (25 ml) was extracted with 5 ml of hexane, and the hexane phase was removed, placed in a glass tube, and reduced to 0.2 ml by passing N₂ at the opening of the tube. The samples (3 μ l of the hexane concentrate) were analyzed by GC-MS.

Molecular mass estimation. Partially purified SAMT was run on a gel filtration column (HR 10/30 Superose 12, Pharmacia,) at a flow rate of 0.3 ml/min in a mobile phase of 50 mM Bis-Tris, pH 7.5, 200 mM KCl, 10 mM EGTA, 10% glycerol, and 10 mM β -mercaptoethanol. Fractions (0.5 ml) were collected for enzyme activity assays. The column was calibrated with the following protein standards: aprotinin (6.5 kDa), cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (68 kDa), alcohol dehydrogenase (150 kDa), and β -amylase (200 kDa). A standard curve was obtained by plotting retention time (V_r/V_0) of the protein standards against the log of the molecular weight. The subunit molecular weight was estimated by denaturing PAGE. The SDS-PAGE was carried out on a 13% polyacrylamide gel and calibrated with molecular weight standards in the range of 14.3–200 kD (GibcoBRL, Grand Island, NY).

Temperature effect on SAMT stability. SAMT was incubated at temperatures ranging from 4 to 65°C for 30 min and then chilled on ice. Samples incubated at each temperature were then used for enzyme assays. At least four independent assays were performed for each point and then an average was taken.

pH optimum of SAMT activity. The optimum pH for SAMT activity was determined using two buffer systems. Reactions were carried out in 50 mM Bis-Tris buffer with pH levels ranging from 6.0 to 7.5 and 50 mM Tris-HCl buffer with pH levels ranging from 7.0 to 10.0. Final results are an average of at least four independent assays.

Effectors. Enzyme assays were performed with one of the following cations present in the assay buffer at the final concentration of 5 mM: Ca²⁺, Cu²⁺, Fe²⁺, Fe³⁺, K⁺, Mg²⁺, Mn²⁺, Na⁺, NH₄⁺, and Zn²⁺. Except for Cu²⁺, Fe²⁺, and Fe³⁺, which precipitate under reducing conditions, all assay buffers also contained 10 mM β -mercaptoethanol. Results presented are averages of at least four experiments.

Determination of K_m values. In all kinetics studies, appropriate enzyme concentration and incubation times were chosen so that the reaction velocity was linear during the incubation time period. To measure the K_m for each substrate, one substrate concentration was fixed at a saturated level and the concentration of the other substrate to be measured was varied. Lineweaver-Burk plots were made to obtain the K_m values.

RESULTS

SAMT protein purification and protein sequencing. Previous work has shown that levels of *C. breweri* SAMT activity were highest in petals compared to the levels of activity in all other floral parts or the vegetative tissue (4). We therefore began SAMT purification from petals of *C. breweri* by several chromatographic steps, including DEAE anion exchange (DE53), HAP, and another anion exchange, Mono-Q, on Pharmacia's FPLC system. Throughout the purification process a reducing agent such as β -mercaptoethanol was used, as SAMT lost all activity within a few hours in solutions devoid of such a reducing agent, and this loss was not reversible. The HAP chromatography step was included, even though it did not result in a substantial increase in purity overall, because it allowed for the efficient separation of SAMT activity from the activities of two other *O*-methyltransferases present in *C. breweri* petals, IEMT and COMT (10), each with a molecular mass similar to that of SAMT (see below).

After these three successive chromatographic steps, the Mono-Q fraction with the highest SAMT activity had SAMT specific activity of 353 pkat/mg protein, representing 93-fold purification over crude extract (Table I). A 40-kDa protein was the major constituent of this fraction, representing about 50% of the total protein, and four other proteins of 45, 75, 105, and 130 kDa were also present in appreciable amounts (Fig. 1). Several lines of evidence suggested that the 40-kDa protein was most likely SAMT. First, the presence and amount of this protein in the various fractions eluting from the Mono-Q column most closely correlated with SAMT activity (both SAMT activity and amount of the 40-kDa protein peaked at the fraction containing 185 mM KCl). On the other hand, the peak amount of the 75-kDa protein was found in the fraction containing 190 mM KCl. The 45-kDa protein was ruled out when a partial sequence obtained through N-terminal sequencing showed it to be cinnamyl alcohol dehydrogenase instead. The two largest proteins (105 and 130 kDa) were excluded based on information concerning

