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Authors

Negre, Florence
Kolosova, Natalia
Knoll, Joseph
et al.

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Novel *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme responsible for biosynthesis of methyl salicylate and methyl benzoate, is not involved in floral scent production in snapdragon flowers☆

Florence Negre, Natalia Kolosova, Joseph Knoll, Christine M. Kish, and Natalia Dudareva*

Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, USA

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Abstract

Using a functional genomic approach we have isolated and characterized a cDNA that encodes a salicylic acid carboxyl methyltransferase (SAMT) from *Antirrhinum majus*. The sequence of the protein encoded by SAMT has higher amino acid identity to *Clarkia breweri* SAMT than to snapdragon benzoic acid carboxyl methyltransferase (BAMT) (55 and 40% amino acid identity, respectively). *Escherichia coli*-expressed SAMT protein catalyzes the formation of the volatile ester methyl salicylate from salicylic acid with a K_m value of 83 μM. It can also methylate benzoic acid to form methyl benzoate, but its K_m value for benzoic acid is 1.72 mM. Snapdragon flowers do not emit methyl salicylate. The potential involvement of SAMT in production and emission of methyl benzoate in snapdragon flowers was analyzed by RNA gel blot analysis. *SAMT* mRNA was not detected in floral tissues by RNA blot hybridization, but low levels of SAMT gene expression were detected after real-time RT-PCR in the presence of SAMT-specific primers, indicating that this gene does not contribute significantly, if at all, in methyl benzoate production and emission in snapdragon flowers. Expression of SAMT in petal tissue was found to be induced by salicylic and jasmonic acid treatments.

Keywords: Floral scent; Methyl salycilate; Methyl benzoate; Carboxyl methyltransferase; Snapdragon; Real-time RT-PCR; Jasmonic acid; Salicylic acid; Secondary metabolism

A recently discovered family of carboxyl methyltransferases consists of enzymes that catalyze the transfer of the methyl group of *S*-adenosyl-L-methionine (SAM)[1] to a free carboxyl group of a corresponding acid. These enzymes are responsible for the formation of a variety of methyl esters of secondary metabolites, which have important biological functions in plants. For example, volatile methyl esters, such as methyl benzoate, methyl cinnamate, methyl jasmonate, and methyl salicylate, are among the widespread fragrant components in the plant kingdom, where they contribute significantly to the total floral scent output [1]. In addition to acting as attractants for pollinators, methyl jasmonate and methyl salicylate also function as airborne signals that mediate inter- and intraplant communication during pathogen infection [2,3]. Methyl jasmonate is also involved in regulation of diverse developmental processes, such as seed germination, flower and fruit development, leaf abscission, and senescence [4]. Methyl salicylate, on the other hand, is recognized as a flavor ingredient found naturally in leaves and flowers of wintergreen [5] and such fruits as plum, strawberry, black cherry, and tomato [6].

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* Corresponding author. Fax: +1-765-494-0391.
E-mail address: dudareva@hort.purdue.edu (N. Dudareva).

[1] *Abbreviations used:* BAMT, *S*-adenosyl-L-methionine: benzoic acid carboxyl methyltransferase; JMT, *S*-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase; SAM, *S*-adenosyl-L-methionine; SAMT, *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase.

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Enzymes responsible for the formation of methyl benzoate (*S*-adenosyl-L- methionine:benzoic acid carboxyl methyltransferase, BAMT), methyl jasmonate (*S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase, JMT), and methyl salicylate (*S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, SAMT) and their corresponding genes have recently been isolated and characterized [7–10]. These carboxyl methyltransferases isolated from different plant species—BAMT from petal tissue of snapdragon flowers, JMT from *Arabidopsis thaliana*, and SAMT from flower petals of *Clarkia breweri*—are homodimers of similar size, with the subunit molecular mass ranging from 40 kDa (SAMT) to 45 kDa (JMT), and share 40% amino acid sequence identity. While BAMT has strict substrate specificity for benzoic acid and no activity with several other structurally related substrates such as salicylic acid, *trans*-cinnamic acid, and their derivatives [8,9], SAMT is specific for salicylic acid but does methylate benzoic acid, although its K_m value for BA is much higher [7]. JMT is specific for jasmonic acid and it does not convert salicylic acid, benzoic acid, cinnamic acid, 12-oxo-phytodienoic acid, or linolenic acid to the corresponding methyl esters, although it can use 9,10-dihydrojasmonic acid with low efficiency [10]. Since these enzymes lack an apparent organelle-specific transit signal peptide, it is believed that they catalyze the formation of corresponding esters in the cytoplasm. Indeed, recent immunogold labeling studies localized BAMT to the cytosol [11].

As part of an ongoing effort to isolate genes involved in floral scent production in snapdragon flowers, we have determined the nucleotide sequence of 792 cDNAs (expressed sequence tags) chosen randomly from a cDNA library constructed from mRNA isolated from petals (upper and lower lobes) of 1- to 5-day-old flowers. In snapdragon, petals are highly specialized for floral scent biosynthesis. Two identical cDNA clones were obtained which had higher amino acid sequence identity to a recently described SAMT from *C. breweri* [7] than to BAMT from snapdragon [8,9]. We found that this putative carboxyl methyltransferase gene encodes an enzyme that catalyzes the transfer of the methyl group of SAM to the carboxyl group of salicylic acid to make the volatile ester methyl salicylate. Here we report the characterization of salicylic acid carboxyl methyltransferase from *Antirrhinum majus* as well as functional and biochemical characterization of the enzyme encoded by this gene.

Materials and methods

cDNA library construction and DNA sequencing

A Lambda-ZAPII cDNA library was constructed from mRNA isolated from upper and lower petal lobes of 1- to 5-day old snapdragon flowers according to manufacturer's protocol (Stratagene, La Jolla, CA) [8]. The titer of the unamplified library was 1.1×10^6 plaque forming units (pfu). This primary library was amplified, and the amplified library had a titer of 1.5×10^{10} pfu. Mass excision of pBluescript phagemids from the amplified library resulted in a stock, which was used for plating and random colony picking for DNA sequencing. The inserts were partially sequenced from one end using T3 primer.

