

An *Arabidopsis thaliana* methyltransferase capable of methylating farnesoic acid [☆]

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Abstract

We previously reported the identification of a new family of plant methyltransferases (MTs), named the SABATH family, that use *S*-adenosyl-L-methionine (SAM) to methylate a carboxyl moiety or a nitrogen-containing functional group on a diverse array of plant compounds. The *Arabidopsis* genome alone contains 24 distinct SABATH genes. To identify the catalytic specificities of members of this protein family in *Arabidopsis*, we screened recombinantly expressed and purified enzymes with a large number of potential substrates. Here, we report that the *Arabidopsis thaliana* gene At3g44860 encodes a protein with high catalytic specificity towards farnesoic acid (FA). Under steady-state conditions, this farnesoic acid carboxyl methyltransferase (FAMT) exhibits K_M values of 41 and 71 μM for FA and SAM, respectively. A three-dimensional model of FAMT constructed based upon similarity to the experimentally determined structure of *Clarkia breweri* salicylic acid methyltransferase (SAMT) suggests a reasonable model for FA recognition in the FAMT active site. In planta, the mRNA levels of At3g44860 increase in response to the exogenous addition of several compounds previously shown to induce plant defense responses at the transcriptional level. Although methyl farnesoate (MeFA) has not yet been detected in *Arabidopsis*, the presence of a FA-specific carboxyl methyltransferase in *Arabidopsis* capable of producing MeFA, an insect juvenile hormone made by some plants as a presumed defense against insect herbivory, suggests that MeFA or chemically similar compounds are likely to serve as new specialized metabolites in *Arabidopsis*.

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A group of plant small molecule methyltransferases (MTs)¹ that share considerable sequence similarity to each other but not to other previously characterized

MTs has recently been identified and named the SABATH family based on the first three members functionally characterized (SAMT, BAMT, theobromine synthase) [1]. Characterized members of this family catalyze transfer of the reactive methyl group from *S*-adenosyl-L-methionine (SAM) to the carboxyl group or a nitrogen-containing moiety of a substrate, forming

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¹ Abbreviations used: MT, methyltransferase; FA, farnesoic acid; MeFA, methyl farnesoate; SAM, *S*-adenosyl-L-methionine; BA, benzoic acid; SA, salicylic acid; MeBA, methylbenzoate; MeSA, methyl

salicylate; MeJA, methyl jasmonate; DMSO, dimethyl sulfoxide; GC-MS, gas chromatography-mass spectrometry; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

S-adenosyl-L-homocysteine and methyl esters such as methylsalicylate (MeSA) [2] or N-methylated compounds such as caffeine [3]. The *Arabidopsis thaliana* genome contains 24 genes encoding members of this MT family collectively termed the *AtSABATH* family.

Thus far, the catalytic specificity for three of these *Arabidopsis* enzymes has been reported. Benzoic acid/salicylic acid carboxyl methyltransferase 1 (AtBSMT1), catalyzing the biosynthesis of MeSA and methylbenzoate (MeBA) from salicylic acid (SA) and benzoic acid (BA), respectively, was identified [4] based upon in vitro steady-state kinetic assays with heterologously expressed and purified protein and in vivo by correlating gene expression with the emission of MeSA and MeBA from *Arabidopsis* plants. Jasmonic acid carboxyl methyltransferase (AtJMT) was identified [5] by testing a heterologously obtained protein with a few acids hypothesized to serve as precursors for widely reported plant methyl esters [2]. Indole-3-acetic acid carboxyl methyltransferase (AtIAMT) was identified [6] based upon protein modeling and biochemical characterization. Yet to date, the substrates of most of the *AtSABATH* enzymes remain unknown.