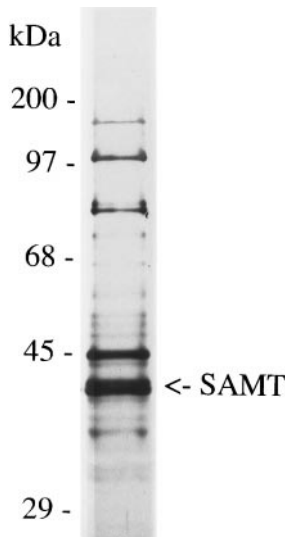


FIG. 1. Purification of SAMT. An aliquot from the Mono-Q fraction with the highest level of SAMT activity was electrophoresed on 10% SDS-PAGE. Position of molecular weight markers is indicated on the left. Approximately 0.15 μ g protein was loaded, and the gel was silver-stained.

the molecular weight of the native SAMT protein, which showed it to be approximately 75 kDa (see below).

Isolation of a SAMT cDNA and analysis of its sequence. The 40-kDa putative SAMT protein was therefore subjected to extensive protein sequence determination. It was found that its N-terminal sequence was blocked, but we were able to determine the sequence of two internal peptides (Fig. 2) obtained from it by cyanogen bromide cleavage, and neither of these was found to have high similarity to any protein in the databases whose function is known. Based on the peptide sequences, oligonucleotides were synthesized (see Materials and Methods) and used to probe a *C. breweri* floral cDNA library. Several cDNA clones were thus identified, all encoding the same protein whose sequence includes the peptide sequences obtained from the 40-kDa putative SAMT protein. The sequence of the longest cDNA clone is shown in Fig. 2. This cDNA has 1321 nucleotides, and it has an open reading frame of 359 codons, starting with the methionine codon at positions 103–105. The calculated molecular weight of the protein encoded by this open reading frame is 40.3 kDa. Upstream of the methionine codon at positions 103–105 there are several stop codons but no methionine codons in-frame, indicating that this cDNA contains the entire open reading frame.

We searched the databases for sequences similar to that of the putative SAMT protein, using the BLAST 2.0 program. No protein of known function was identi-

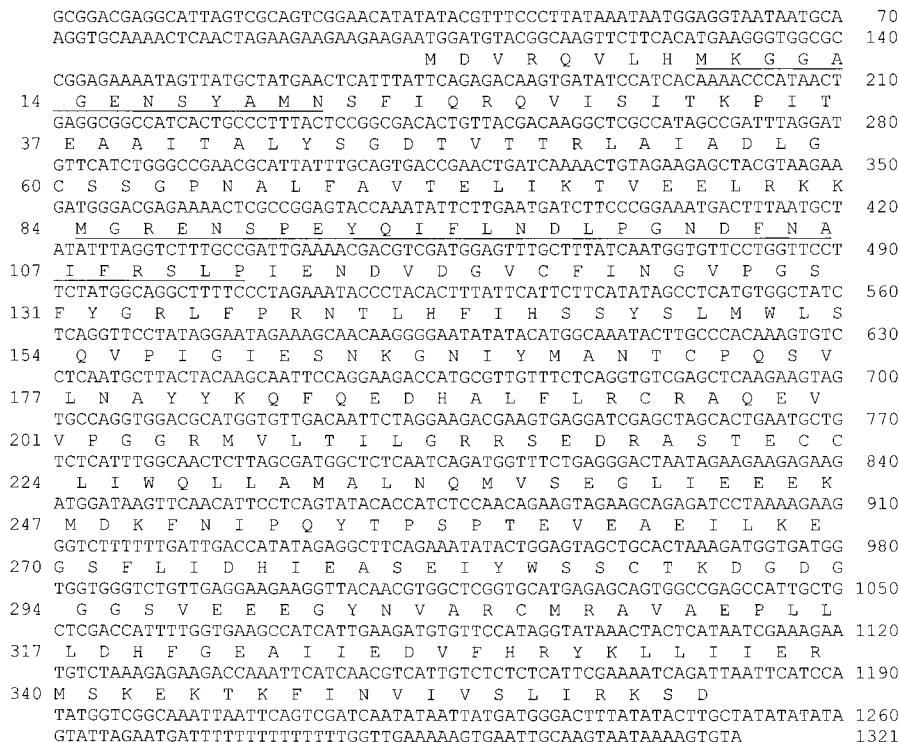


FIG. 2. DNA and protein sequence of *C. breweri* SAMT. The DNA sequence of SAMT cDNA is shown with numbering on the right. The sequence of the encoded protein is shown below the corresponding DNA sequence, with the numbering of the amino acids on the left. The peptides whose sequences were determined experimentally are underlined. The Accession No. of the *C. breweri* SAMT cDNA is AF133053.