Expression of SAMT in Escherichia coli and purification of recombinant protein

The coding region of SAMT was amplified using the sense 28-mer oligonucleotide 5′-GACCATATGACAA AACAAACACAAAAGC-3′, which introduced an *Nde*I site at the initiating ATG codon, and the antisense 26-mer oligonucleotide 5′-TTGGATCCTGTCACTC TCGCCTTGTC-3′, which introduced a *Bam*HI site downstream of the stop codon as primers. The PCR-amplified 1.15-kb fragment was cloned into the *Nde*I–*Bam*HI site of the expression vector pET-28a, which contains an N-terminal polyhistidine (6× His) tag (Novagen). *E. coli* BL21(DE3) cells were transformed with recombinant plasmid and grown in LB medium with 50 μg/ml kanamycin at 37 °C. When the culture density reached OD_{600} of 0.5, the expression of SAMT cDNA was induced by addition of IPTG to a final concentration of 0.4 mM. After 20 h incubation with shaking (200 rpm) at 20 °C, *E. coli* cells were harvested by centrifugation and sonicated in lysis buffer containing 10 mM NaCl, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10% glycerol, and 10 mM β-mercaptoethanol. SAMT activity was measured in soluble and insoluble fractions.

For protein purification, *E. coli* cells were grown as described above. After centrifugation, cells were resuspended in binding buffer [3:1 (v/w) buffer:cells] containing 20 mM Tris–HCl, pH 7.9, and 500 mM NaCl. Resuspended cells were broken by French press (three passes at 1200 psi), and the cell debris was removed by centrifugation (30 min at 24,000*g*). The supernatant was incubated with protamine sulfate [1% (w/v)] for 30 min at 4 °C. After centrifugation (30 min at 24,000*g*), supernatant was dialyzed against 2 L of Binding buffer overnight at 4 °C. The *E. coli*-expressed SAMT protein was purified by nickel-based affinity chromatography (bed volume 2.5 ml) according to manufacturer's protocol (Novagen). Protein was eluted with 10 ml of stripping buffer (0.5 M NaCl, 20 mM Tris–HCl, pH 7.9, and 100 mM EDTA). The fractions containing SAMT activity were pooled and dialyzed against buffer containing 50 mM Bis–Tris–HCl, pH 6.9, 10% glycerol, and 10 mM β-mercaptoethanol.

Enzyme assays and product identification

Enzyme assays were performed with extracts of IPTG-induced cells containing pET-28a vector with

SAMT insert, vector without insert (control samples), and purified SAMT protein. Enzyme activity was determined by measuring transfer of the ^{14}C-labeled methyl group of SAM to the carboxyl group of salicylic acid. The standard reaction mixture (100 μl) consisted of 20 μl of cell extract (25–40 μg of protein) or pure protein (1.4 μg) and 2 μl (4×10^{-5} mCi) of 0.34 mM *S*-[methyl-^{14}C]adenosyl-L-methionine (SAM) (NEN Life Science Products, Boston, MA) in assay buffer (50 mM Tris–HCl, pH 7.5, and 3 mM β-mercaptoethanol) containing 2 mM salicylic acid and 0.5 mM EDTA. After incubation for 30 min at 20 °C, the radioactively labeled methylated product was extracted by the addition of 100 μl hexane and 50 μl of the organic phase was counted in a liquid scintillation counter (model LS 3801, Beckman, Fullerton, CA). The raw data (counts per minute) were converted to picokatals (picomoles of product produced per second) based on the specific activity of the substrate and the efficiency of counting. Controls included assays with boiled extracts and without substrate, and background radioactivity produced in such assays was subtracted from all of the results. In assays for pH optimum, cofactor requirements, and K_m measurements, proper concentrations of purified SAMT were chosen so that the reaction velocity was proportional to enzyme concentration and was linear with respect to time for at least 30 min. Protein content of samples was determined by the Bradford method [12] using the Bio-Rad protein reagent (Hercules, CA) and bovine serum albumin as a standard.

Product verification was performed by growing BL21 (DE3) cells expressing SAMT and those containing pET-28a vector (controls) in the presence (5 μg/ml) and absence of salicylic acid and benzoic acid under the conditions described above. After the cells were harvested by centrifugation, the cultured medium (25 ml) was extracted with 5 mL of hexane, and the hexane phase was concentrated to 200 μl and analyzed by gas chromatography–mass spectrometry [8].

Ion requirements, pH optimum, and stability of SAMT protein

Enzyme assays were performed with one of the following cations present in the assay buffer at a final concentration of 5 mM: Ca^{2+}, Cu^{2+}, Fe^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, NH_4^+, or Zn^{2+}. Except for Cu^{2+} and Fe^{2+}, which precipitate under reducing conditions, all assay buffers also contained 10 mM β-mercaptoethanol. Final results are an average of three independent assays.

The optimum pH for SAMT activity was determined using two buffer systems. Reactions were carried out in 50 mM Tris–HCl buffer with pH ranging from 6.5 to 9.0 and in 50 mM Tris–Na phosphate–Na citrate buffer with pH ranging from 4.0 to 9.5. Final results are an average of three independent assays.

Temperature effect on SAMT stability was determined by incubating purified SAMT protein at temperatures ranging from 4 to 65 °C for 30 min, chilling samples on ice, and then using them for enzyme assays. Two independent assays were performed for each point and then an average was taken.

Molecular mass determination

The molecular mass of the recombinant SAMT protein was determined by gel filtration on a Sephacryl 200-HR (Pharmacia Biotech, NJ) column (1.6 × 60 cm) calibrated with the following markers: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). Buffer containing 200 mM Hepes, pH 7.0, 100 mM KCl, 0.1% Triton X-100, 5% glycerol, and 2 mM β-mercaptoethanol was used for column equilibration and elution. Fractions of 1 ml were collected at a flow rate of 0.7 ml/min and analyzed for SAMT activity. Denaturing SDS–PAGE was performed on 10% gels to determine the subunit molecular weight. The gels were calibrated with molecular weight standards in the range 7.4–208 kDa (Bio-Rad, CA).

Determination of kinetic properties

Alternative substrate competition experiments were performed by varying the concentration of one substrate at each of a series of concentrations of the other. Data were presented as double-reciprocal plots of initial velocity (v) versus varying substrate concentration (S). In all experiments, appropriate enzyme concentration was chosen so that the reaction velocity was linear during the incubation time period. Substrate interaction studies were done by fixing the concentration of one substrate while changing that of the other. Linear regressions were fitted to the data in double-reciprocal plots. Replots of the data were used to determine the kinetic parameters.