Here, we report a large-scale biochemical approach to identify the catalytic specificities of the *AtSABATH* family of MTs. By qualitatively assaying recombinantly produced *AtSABATH* family members in parallel against a large number of potential substrates, chosen based upon their putative presence in planta, at relatively high concentrations in vitro, we identified the fatty acid lauric acid as a substrate for the protein encoded by the *AtSABATH* gene At3g44860. Additional assays with chemically related carboxyl-bearing substrates showed that the At3g44860-encoded MT displayed the highest activity when assayed with farnesoic acid (FA). Structure-based modeling of the protein encoded by At3g44860 based on the related salicylic acid carboxyl methyltransferase (SAMT) from *Clarkia breweri* [6] produced a three-dimensional model and active site geometry consistent with the efficient recognition of the 15-carbon isoprenoid carbon chain of FA. Analysis of the expression pattern of this gene indicated that its mRNA levels increased upon induction of the plant defense response.

Materials and methods

Plant material and treatments

Arabidopsis thaliana (ecotype Columbia) plants were grown in soil at 22 °C under 16 h light/8 h dark cycles. The 8-week-old flowering plants were used to collect inflorescences, siliques, stems, rosette leaves, and roots for tissue-specific expression analysis. The 6-week-old non-bolting plants were treated with several chemicals

known to induce defense-like responses, including SA, methyl jasmonate (MeJA), and alamethicin, or were injured by physical wounding as previously reported [4]. Briefly, for SA treatment, plants were sprayed with a solution of SA dissolved in 0.1% Tween (pH 7.0) at a final concentration of 5 mM. For MeJA treatment, plants were placed in a 1 liter glass jar and a solution of MeJA (1 μ l MeJA dissolved in 200 μ l ethanol) was applied to cotton tips placed in the jar. Distinct from the phytochemical treatments with SA and MeJA wherein whole plants were employed, alamethicin was applied to leaves cut from the base of the petiole and then placed upright in a small glass beaker containing 10 ml of a 5 μ g/ml alamethicin solution (diluted 1000-fold in tap water from a 5 mg/ml stock solution dissolved in 100% methanol). Care was taken to ensure that only the petiole of each leaf was submerged in the solution. The glass beaker was then sealed with Saran wrap and placed in a growth chamber. As a control, detached leaves were placed in 0.1% methanol. To examine the metabolic response following physical wounding, leaves were cut with a sterile razor blade producing two lateral incisions on each side of the mid vein. Two sets of samples were initially treated for each experiment. Following treatment, leaf tissue was harvested from one sample of each at 2 h and the second set of samples was collected at 24 h.

Chemicals and reagents

FA and MeFA were purchased from Echelon (Salt Lake City, UT). Geranylgeranoic acid was purchased from BIOMOL Research Lab (Plymouth Meeting, PA). Mycorradicin was collected from HPLC-purified fractions of alkaline-treated yellow pigment obtained from arbuscular mycorrhizal maize roots (a gift from Thomas Fester) following methods described previously [7]. Camalexin was a gift from Jane Glazebrook. All other chemicals were purchased from Sigma–Aldrich.

cDNA isolation and protein expression in Escherichia coli

Total RNA was extracted from *Arabidopsis* leaves with a RNeasy Plant Mini Kit (Qiagen, Valencia, CA). RT-PCR was performed as previously described [4] using the forward primer 5'-AATGTCGACTTCATTCACAATGATCG-3' and the reverse primer 5'-CTTTCGCTCTTCTCTTTCACAACG-3'. The resulting PCR fragment was cloned into T7/CT-TOPO vector (Invitrogen, Carlsbad, CA). The resulting plasmid was transformed into *Escherichia coli* BL21 Codon Plus cells and expressed as previously described [8].

Protein purification

Escherichia coli cells induced with IPTG and expressing a full-length At3g44860 cDNA were har-

vested and lysed, and the lysate was loaded onto a 10 ml DEAE anion-exchange column pre-equilibrated with buffer A (50 mM Tris-HCl, pH 7.0, 10% glycerol, and 5 mM β -mercaptoethanol) as previously described [8]. The column was washed with 30 ml buffer A and eluted with 35 ml of a linear gradient of 0.1–1 M KCl in buffer A. Fractions of 3 ml each were collected and 5 μ l of each fraction was assayed using FA as substrate. Fractions with the highest activity (eluted between 267 and 349 mM KCl) were pooled, dialysed against buffer A for 5 h and then loaded onto a 1 ml Mono-Q FPLC column. The column was eluted with 35 ml of a linear gradient of 0–0.7 M KCl in buffer A. Fractions (1 ml each) were collected and assayed for methyltransferase activity with FA. The majority of FAMT activity eluted between 184 and 224 mM KCl. Peak fractions from Mono-Q were examined by SDS-PAGE and stained with Coomassie blue.