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SAMT:      MDVR---QVLHMKGAGENSSYAMNSFIQRQVISITKPITEAAITALYSGDTVTRLAIADLGC 60
AB013389:  MYSFV--NALRMSGGDGDNSYSTNSLLQKKVLSKAKPVLVKNTKGMMINLNFPNYIKVADLGC
Z99708:    MDKKDMEREFYMTGGDGKTSYARNSSLQKKASDTAKHITLETLQQLYKETRPKS-LGIADLGC

SAMT:      SSGPNALFAVTELIKTVEELRKKM-GRENSPEYQIFLNDLPGNDFNAIFRSLPDIENDVD--- 118
AB013389:  ATGENTFLTMAEIVNTINVLCQQC--NQKPEIDCCLNDLPDNDFNTTFKFVPFFNKRVKSK-
Z99708:    SSGPNTLSTITDFIKTVQVAHREIPIQPLPEFSIFLNDLPGNDFNFIFKSLPDFHIELKRDN

SAMT:      -----GVCFINGVPGSFYGRLFPRNTLHFIHSSYSLMWLSQVPIGIESNKGNIYMANTCPQSV 176
AB013389:  -----RLCFVSGVPGSFYSRLFPRKSLHFVHSSYSLHWLSKVPKGLEKNSSSVYITTSSPPNA
Z99708:    NNGDCPSVFIAAYPGSFYGRLFPENTIHFVYASHSLHWLSK--QGKSINKGCVSICSLSSEAV

SAMT:      LNAYKQFQEDHALFLRCRAQEVVPGGRMVLTILGRRS-EDRASTECCLIWQLLAMALNQMVS 238
AB013389:  YKAYLNQFQSDFKSFLEMRSEMVSNGRMVLTFIGRKTLDDPLHRDCHFWTLLSTSLRDLVY
Z99708:    SKAYCSQFKEDFSIFLRCRSKEMVSAGRMVLIILGREG-PDHVDRGNSFFWELLSRSIADLVA

SAMT:      EGLIEEEKMDKFNIPQYTPSPTEVEAEILKEGSFLIDHIEASEIYWSSCTKDGDGGG--SVEE 297
AB013389:  EGLVSASKVDSFNIPFYDPSKEVMEMIRNEGSFEINDLEIHGFELGLSNHDEDYMLHSQISK
Z99708:    QGETEEEKLDSYDMHFYAPSADEIEGEVDKEGSFELERLEMLEVKKDKGNTEGDISY-----

SAMT:      EGYNVARCMRAVAEPLLLDHFGEAIEDVFHRYKLLIERMSKEKTKFINVIVSLIRKSD 359
AB013389:  AGQREANCIRAVSESMLVADFGVDIMDTLFKKFAYHVSQHASCTNKTTVTLVVSLIRK
Z99708:    -GKAVAKTVRAVQESMLVQHFGEKILDKLFDTYCRMVDDELSKEDIRPITFVVLRKKL

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FIG. 3. Comparison of *C. breweri* SAMT with the two most similar proteins in the databank. Both these proteins are encoded by *Arabidopsis* genes, whose Accession Nos. are indicated on the left. Residues in red are identical in all three proteins. Numbering of the residues refers to SAMT. The second conserved M (see text) is underlined.

fied in this search. In particular, it is noteworthy that no sequence similarity to any other known plant methyltransferase (15) was found. However, the search identified seven proteins encoded by *Arabidopsis* genes of unknown function that were 20–40% identical to SAMT. In addition, a short (454 nucleotides) genomic fragment from barley (Accession No. AJ234779) which potentially encodes, partially, a protein with significant sequence similarity to SAMT was also identified. Several Expressed Sequence Tags (ESTs) from *Arabidopsis* and other species with sequence similarity to *C. breweri* SAMT were also found in our search. The sequences of the two *Arabidopsis* proteins with the highest identity (40% each) to SAMT are shown in Fig. 3. We note the fact that all these genes have a conserved methionine codon downstream nearby to the first methionine codon. In the *C. breweri* SAMT cDNA, this downstream codon is found at positions 127–129 (Fig. 2, and see underlined M in Fig. 3).