Quantitative real-time RT-PCR

Total RNA from leaves and floral tissues of 3-day-old snapdragon flowers was isolated as previously described [8,13–15]. Real-time quantitative reverse transcriptase-polymerase chain reaction (real-time RT-PCR) was performed on a Gene-Amp 5700 sequence detection system (PE Applied Biosystems, Foster City, CA) using SYBR Green fluorescent dye. Ubiquitin was used as an endogenous control, and reactions for SAMT and ubiquitin were carried out in separate tubes. The primers for SAMT were designed to amplify a 200-bp amplicon in the 3′ region of the coding sequence and were as follows: forward primer 5′-TAGTAGCGAGCGG CAATGAGT A-3′ and reverse primer 5′-TTGTTTCCT CTCTGGAC ATGCGA-3′. These primers were specific for SAMT and

did not amplify BAMT in PCR. For ubiquitin, the primers were designed to amplify a fragment of approximately 400 bp and were as follows: 5′-ACTTGGTGCTGAGGTTGAGG-3′ (forward) and 5′-ACAACTGACTCCAGCAAACGAGA-3′ (reverse). Real-time RT-PCRs were carried out in a 50 μl final volume. The reaction mixture contained 500 ng total RNA template, 0.1 μM of forward and reverse primers, 1× SYBR Green PCR buffer, 1.25 U Ampli-Taq Gold DNA polymerase, 12.5 U Multi-Scribe reverse transcriptase, 1 μl RNase inhibitor, 3.0 mM $MgCl_2$, 0.6 mM dUTP, and 0.3 mM each of dATP, dCTP, and dGTP. The 10× SYBR Green PCR buffer contains the passive reference, a dye that does not participate in the PCR and provides an internal reference to which the SYBR Green–dsDNA complex signal can be normalized during data analysis to overcome the fluorescence fluctuations caused by differences in concentration or volume. All reagents were purchased from PE Applied Biosystems (Warrington, UK). Real-time RT-PCR conditions were as follows: 48 °C for 30 min and 94 °C for 10 min for one cycle, followed by 40 cycles of 94 °C for 15 s and 54 °C for 60 s. Several real-time RT-PCRs were run per sample in triplicate per run. After the real-time RT-PCR, the amplified products were run on 2% agarose gel to check the size of the amplified fragment and the presence of other bands.

Relative quantification of SAMT gene expression in different floral tissues was performed using the comparative C_t method according to manufacturer's protocol (Applied Biosystems). The comparative C_t method allows one to normalize the quantity of SAMT transcripts to an endogenous control, such as ubiquitin in our experiments, in order to overcome the variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. Prior to data analysis, the threshold cycle, or C_t value, for each sample was generated, which corresponds to the cycle number when the sequence detection application began to detect the increase in signal associated with an exponential growth of PCR product. The difference between the average C_t values for SAMT and ubiquitin within a given sample gives the ΔC_t value for that sample and shows *SAMT* mRNA expression relative to ubiquitin mRNA. Fold differences between samples in relative *SAMT* mRNA expression were calculated using the differences in ΔC_t values of each sample and the highest ΔC_t value in the experiment ($\Delta\Delta C_t$) and the equation: fold differences $= 2^{-\Delta\Delta C_t}$. The highest ΔC_t value belongs to the sample with the lowest level of SAMT expression. The $\Delta\Delta C_t$ value for this sample equals 0 and correspondingly the level of SAMT expression equals 1.

Jasmonic and salicylic acid treatments

Maryland True Pink snapdragon cultivar (*A. majus*) (Ball Seed, IL, USA) was grown under standard greenhouse conditions, as previously described [8]. Snapdragon plants were sprayed with an aqueous solution (in 0.05% Tween) of either 50 μM jasmonic acid (mixed isomers, Sigma), 5 mM salicylic acid, or 0.05% Tween as a control once per day on two consecutive days. Snapdragon flowers were 3 days old on the first day of treatment. Petal tissue (upper and lower petal lobes) and leaves were harvested before treatment and 24 and 48 h after last treatment, frozen in liquid N_2, and stored at −80 °C prior to extraction of RNA. Total RNA was isolated as previously described [8,13–15] and analyzed by RT-PCR. One microgram of total RNA was used to make cDNA using random hexamer primers with the Advantage RT-for-PCR kit according to manufacturer's instructions (CLONTECH, Palo Alto, CA). The SAMT-specific primers for PCR were the same as those used for the amplification of the SAMT coding region for subcloning in the expression vector and amplified a product of 1.1 kb in size. As a control for RT-PCR reactions we used the18S rRNA gene, a 1.1-kb fragment of which was amplified using the primers 5′-GATA AAAGGTCGACACGGGCTCTGC-3′ (forward) and 5′-AACGGAATTAACCAGACAAATCGCTCC-3′ (reverse). Initially, PCR reactions were carried out with SAMT-specific primers and 5, 10, 15, and 20 μl of cDNA for 40 cycles with the annealing temperature at 60 °C. The amplified products were run on 1.2% agarose gel, blotted onto nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad, Hercules, CA), and hybridized with the corresponding DNA probe. Quantitation was performed using a Storm 860 Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Analysis of products obtained with different amounts of cDNA showed that product increased linearly. Fifteen microliters of cDNA was chosen as optimal for further PCR reactions. For rDNA, PCR reactions were carried out for 15 cycles. For quantitative analysis of SAMT expression, RT-PCR products were hybridized with the corresponding DNA probe and normalized to the 18S rRNA level.

Results and discussion

Isolation and characterization of snapdragon SAMT cDNA

In snapdragon, upper and lower petal lobes of the flowers are the principal emitters of scent volatiles [8]. Sequencing of 792 random clones (expressed sequence tags) from a cDNA library constructed from mRNA isolated from upper and lower petal lobes of 1- to 5-day-old flowers revealed two cDNA clones (designated ama1d21 and ama2e03) with amino acid sequence similarity to a recently described SAMT from *C. breweri* [7]. These full-length clones were identical and contained a

total of 1333 nucleotides. They encode an ORF of 1149 nt corresponding to a protein of 383 amino acids with a calculated molecular mass of 43,613 and p*I* of 8.04 (GenBank Accession No. AF515284). The protein encoded by these cDNAs has 54–56% amino acid identity to SAMT from *C. breweri* [7], *Atropa belladonna* (AB049752), and *Stephanotis floribunda* (AJ308570) (Fig. 1) and 49% identity to SAMT-like protein from *Cucumis sativus* (AB046595). Although four salicylic acid carboxyl methyltransferases from *C. breweri*, *A. belladonna* (AB049752), *S. floribunda* (AJ308570), and *C. sativus* (AB046595) are catalogued in GenBank, the experimentally determined biochemical function was reported only for *C. breweri* SAMT [7]. The tentative snapdragon SAMT also exhibits from 28 to 43% amino acid identity to recently isolated *Arabidopsis* JMT [10]