Screen for MT substrates

We designed a rapid biochemical assay system for the initial screening of potential substrates for individual AtSABATH proteins. We initially chose 59 compounds known to exist in plants that contain either a free carboxyl group or a nitrogen-containing moiety. Depending on compound solubility properties, each compound was dissolved in an appropriate solvent to make a 50 mM stock solution (Table 1). Compounds were pooled into 11 groups, each group containing five or six putative substrates, with each compound present at a concentration of 8.3 mM (six compounds) or 10 mM (five compounds), except for group 7, which contained two compounds at 25 mM

each (Table 1). Parallel assays of a total volume of 50 μ l each contained 50 mM Tris-HCl, pH 7.5, 1 μ l purified protein (1 mg/ml) or 5 μ l unpurified protein expression crude extract, 0.5 μ l [14 C]SAM (specific activity 52.7 mCi/mmol, Perkin Elmer Instruments, Shelton, CT), and 1 μ l substrate pool dissolved in the appropriate solvent (Table 1). Solvents were chosen that had been determined in previous work not to affect the activity of SABATH methyltransferases at their final concentration in the assay (2%). In some cases, several solvents were tested to assess their effect on enzymatic activity. In all cases, control assays were conducted containing only solvent but no substrates. Assays were performed in triplicate for each group of substrates. Reactions were incubated at room temperature for 30 min, the products were extracted with 180 μ l ethyl acetate, and finally counted in a scintillation counter as previously described [8].

Identification of methyl farnesoate

A 1 ml reaction containing 150 μ g purified FAMT, 1 mM FA, and 600 μ M unlabelled SAM was incubated in a buffer containing 50 mM Bis-Tris propane, pH 8.0, for 4 h at room temperature. The reaction was then extracted with 1.5 ml hexane, the upper hexane layer was collected, concentrated under N₂ gas, and the hexane concentrate was analyzed by GC-MS as described [9]. As a control, an identical reaction mixture was formulated, except that FAMT was denatured by boiling at 100 °C for 10 min before adding to the assay mix. A MeFA authentic standard was dissolved in ethanol and 1 μ g MeFA was loaded into the GC-MS using a split (1/30) mode injection.

Table 1
Compounds screened for MT enzymatic activity

Group	Compounds	Solvent
1	Benzoic acid, caffeic acid, 3,5-dimethoxy-4-hydroxy-cinnamic acid, chlorogenic acid, phenylpyruvic acid	Ethanol
2	Salicylic acid, ferulic acid, vanillic acid, gallic acid, 4-hydroxy-phenylpyruvic acid	Ethanol
3	3-Hydroxy-benzoic acid, anthranilic acid, <i>p</i> -coumaric acid, jasmonic acid, shikimic acid	Ethanol
4	4-Hydroxy-benzoic acid, 4-amino-benzoic acid, cinnamic acid, 3-hydroxy-4-methoxy-cinnamic acid, rosmarinic acid 4-hydroxy-phenyllactic acid	Ethanol
5	Abscisic acid, gibberellic acid, indole-3-acetic acid, indole-3-butyric acid, 2,4-dichlorophenoxyacetic acid (2,4-D), indole	Ethanol
6	Zeatin, kinetin, 6-benzylaminopurine, γ -amino butyric acid (GABA), 3,4-dihydroxyphenylalanine (DOPA), tryptophan	DMSO
7	Trigalacturonic acid, muramic acid	DMSO
8	Octanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid, stearic acid	Ethanol/chloroform (1:1)
9	Xanthosine, 7-methyl-xanthine, theobromine, paraxanthine, β -alanine, camalexin	DMSO
10	Glutamic acid, valine, alanine, phenylalanine, aspartic acid, asparagine	H ₂ O
11	Nicotinic acid, nicotinamide, anabasine, normicotine, tryptamine, tyramine	Ethanol

A total of 59 potential substrates were pooled into 11 groups from which appropriate solvents were used to create a master stock solution.