Expression of SAMT cDNA in *E. coli*. We amplified the open reading frame starting with the first methionine codon and ligated it into the *Nde*I–*Bam*HI sites of the *E. coli* expression vector pET-11a, which allows for the expression of a non-tagged polypeptide. We did the same with the open reading frame starting with the second methionine codon. We also ligated the larger open reading frame into the *Nde*I–*Bam*HI sites of the pET-28 vector. This vector has a short open reading

frame that includes six consecutive histidine codons upstream of the *Nde*I site. Ligating the SAMT open reading frame into the *Nde*I site of pET-28 results in an in-frame fusion, and therefore the SAMT protein produced by this vector will have a short N-terminal extension of 20 residues that includes six histidines (i.e., a “His tag”). All of these constructs (and a pET-11a control plasmid without an insert) were then mobilized into *E. coli* cells. Cells were grown with or without 5 μ g/ml SA, under the specified conditions, and induced by IPTG as described under Materials and Methods. The cells were then harvested and sonicated in lysis buffer to extract the proteins. The spent medium was extracted with hexane and the hexane fraction concentrated and analyzed by GC-MS.

Lysates of cells carrying either the pET-11a or the pET-28 constructs with the complete open reading frame of 359 codons had substantial SAMT activity after IPTG induction, while cells carrying the same plasmids had very little SAMT activity when induction with IPTG was not carried out (Table II). Cells expressing His-tagged SAMT had greater than fourfold more SAMT activity. This is probably due to the fact that the His-tagged protein has a specific activity fivefold higher than that of native SAMT (see below) and not to a higher level of protein synthesis. The spent medium of *E. coli* cells containing SAMT activity had high levels of MSA (0.4 μ g/ml) when the growing medium was

TABLE II

Plant SAMT Gene Expression in *E. coli* BL21 Cells^a

Construct	SAMT specific activity in crude lysates (pkat/mg protein)	
	No IPTG added	IPTG added
pET-11a	0	0
pET-11a-SAMT359 ^b	0.02	1.6
pET-11a-SAMT351 ^c	0	0
pET-28-SAMT359 ^d	0.12	6.9

^a Growing conditions are described under Materials and Methods. Values are averages of three measurements.

^b This construct contains the entire open reading frame of the *C. breweri* SAMT cDNA, starting with the methionine codon at positions 103–105.

^c This construct contains an open reading frame of 351 residues from the SAMT cDNA starting with the methionine codon at positions 127–129.

^d This construct contains the entire open reading frame of the SAMT cDNA, starting with the methionine codon at positions 103–105 and fused in-frame downstream to a short open reading frame of 20 residues.

supplemented with 5 μ g/ml SA (Fig. 4B), but no MSA was detected in the spent medium when the growing medium was not supplemented with SA (Fig. 4C). The lysates of cells containing the pET-11a constructs with the shorter open reading frame or no insert had no SAMT activity (Table II), and no MSA was found in their spent medium, under any conditions (Fig. 4D).

Enzymatic characterization of SAMT. Plant SAMT protein purified from both *C. breweri* petals and from *E. coli* expressing the complete SAMT gene (with and without the N-terminal His tag) were further characterized with respect to several physical and biochemical properties. The properties of the SAMT protein purified from petals and the nontagged protein produced in *E. coli* were virtually identical. The SAMT protein with the His tag was also virtually identical to the plant-purified SAMT in its properties, with a few exceptions noted below.

Molecular weight of the native protein. The molecular weight of the native protein was found to be 74.5 kDa. Since the molecular weight of the monomer is 40.3 kDa, this result indicates that the native protein is a dimer.

pH optima. SAMT has a pH optimum of 7.5. At pH 6.0, activity was 80% of optimal activity, at pH 8.0 it was 75%, and at pH 9 it was less than 50%.

Temperature effect on protein stability. SAMT was 100% stable for 30 min at 20°C and 80% stable for 30 min at 30°C, but after 30 min incubation at 40°C it completely lost activity.

Effects of metal ions. The effects of various metal ions on the activity of SAMT were tested. K⁺ was found

to stimulate SAMT activity by a factor of 2. The monovalent cations Na⁺ and NH₄⁺ had no effect on activity. Mg²⁺, Mn²⁺, and Ca²⁺ had a mild inhibitory effect (<35%). The cations Cu²⁺, Fe²⁺, Fe³⁺, and Zn²⁺ had a strong inhibitory effect (50–100% inhibition).