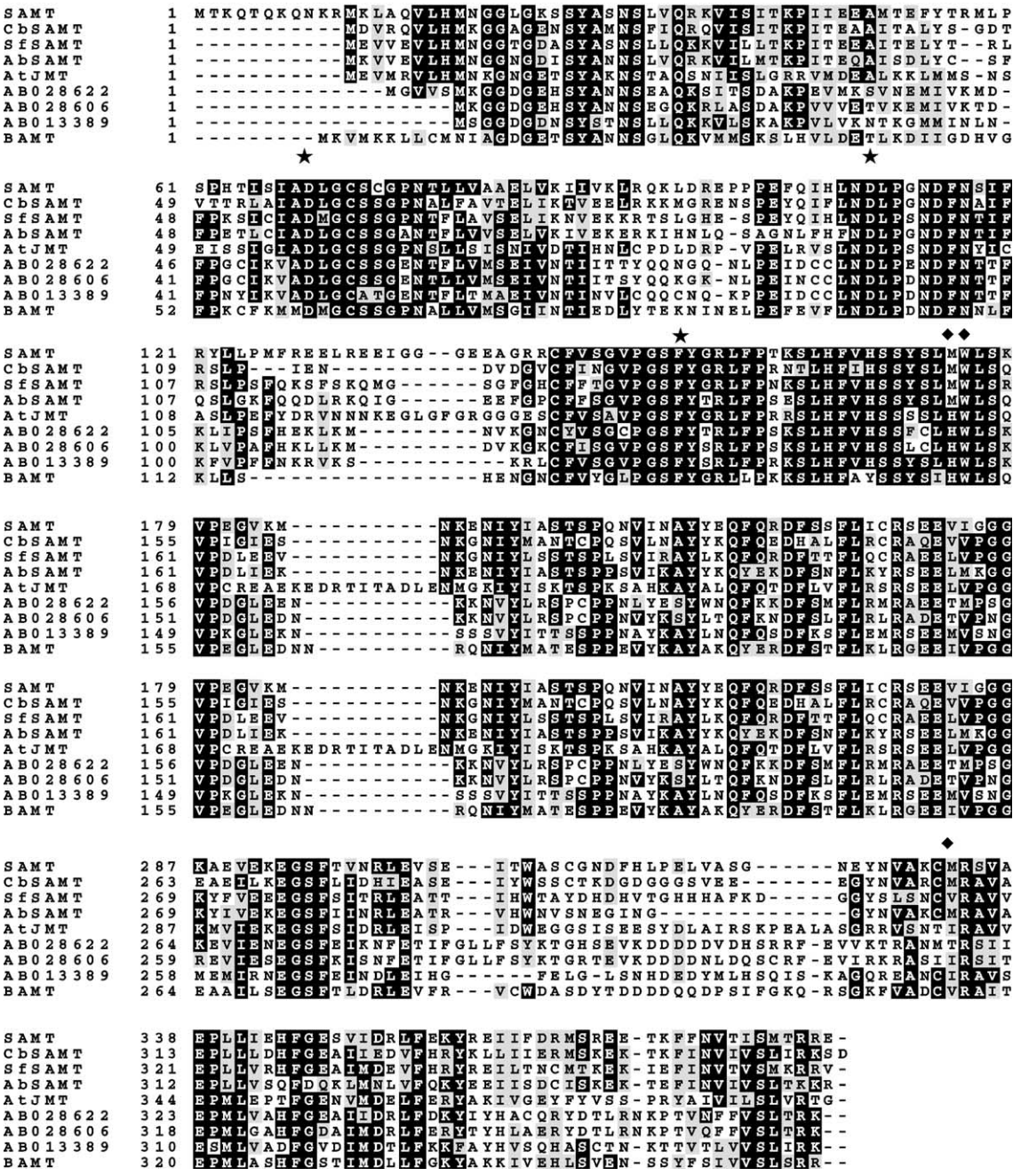

Fig. 1. Comparison of the predicted amino acid sequences of snapdragon SAMT and related proteins. Snapdragon SAMT sequence was aligned with SAMT from *C. breweri* (CbSAMT, AF133053), SAMT from *S. floribunda* (SfSAMT, AJ308570), SAMT from *A. belladonna* (AbSAMT, AB049752), JMT from *A. thaliana* (AtJMT, AY008434), SAMT-like proteins from *Arabidopsis* (AB013389, AB028622, and AB028606), and BAMT from snapdragon (AF198492) using ClustalW. This alignment was shaded using Boxshade 3.21 software program (Human Genome Sequencing Center, Houston, TX) to show conserved amino acid residues in black and similar residues in gray. Dashes indicate gaps that have been inserted for optimal alignment.

and to 24 other SAMT-like *Arabidopsis* proteins identified by the genome project, based on sequence comparison. However, with the exception of JMT, none of the reactions catalyzed by these proteins have been determined. Three *Arabidopsis* SAMT-like proteins (AB013389; AB028622; AB028606) showing the highest levels of identity with snapdragon SAMT (43–40% amino acid identity) were aligned in Fig. 1. Snapdragon SAMT shows 40% amino acid identity to snapdragon BAMT [8,9] (Fig. 1).

The recently obtained crystal structure of *C. breweri* SAMT has identified the specific residues responsible for substrate binding in carboxyl methyltransferases (Zubieta et al., manuscript in preparation). Asp-57, Asp-98, and Phe-130 (labeled in Fig. 1 with stars) were found to be involved in SAM binding, whereas Gln-25, Met-150, Trp-151, and Met-308 (labeled with diamonds) are responsible for salicylic acid binding. While amino acid residues involved in SAM binding are conserved in the aligned sequences shown in Fig. 1, there are some variations in amino acid residues involved in the acceptor molecule site. These variations most likely determine the substrate specificity of carboxyl methyltransferases. We note that two amino acid residues, Gln-25 and Trp-151, were present in all aligned sequences, whereas two methionine residues, Met-150 and Met-308, were highly conserved only among SAMT isolated from snapdragon, *C. breweri*, *A. belladonna*, and *S. floribunda*.