Steady-state kinetic analysis of *FAMT*

Appropriate enzyme concentrations and incubation time were chosen so that the reaction velocity was in linear with time with no more than 10% of the substrate consumed during the time period. Incubation times for all kinetic analyses were 30 min. The determination of kinetic properties was as described [8] except that 50 mM Bis-Tris propane, pH 8.0, was used as assay buffer when examining K_M values, the effect of metal ions, and the temperature dependence of the steady-state kinetic parameters. Lineweaver–Burk, Eadie–Hofstee, and Hanes plots were constructed for each measurement, and an average of their K_M values and maximum velocity values were used. When examining effect of metal ions, 5 mM chloride salt of each ion was used, except for Cu^{2+} and Fe^{2+} in which sulfate salts were used.

Real-time quantitative PCR

Total RNA was isolated with a RNeasy Plant Mini Kit (Qiagen) and DNA contamination was removed with an on-column DNase (Qiagen) treatment. One microgram of total RNA was transcribed into first strand cDNA in a 20 μl reaction using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time quantitative PCR (RT-PCR) was performed on an ABI7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR green fluorescence dye (Bio-Rad Laboratories). For RT-PCR analysis of *FAMT* gene expression, gene-specific primers designed to amplify a fragment of approximately 100 bp were as follows: (forward primer) 5'-CCGCGGTACAAACCTTAATT-3' and (reverse primer) 5-TGGAAGCTCGATTCCCTCAAT-3'. Amplification of the ubiquitin gene using the forward primer, 5'-CACACTCCACTTGGTCTTGCG-3', and the reverse primer, 5'-TGGTCTTTCCGGTGAGAGTCTTCA-3'), was used as an internal control. Each 25 μl RT-PCR contained 12.5 μl iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories), 900 nM of the forward primers and 300 nM of the reverse primers listed above for *FAMT* and ubiquitin, respectively. Three different concentrations of each cDNA were used as templates for the RT-PCR assays to ensure 100% efficiency. RT-PCRs were carried out using the following conditions: 50 °C for 5 min and 95 °C for 5 min for one cycle, followed by 44 cycles at 95 °C for 25 s; and 54 °C for 90 s.

The relative quantifications of *FAMT* gene expression in different tissues and in different treated samples were performed using the comparative threshold cycle (C_t) method. The C_t for each reaction was generated during the PCR assays. The difference between the C_t values for *FAMT* and ubiquitin within a given sample provided the value of C_t for that sample, reflecting the

FAMT mRNA expression relative to the ubiquitin mRNA expression level. To compare the relative abundance of *FAMT* mRNA in different tissues and in different treated samples, the $2^{-\Delta\Delta C_{mt}}$ method [10] was employed. In this method, $\Delta\Delta C_t$, the difference in C_t values of each sample and an arbitrarily chosen control (in this case leaf tissue in tissue-specific analysis and untreated leaves in treatment assays), was used to calculate differences in relative levels of *FAMT* mRNA expression.

Structural modeling

Molecular modeling of the *FAMT* active site based on the experimentally determined structure of the *C. breweri* SAMT [6] was carried out using the program MODELLER [11] and the UCSF Chimera package [12] (<http://www.cgl.ucsf.edu/chimera>, supported by NIH P41 RR-01081). Three-dimensional images were rendered using the program POV-Ray (Persistence of vision, <http://www.povray.org/download>).

Results

Identification of *At3g44860* as farnesoic acid methyltransferase (*FAMT*)

The *AtSABATH* gene *At3g44860* encodes a protein of 348 amino acids with a calculated molecular weight of 38.5 kDa. The sequence alignment of this protein with known *AtSABATH* proteins (*AtBSMT1*, *AtJMT*, and *AtIAMT*) and *C. breweri* SAMT (Fig. 1) indicates a 24–31% sequence identity among these proteins.