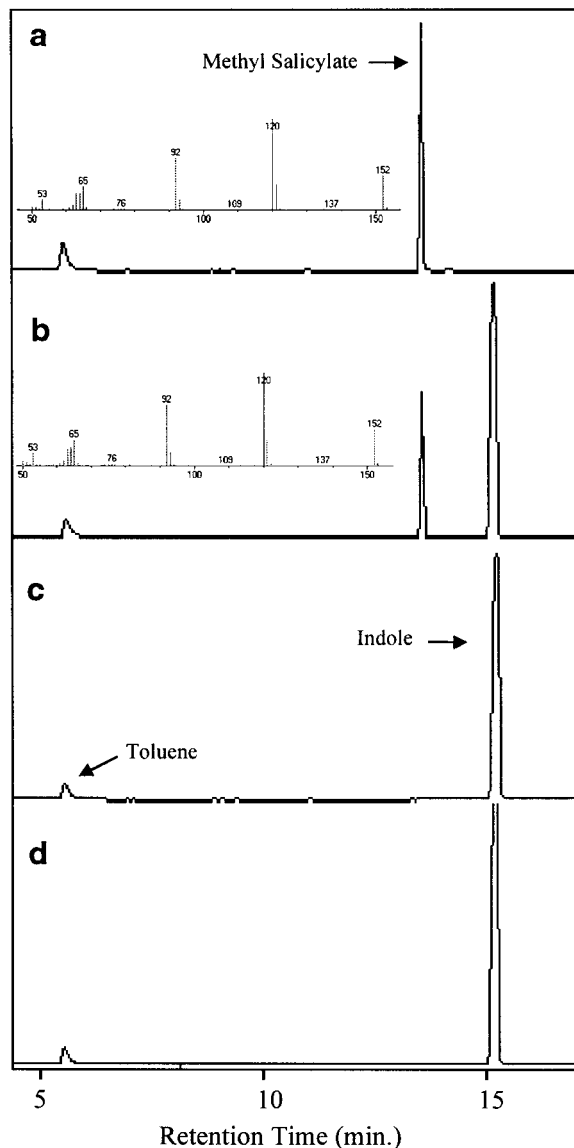


FIG. 4. Detection of MSA in the spent medium of *E. coli* cells expressing *C. breweri* SAMT. (A) GC-MS analysis of MSA standard. (B) Analysis of the spent medium of *E. coli* cells expressing nontagged SAMT after induction with IPTG. The growing medium was supplemented with 5 μ g/ml SA. The MS shown is that of the peak which elutes at the same time as the authentic MSA standard. (C) Analysis of the spent medium of *E. coli* cells expressing nontagged SAMT after induction with IPTG. The growing medium was *not* supplemented with SA. (D) The lysates of cells containing the pET-11a with no insert after induction with IPTG. The growing medium was supplemented with 5 μ g/ml SA. Toluene was added to all samples as an internal control. Indole is produced by all *E. coli* cells (11).

TABLE III

Relative Activity of *C. breweri* SAMT with Salicylate and Related Substrates

Substrate	Relative activity ^a (%)
Salicylic acid	100
Benzoic acid	69
3-Hydroxybenzoic acid	<2
4-Hydroxybenzoic acid	0
Vanillic acid	0
Caffeic acid	0
ρ -Coumaric acid	0
Cinnamic acid	<2
(\pm)Jasmonic acid	0

^a Values are averages of at least four independent measurements. All substrates were tested at a 1 mM concentration. The activity level of SAMT with salicylic acid was set arbitrarily at 100%.

K_m for SAM and SA and turnover number. The reaction catalyzed by SAMT exhibited Michaelis–Menten kinetics with respect to saturation responses. For the nontagged protein, apparent *K_m* values of 24 μ M for SA and 9 μ M for SAM were calculated. The *K_m* value for SAM for the His-tagged protein was the same as that for the nontagged protein, but its *K_m* value for SA was 1.7 μ M. The turnover number for the nontagged protein under saturating conditions was 2.8 s⁻¹, while the turnover number for the His-tagged protein was fivefold higher.

Activity with other substrates. The activity of SAMT with various acids in addition to SA was examined (Table III). Of the substrates tested, only benzoic acid could serve in the reaction catalyzed by SAMT, at 69% efficiency compared to SA. The *K_m* value of SAMT for benzoic acid was 190 μ M.