Functional characterization of snapdragon SAMT

To examine the possible function of the isolated cDNA clone, the coding region of the gene was subcloned into the expression vector pET-28a, which contains an N-terminal polyhistidine (6× His) tag, and expressed in *E. coli*. Gene products were evaluated using bacterial lysates as the source of the enzyme. When crude extracts of sonicated transformed cells after IPTG induction were tested with benzoic acid, cinnamic acid, salicylic acid, and their derivatives, the highest activity (2.1 pkat/mg protein) was detected with salicylic acid as the substrate, 2.3-fold lower activity (0.9 pkat/mg protein) was detected with benzoic acid, and no activity was detected with any of the others (Table 1). Moreover, the culture medium of the *E. coli* cells expressing SAMT contained methyl salicylate (2.1 μg/ml) (Fig. 2B) when the growing medium was supplemented with 5 μg/ml salicylic acid, and methyl benzoate (0.86 μg/ml) when the growing medium was supplemented with 5 μg/ml benzoic acid (Fig. 2C). *E. coli* cells that contained a pET-28a plasmid without the SAMT coding region did not have any detectable enzyme activity and did not produce methyl salicylate or methyl benzoate (Fig. 2D). These results show that the isolated cDNA clone encodes a salicylic carboxyl methyltransferase and that, similar to SAMT from *C. breweri*, it can use both salicylic and benzoic acids as substrates, although the latter with lower efficiency.

Table 1
Relative activity of snapdragon SAMT with salicylic acid and related substrates

Substrates	Relative activity[a] (%)
Salicylic acid	100
Benzoic acid	45
3-Hydroxybenzoic acid	0
4-Hydroxybenzoic acid	0
trans-Cinnamic acid	0
p-Coumaric acid	0
m-Coumaric acid	0
o-Coumaric acid	0
Benzylalcohol	0

[a] Values are the averages of four independent measurements. All substrates were tested at a 2 mM concentration. The SAMT activity with salicylic acid, set up as 100%, was 2.1 pkat/mg protein.

Biochemical characterization of snapdragon SAMT

To characterize the biochemical properties of SAMT the *E. coli* expressed protein was purified by nickel-based affinity chromatography. The purified protein was catalytically stable for more than a year when stored at −80 °C. It was also stable at temperatures up to 42 °C for 30 min; however, the 30-min incubation at 65 °C led to a 70% loss of activity. The expressed recombinant SAMT enzyme possesses a pH optimum from 7.0 to 7.5. It was also active in a broad pH range from 5.0 to 8.0 with 80–90% of maximum activity. At pH 4.0 and 9.5 the enzyme activity fell to about 20% of the maximal value. The enzyme was active in both Tris- and phosphate–citrate-based buffers, although its activity in phosphate-citrate buffer was about 20% lower than in Tris-buffer. SAMT activity was not affected by the presence of 5 mM Mg^{2+} in the assay reaction. The addition of K^+, NH_4^+, and Ca^{2+} also does not affect the SAMT activity, whereas the addition of Fe^{2+} and Cu^{2+} has a strong inhibitory effect (>95% inhibition). Other cations such as Zn^{2+}, Na^+, and Mn^{2+} inhibit SAMT activity less than 30%. The approximate molecular mass of the SAMT was determined on a calibrated Sephacryl 200-HR column to be 107 kDa, whereas on the SDS-PAGE gels the denatured enzyme exhibited a single band corresponding to a molecular mass of 49 kDa, indicating that the active enzyme exists as a homodimer.

Kinetic parameters of SAMT for salicylic acid, benzoic acid, and methyl donor SAM were determined using substrate interaction and saturation kinetics. K_m values for salicylic and benzoic acid were 83 μM and 1.72 mM, respectively, indicating that salicylic acid is the favored substrate (Table 2). Depending on the substrate used, K_m values for SAM was 4 μM with salicylic acid and 3 μM with benzoic acid.

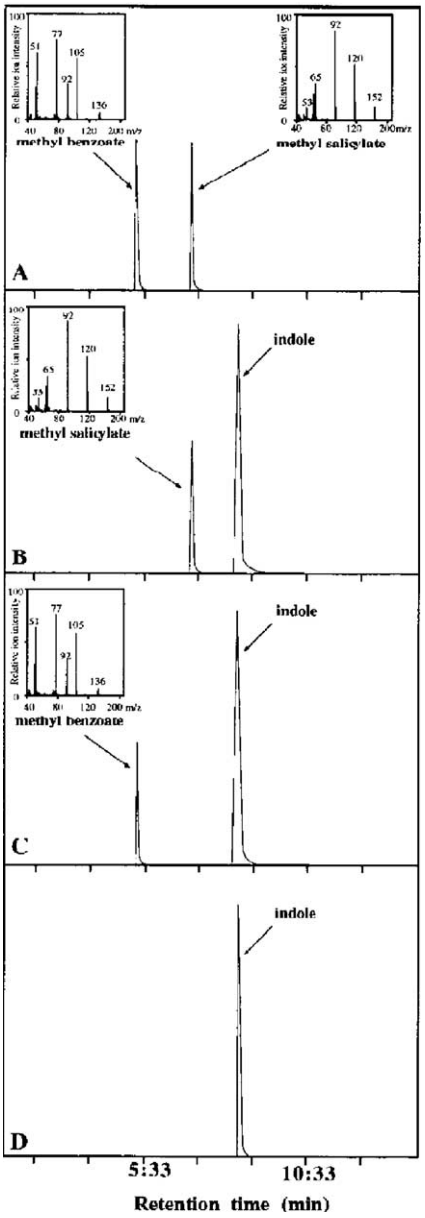

Fig. 2. Analysis of products formed in vivo by *E. coli* expressed SAMT. (A) Gas chromatography–mass spectrometry analysis of methyl salicylate and methyl benzoate standards. Electron impact mass spectrum of methyl benzoate is shown in the upper left-hand corner and of methyl salicylate in the upper right-hand corner. Numbered peaks represent mass-to-charge ratios of molecular ions and fragment ions of methyl benzoate and methyl salicylate. (B) Analysis of the medium of *E. coli* cells expressing snapdragon SAMT after induction with isopropyl β-D-thiogalactopyranoside when the growing medium was supplemented with 5 μg/ml salicylic acid. The mass spectrum is that of the peak eluted at the same retention time as the authentic methyl salicylate standard (A). (C) Analysis of the medium of *E. coli* cells expressing snapdragon SAMT after induction with isopropyl β-D-thiogalactopyranoside when the growing medium was supplemented with 5 μg/ml benzoic acid. The mass spectrum is that of the peak eluted at the same retention time as the authentic methyl benzoate standard (A). (D) Analysis of the medium of *E. coli* cells expressing pET-28a vector with no insert after induction with isopropyl β-D-thiogalactopyranoside. Indole is produced by all *E. coli* cells [7,8,14].