A full-length cDNA of *At3g44860* was obtained by RT-PCR from *Arabidopsis*, inserted into the expression vector T7/CT-TOPO, and recombinant protein obtained from over-expression in *E. coli*, as described under Materials and methods. The full-length, non-fusion protein was purified to near homogeneity in two ion-exchange chromatographic steps (Fig. 2).

The protein encoded by *At3g44860* was examined using our in vitro biochemical assays for potential substrates. Since previously characterized MTs belonging to this class have been shown to methylate either a carboxyl group or a nitrogen-containing moiety, we focused our attention on a list of such compounds known to occur in plants. To simplify and accelerate our screening process, we first pooled chemicals into 11 groups of 5–6 compounds each (except for group 7 which contained 2 compounds) (Table 1). The substrate pools were then assayed en masse. The *At3g44860*-encoded protein showed methyltransferase activity with pool #8, but not with any other substrate pools. Compounds in this group included the saturated fatty acids octanoic acid ($\text{C}_8:0$), decanoic acid ($\text{C}_{10:0}$), lauric acid

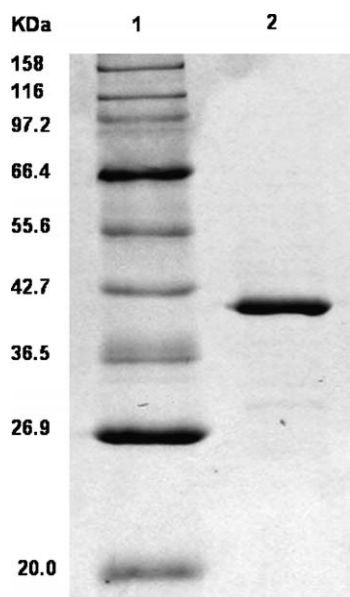


Fig. 2. SDS-PAGE analysis of purified recombinant FAMT. FAMT produced in *E. coli* was purified by a two-step chromatographic ion-exchange procedure as described under Materials and methods. Lane 1 contains molecular weight markers. Lane 2 contains 1 μ g FAMT from the final chromatographic step. The gel was stained with Coomassie brilliant blue R250.

Mn^{2+} present at 5 mM increased the specific activity by 38%. K^+ , NH_4^+ , or Mg^{2+} at 5 mM had mild effect on FAMT activity.

The enzyme retained 90% of its activity when incubated for 30 min at 30 $^{\circ}C$, but lost 50% of its activity when incubated for 30 min at 40 $^{\circ}C$. When kept at 50 $^{\circ}C$ for 30 min, FAMT lost all of its activity. Steady-state kinetic analysis of FAMT gave K_M values of 41 and 71 μM for FA and SAM, respectively, with a k_{cat} of 0.004 s^{-1} for FA (Fig. 5).

Molecular modeling of Arabidopsis FAMT

C. breweri SAMT was the first SABATH family member to have its crystal structure experimentally determined [6]. Since *C. breweri* SAMT and *A. thaliana* FAMT are 31% identical, a reasonable three-dimensional model of FAMT, including its active site, was constructed using *Clarkia* SAMT as a template (Fig. 6). The position of the FA substrate in the model presented in Fig. 6 was arrived at by aligning the carboxyl group of FA with the carboxyl moiety of the SA substrate bound in the active site of *Clarkia* SAMT. Moreover, a similar result was obtained in 7 out of 10 solutions calculated by the automated small molecule docking program GOLD (software package for the computer-aided modeling of protein-small molecule interactions, CCDC Software, 2004) [13,14].