DISCUSSION

Characterization of SAMT. SAMT activity was previously demonstrated in different parts of scented *C. breweri* flowers, with the highest activity found in petals (4). Increased MSA levels have also been detected in pathogen-infected tobacco leaves (2, 3), although a specific SAMT enzymatic activity has not yet been reported in that species. However, the enzyme catalyzing the earlier step in this pathway, the synthesis of SA, has been detected in tobacco and shown to be upregulated upon infection (16, 17). We have partially purified SAMT from *C. breweri* petals and also from *E. coli* cells expressing the *C. breweri* SAMT cDNA. The enzyme is specific for SA and does not react with other structurally related compounds, with the exception of benzoic acid. At high substrate concentration (1 mM), SAMT reacts with benzoic acid at about 70% efficiency compared to its activity with SA (Table III); however, its *K_m* value for benzoic acid, 190 μ M, is eightfold higher

than its *K_m* value for SA. That SAMT can react to some extent with benzoic acid but not with other substrates is perhaps not surprising, as benzoic acid is very similar to SA (SA = 2-hydroxybenzoic acid), lacking only the 2-hydroxyl group (and thus being smaller than SA). Thus, if the active site is designed to accommodate a benzene ring with a hydroxyl group at the *ortho* position, a similar compound missing this hydroxyl group should also fit, although perhaps with reduced affinity. The other substrates tested either have additional functionalities on the benzene ring at the *meta* and/or *para* positions that SA does not have, which could reduce their fit in the binding site and which also could change the electron density around the carboxyl group (e.g., 3- and 4-hydroxybenzoic acids and vanillic acid), or are simply longer than SA (e.g., cinnamic, ρ -coumaric, and jasmonic acids).

SAMT represents a new type of methyltransferase. Plants produce many methyl esters of secondary metabolites, and some of them, such as methylsalicylate and methyljasmonate, are known to be key compounds in several signal transduction pathways that lead to systemic and local defense responses (3, 18). Yet the enzymes that catalyze their formation have not previously been purified and characterized. Here we show that the sequence of the SAMT protein does not show significant similarity to any proteins whose functions have been identified, including a large number of plant methyltransferases that use SAM to methylate the hydroxyl group of intermediates in the lignin and flavonoid pathways and another type of enzymes, found both in plants and animals, that use SAM to methylate the free carboxyl group of aspartic acids within the polypeptide chain of a damaged protein (15, 19).

These OMTs range in size from 25 to 45 kDa, and sequence analyses have identified several conserved motifs in the C-terminal half of these proteins that could be involved in the binding of the methyl donor SAM (15, 20). Visual inspection of the sequence of SAMT reveals that segments with limited similarity to Motifs I and III described by Kagan and Clarke (20) are present in SAMT: Residues 55–62 [IADLGSSG] resemble Motif I [(LIV)(VL)(ED)(VI)G(GC)G(PT)G, a bold letter indicates a match], and residues 199–208 [EVVPGGRMVL] resemble Motif III [LL(KR)PGG(LIR)(IL)(VIFL)(VLI)]. However, the limited sequence similarity (over short stretches of residues), the fact that these sequences in SAMT occur in the N-terminal half of the protein, and the observation that their spacing (137 residues apart) is also beyond the range found in other plant OMTs all suggest that even if these sequences in SAMT are involved in SAM binding, they could have evolved independently.

The sequence of SAMT does show significant similarity throughout the protein to at least seven proteins encoded

by the *Arabidopsis* genome (it is noteworthy that the two short segments in SAMT with similarity to OMT's Motifs I and III, discussed above, are particularly conserved in these *Arabidopsis* proteins; see Fig. 3). Additional genes with homology to SAMT are likely to be discovered when the *Arabidopsis* genome project is completed. These *Arabidopsis* genes (and the barley gene whose partial sequence has been reported) may encode SAMT and other related activities such as the hypothetical SAM:jasmonate carboxyl methyltransferase (JAMT). Similar genes are likely to be discovered in other species as well, as demonstrated by the recent isolation of a SAM:benzoic acid carboxyl methyltransferase (BAMT) from snapdragon (N. Dudareva, personal communication). The fact that all the *Arabidopsis* sequences are at most 40% identical to each other and to SAMT may indicate that they all have different substrate specificities. Alternatively, the orthologous proteins from different species may not be highly conserved. It would be a challenge to discover the role of each of these proteins and to determine which carboxyl methyltransferases and their products are common to all plants and which members of this protein family produce specialized products and are found in only in a subset of plant lineages.

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