Snapdragon SAMT belongs to a novel class of carboxyl methyltransferases that catalyze the methylation of a free carboxyl group of corresponding acids. The biochemical properties of SAMT show that it has many features in common with the other members of this family of enzymes such as *C. breweri* SAMT [7], snapdragon BAMT [9], and JMT from *Arabidopsis* [10]. They all function as homodimers with subunit molecular mass 40–45 kDa and have a pH optimum from 7 to 8. They do not require any metal ions as cofactors and they are strongly inhibited by Fe^{2+} and Cu^{2+}. Similarly to *Arabidopsis* JMT, the addition of K^+ does not enhance snapdragon SAMT activity, whereas it stimulates BAMT and *Clarkia* SAMT activities by a factor of 2. Snapdragon SAMT has a broader temperature stability profile than the other members of this family. Incubation for 30 min at 42 °C led to no loss of activity, whereas *Clarkia* SAMT completely lost its activity and BAMT retained only 20% after such treatment. All enzymes use SAM as the methyl donor with similar K_m values ranging from 4 to 9 μM with the exception of BAMT which has values of 28 and 87 μM for plant-purified and *E. coli*-expressed proteins, respectively [9].

Snapdragon SAMT has the same substrate specificity as *Clarkia* SAMT. Salicylic acid is the preferred substrate, although benzoic acid can also be used. Both enzymes have comparable K_m values for salicylic acid (24 μM for *Clarkia* SAMT and 83 μM for snapdragon SAMT), whereas snapdragon SAMT has a much higher K_m value (9-fold higher) for benzoic acid than *Clarkia* SAMT (190 μM for *Clarkia* SAMT and 1.72 mM for snapdragon SAMT). This apparent K_m value for benzoic acid is very similar to that of BAMT (1.72 and 1.5 mM for recombinant proteins, respectively). Although K_m values for benzoic acid of snapdragon SAMT and BAMT are very close, the SAMT V_{max} value for benzoic acid and k_{cat}/K_m ratio are almost 6-fold lower than the BAMT kinetic parameters ([9] and Table 2), indicating that SAMT has a lower catalytic efficiency for benzoic acid than BAMT.

Groups of enzymes of secondary metabolism that carry out similar reactions (e.g., methylation, hydroxylation) but with slightly different substrates arise by gene duplication and divergence and therefore may share high levels of amino acid identity with one another. In several cases, it has been shown that one or very few amino acid changes can alter the susbtrate specificity of the enzyme. For example, *C. breweri* (iso)eugenol *O*-methytransferase (IEMT), which methylates the 4-hydroxyl group of (iso)eugenol to make (iso)methyleugenol, and caffeic acid *O*-methytransferase (COMT), which catalyzes the methylation of the 3-hydroxyl group of caffeic acid and the 5-hydroxyl group of 5-hydroxyferulic acid to give ferulic and sinapic acids, respectively, are 83% identical at the protein level [16,17], and seven amino acid substitutions can switch the substrate preference of these methyltranasferases to that of the other [17].

Table 2
Kinetic parameters of snapdragon SAMT protein

Substrate	K_m(μM)	V_{max} (pkat/mg)	k_{cat} (s^{-1})[a]	k_{cat}/v_m ($nM^{-1}\,s^{-1}$)
S-adenosyl-L-methionine[b]	4.05	128	0.013	3.2
S-adenosyl-L-methionine[c]	3.06	63	0.006	1.9
Salicylic acid	83	108	0.011	0.132
Benzoic acid	1720	55	0.0058	3.37×10^{-3}

[a] k_{cat}, turnover number of enzyme.
[b] *S*-adenosyl-L-methionine with salicylic acid.
[c] *S*-adenosyl-L-methionine with benzoic acid.

Another example is represented by two phenylpropene *O*-methytransferases from basil: chavicol *O*-methytransferase (CVOMT), which catalyzes the *O*-methylation of chavicol, and eugenol *O*-methytransferase (EOMT), which uses eugenol as the preferred substrate, although it can methylate chavicol [18]. These two enzymes are 90% identical and a single amino acid difference is responsible for the difference in substrate specificity [18]. Similarly, a difference of a single amino acid residue was sufficient to alter the substrate specificity of *O*-methyltransferase from *Thalictrum tuberosum* [19]. In the case of snapdragon SAMT and BAMT, the same pattern is not observed. Although SAMT and BAMT react with structurally similar substrates (salicylic acid is 2-hydroxybenzoic acid) and in addition SAMT can also use benzoic acid, these two proteins share only 40% of sequence identity. Moreover, snapdragon SAMT has higher sequence identity to *C. breweri* SAMT (55% of amino acid identity). These results indicate that snapdragon SAMT may be the ortholog of *Clarkia* SAMT, and that snapdragon SAMT and BAMT diverged from each other prior to the divergence of snapdragon-Clarkia lineages.

Characterization of SAMT expression in snapdragon

SAMT from petal tissue of *C. breweri* flowers catalyzes the formation of methyl salicylate, one of the components of *C. breweri* floral scent [7,20]. Snapdragon flowers do not emit methyl salicylate, but they do emit methyl benzoate, which is one of the most abundant scent compounds [8]. Since the concentration of free benzoic acid in snapdragon petal tissue (0.2–2 mM) [8] is in the range of the K_m value of snapdragon SAMT for benzoic acid, it can potentially participate in methyl benzoate synthesis in the snapdragon flower. We therefore analyzed expression of the SAMT gene in different floral parts of 3-day-old flowers (sepals, pistils, stamens, and different regions of the corolla—the upper petal lobes, the lower petal lobes, and the tube) by RNA gel blot analysis. Expression of SAMT in leaves was also analyzed.

SAMT transcripts were undetectable in floral and leaf tissues by RNA-blot hybridization, but low levels of SAMT gene expression were detected after real-time RT-PCR [21,22] in the presence of SAMT-specific primers. Typical amplification plots for SAMT and ubiquitin fragments in floral and leaf tissues during real-time RT-PCR analysis are shown in Fig. 3A. When SAMT transcript values in samples were normalized to the expression levels of ubiquitin in respective tissue, the lowest level of *SAMT* mRNA expression was found in pistil. Its value was set up as 1 and the SAMT expression in other tissues was evaluated relative to the pistil level (Fig. 3B). Expression of SAMT in stamens was also very low and only 2-fold higher than in the pistil. The highest levels of *SAMT* mRNA were observed in the upper and lower petal lobes, about 200-fold higher than in the pistil. Expression of *SAMT* mRNA in sepals was about half the level in petal lobes and 2-fold higher than in the tube and leaf tissue (Fig. 3B). These results clearly show that expression of SAMT is very low in all tissues examined. Low expression of SAMT in petal tissue along with low catalytic efficiency of the enzyme for benzoic acid suggest that this gene cannot make a significant, if any, contribution to methyl benzoate production and emission in snapdragon flowers.