In contrast to the relatively open active site architecture of *Clarkia* SAMT in and around the SA binding site, the FAMT model suggests that the residues comprising the likely FA binding site narrow the active site cavity and sit comfortably nestled around the extended

Substrate	Structure	Relative Activity (%)
Farnesoic Acid		100
Geranic Acid		28+/-3
Geranylgeranoic acid		0
Octanoic Acid		0
Decanoic Acid		0
Lauric Acid		18+/-6
Myristic Acid		0
Palmitic Acid		0
Stearic Acid		0
Traumatic Acid		0
Dodecanedioic Acid		0
3-Hydroxydodecanoic Acid		0
11-Methyldodecanoic Acid		0
11-Dodecenoic Acid		0
Mycorradicin		0

Fig. 3. Relative activities of FAMT with farnesoic acid and chemically related compounds. All compounds were assayed at 1 mM final concentration using 3 μM SAM and 2 μg of purified FAMT. The specific activity of FAMT with 1 mM FA, 6.7×10^{-11} katal, was set at 100%. Each value of relative activity is an average of three independent measurements.

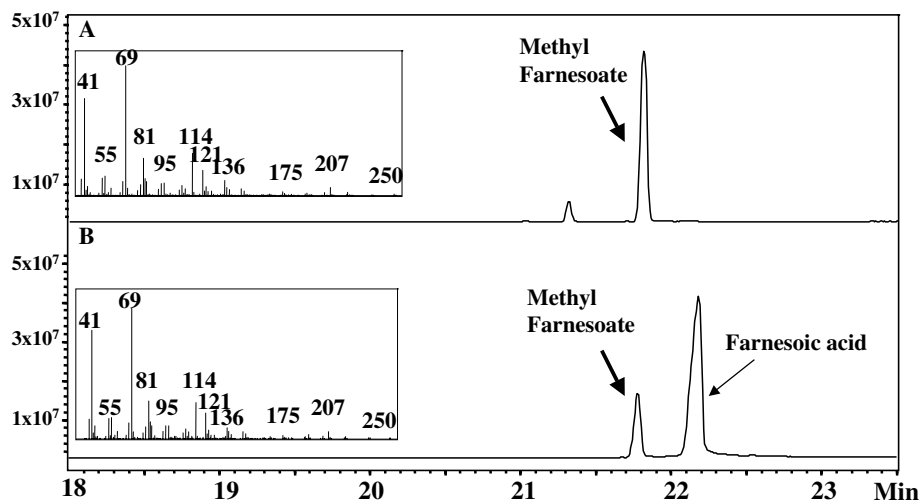


Fig. 4. GC–MS analysis of the product of the enzyme assay catalyzed by recombinant FAMT with FA as the substrate. (A) GC–MS analysis of methyl farnesoate standard. The mass fragmentation pattern of the methyl farnesoate peak is shown in the inset. (B) GC–MS analysis of the hexane extract of FAMT enzyme assay with FA, as described under Materials and methods. The Y-axis is given in arbitrary units.

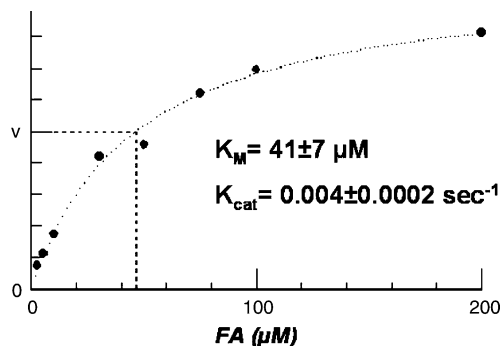


Fig. 5. An example of steady-state kinetics measurements of FAMT with FA as the substrate. Average K_M and k_{cat} values shown were obtained from three independent measurements of initial velocity versus substrate concentration plots fit by non-linear least-squares analysis using the Michaelis–Menten equation.

isoprenoid hydrocarbon chain of FA. A crown of aromatic residues including Tyr254, Phe145, Tyr16 (not shown to reveal the SAH beneath), Tyr141, and Tyr22 surround the carboxyl end of FA. This aromatic-rich active site pocket most likely provides the hydrophobic environment necessary for the exclusion of water that enables the desolvation of the carboxyl group to facilitate transfer of a methyl group from the sulfur of SAM to the methyl acceptor. In *Clarkia* SAMT, a Val311/Leu210 clamp (*Clarkia* numbering) brackets the SA substrate; a similar clamp composed of Thr300 and Ser208 constrains the carboxyl group of FA.