Further experiments were performed to determine the possible function of the SAMT gene in snapdragon. It has been previously shown that methyl salicylate is a major volatile compound produced by tobacco plants inoculated with tobacco mosaic virus (TMV) [3]. It is also released from leaves of some plant species in response to herbivore damage [23–26]. Since exogenously applied salicylic acid induces the same set of genes that are activated systemically upon TMV infection [27], and jasmonic acid activates the natural defense response of plants and also induces the same chemicals that are associated with herbivore damage [23,28–30], we used salicylic and jasmonic acids to test if these compounds induce the SAMT gene in snapdragon. Plants were sprayed with jasmonic or salicylic acids once per day for two consecutive days; total RNA was isolated from petal tissue as well as from leaves 24 and 48 h after the last treatment and analyzed by RT-PCR. Our results show that the salicylic and jasmonic acid treatments induce the expression of SAMT in petal tissue 48 h after the last treatment, although a slight decrease in the level of expression was observed after 24 h (Fig. 4). No induction of SAMT expression was detected in leaf tissue (data not shown). These results suggest that the SAMT

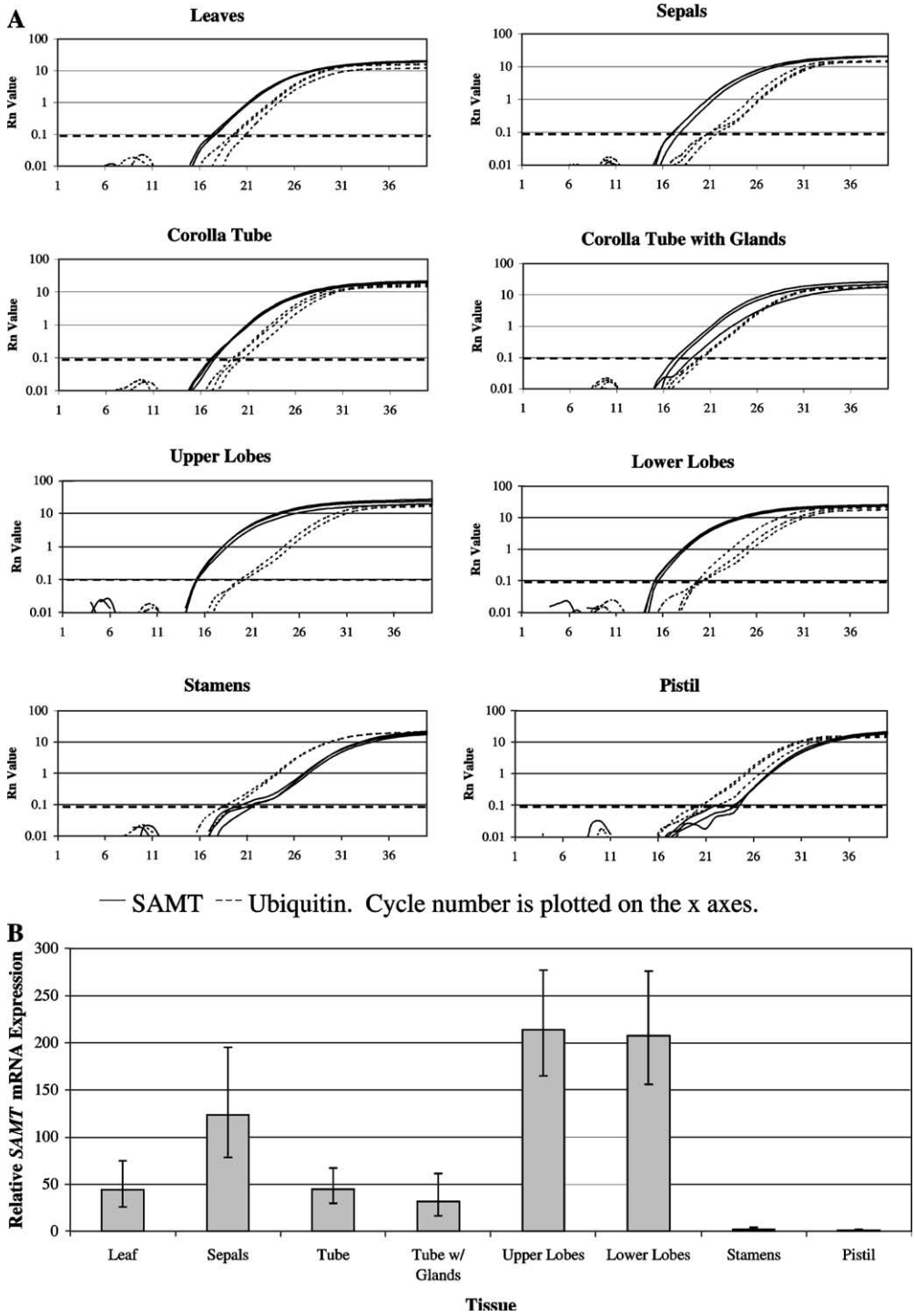

Fig. 3. Real-time quantitative RT-PCR analysis of tissue-specific *SAMT* mRNA expression in snapdragon. Quantitative real-time RT-PCR was performed on total RNA isolated from different floral parts of 3-day-old flowers and leaves as described in Materials and methods. (A) Graphs of SAMT and ubiqiutin PCR fluorescence profiles against cycle number with the Gene-Amp 5700 in leaves and different floral parts in triplicates. R_n is the normalized reporter value, which is the ratio of SYBR Green fluorescence versus that of the passive reference dye (included in the SYBR Green buffer). The horizontal dashed line in each panel is the threshold R_n value, set up arbitrarily in the lower linear range, at which C_t (threshold cycle) values for samples were determined. (B) Quantification of SAMT expression in different floral parts and leaves of snapdragon. SAMT expression values were normalized to the levels of ubiquitin expression in respective tissues. SAMT expression in pistils was set up as 1. Values are means ± SE from three separate real-time RT-PCR assays.

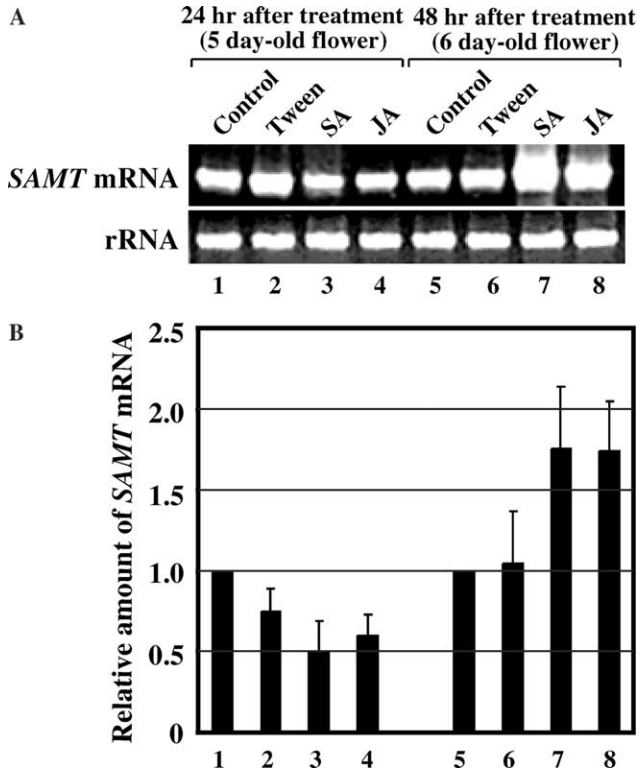

Fig. 4. Induction of SAMT expression in petal tissue after treatment with salicylic and jasmonic acids. (A) RT-PCR products stained with ethidium bromide. RT-PCR was performed on RNA isolated from upper and lower flower petal lobes 24 (lanes 1–4) and 48 (lanes 5–8) h after the second treatment with salicylic and jasmonic acids. RNA isolated from upper and lower flower petal lobes of untreated plants and plants treated with an aqueous solution containing 0.05% Tween were used as controls. Lanes 1 and 5—RNA from petal tissue of untreated plants (flowers were 5 and 6 days old, correspondingly); lanes 2 and 6—RNA from petal tissue of plants treated with aqueous solution containing 0.05% Tween (flowers were 5 and 6 days old, correspondingly); lanes 3 and 7—RNA from petal tissue of plants treated with 5 mM salicylic acid; lanes 4 and 8—RNA from petal tissue of plants treated with 50 μM jasmonic acid (flowers were 5 and 6 days old, correspondingly). (B) Quantification of SAMT expression in petal tissue after treatment with salicylic and jasmonic acids. Relative expression was estimated after hybridization of RT-PCR products with gene probes (SAMT and 18S rRNA) and normalization to the18S rRNA levels. Hybridization signals were analyzed using a Storm 860 Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The SAMT transcript levels in control samples were set at 1. Each point is the average of four to six independent experiments. Standard deviations are indicated by vertical bars.

gene may serve a physiological role in plant defense by producing methyl salicylate, a volatile signal molecule.

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References

[1] J.T. Knudsen, L. Tollsten, Bot. J. Linn. Soc. 113 (1993) 263–284.
[2] E.E. Farmer, C.A. Rayan, Proc. Natl. Acad. Sci. USA 87 (1990) 7713–7716.
[3] V. Shulaev, P. Silverman, I. Raskin, Nature 385 (1997) 718–721.
[4] R.A. Creelman, J.E. Mullet, Proc. Natl. Acad. Sci. USA 92 (1995) 4114–4119.
[5] W.L. Cauthen, W.H. Hester, J. Fam. Practice 29 (1989) 680–681.
[6] K. Herrmann, Ernahrungs-Umschau 37 (1990) 108–112.
[7] J.R. Ross, K.H. Nam, J.C. D'Auria, E. Pichersky, Arch. Biochem. Bioiphys. 367 (1999) 9–16.
[8] N. Dudareva, L.M. Murfitt, C.J. Mann, N. Gorenstein, N. Kolosova, C.M. Kish, C. Bonham, K. Wood, Plant Cell 12 (2000) 949–961.
[9] L.M. Murfitt, N. Kolosova, C.J. Mann, N. Dudareva, Arch. Biochem. Biophys. 382 (2000) 145–151.
[10] H.S. Seo, J.T. Song, J.-J. Cheong, Y.-H. Lee, Y.-W. Lee, I. Hwang, J.S. Lee, Y.D. Choi, Proc. Natl. Acad. Sci. USA 98 (2001) 4788–4793.
[11] N. Kolosova, D. Sherman, D. Karlson, N. Dudareva, Plant Physiol. 126 (2001) 956–964.
[12] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[13] N. Dudareva, L. Cseke, V.M. Blanc, E. Pichersky, Plant Cell 8 (1996) 1137–1148.
[14] N. Dudareva, J.C. D'Auria, K.H. Nam, R.A. Raguso, E. Pichersky, Plant J. 14 (1998) 297–304.
[15] N. Kolosova, N. Gorenstein, C.M. Kish, N. Dudareva, Plant Cell 13 (2001) 2333–2347.
[16] J. Wang, E. Pichersky, Arch. Biochem. Biophys. 349 (1998) 153–160.
[17] J. Wang, E. Pichersky, Arch. Biochem. Biophys. 368 (1999) 172–180.
[18] D.R. Gang, N. Lavid, C. Zubieta, F. Chen, T. Beuerle, E. Lewinsohn, J.P. Noel, E. Pichersky, Plant Cell 14 (2002) 505–519.
[19] S. Frick, T.M. Kutchan, Plant J. 17 (1999) 329–339.
[20] N. Dudareva, R.A. Raguso, J. Wang, J.R. Ross, E. Pichersky, Plant Physiol. 116 (1998) 599–604.
[21] M.G. Paton, S.H.P.P. Karunaratne, E. Giakoumaki, N. Roberts, J. Hemingway, Biochem. J. 346 (2000) 17–24.
[22] J. Nilsson, C. Vallbo, D. Guo, I. Golovleva, B. Hallberg, R. Henriksson, H. Hedman, Biochem. Biophys. Res. Commun. 284 (2001) 1155–1161.
[23] M. Dicke, R. Gols, D. Ludeking, M.A. Posthumus, J. Chem. Ecol. 25 (1999) 1907–1922.
[24] G. Arimura, R. Ozawa, J. Horiuchi, T. Nishioka, J. Takabayashi, Biochem. System. Ecol. 29 (2001) 1049–1061.
[25] A. Kessler, T. Baldwin, Science 291 (2001) 2141–2144.
[26] R.M.P. Van Poecke, M.A. Posthumus, M. Dicke, J. Chem. Ecol. 27 (2001) 1911–1928.
[27] J. Durner, J. Shah, D.F. Klessig, Trends Plant Sci. 2 (1997) 266–274.
[28] J. Hopke, J. Donath, S. Blechert, W. Boland, FEBS Lett. 352 (1994) 146–150.
[29] J.S. Thaler, M.J. Stout, R. Karban, S.S. Duffey, J. Chem. Ecol. 22 (1996) 1767–1781.
[30] R. Ozawa, G. Arimura, J. Takabayashi, T. Shimoda, T. Nishioka, Plant Cell Physiol. 41 (2000) 391–398.