While SA bears a 2-hydroxyl group with which it can form an intra-molecular hydrogen bond with its own carboxyl, causing the carboxyl group to remain in the same geometric plane formed by the aromatic ring, FA possesses no such stabilizing internal hydroxyl but-ress. The hydroxyl groups of Thr300 and Ser208 are

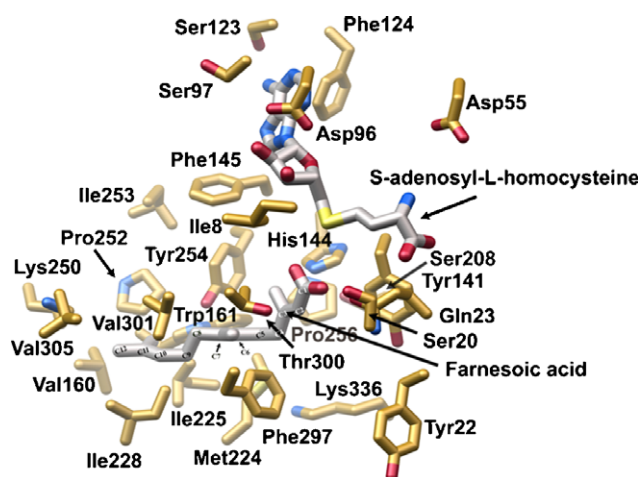


Fig. 6. Active site architectural model of FAMT. The active site model of *Arabidopsis* FAMT with FA substrate bound was constructed by using the experimentally determined structure of *C. breweri* SAMT with SA as the template, and aligning the carboxyl group of FA with that of SA. A similar result was found in 7 out of 10 solutions produced by computer-aided docking of the farnesoic acid substrate into the modelled active site of FAMT using the program GOLD (Genetic Algorithm for Ligand Docking—CCDC Software, 2004). In contrast to the more open active site of *Clarkia* SAMT, the residues in the proposed FAMT active site form a pocket that nicely encapsulates the farnesyl chain of FA.

both within hydrogen bonding distance of the carboxyl moiety of FA, and hence these residues may serve to sequester the carboxyl group of the substrate for proper positioning near SAM. In *Clarkia* SAMT, a methionine “sandwich” (Met308/Met150) further brackets the phenyl ring of salicylic acid, while in FAMT this partially hydrophobic “molecular sandwich” is composed of Phe297 and His144. Hydrophobic pairs of residues, including Trp161/Met224, Val301/Ile225, and Val160/

Ile228, are positioned along the remainder of the hydrocarbon chain of FA with one member of each pair cradling either side of the FA isoprenoid tail. Val160 is positioned just beyond the C12 carbon of the FA chain, possibly dictating the specificity of this hydrophobic pocket for substrates no more than 12 carbons in backbone linear length. Interestingly, there is a somewhat spacious “crawl space” directly above this 12 carbon chain of FA, which in turn is bordered by Val305 (Fig. 6). While Val305 may serve to regulate access of longer isoprenoid or fatty acid derived substrates, a smaller residue at this position as well as at the position equivalent to Val160 may open up additional crawl space to allow access of longer substrates such as geranylgeranoic acid (C₂₀).

Characterization of *FAMT* gene expression in different organs and under various treatments

To determine the tissue-specific expression pattern of *FAMT* in mature *Arabidopsis* plants, total RNA from different organs was isolated as described under Materials and methods and used in real-time quantitative RT-PCR analyses. The highest level of the *FAMT* transcript was observed in leaves. By contrast, almost no expression was detected in roots, stems, flowers or siliques (Fig. 7A). In addition, the expression of *FAMT* in leaf and root tissues of young *Arabidopsis* plants was not significantly different from that in mature leaves and roots, respectively (data not shown).

Additional real-time quantitative RT-PCR experiments were performed to identify conditions in which *FAMT* expression is induced in the *Arabidopsis* leaves. Four treatments, including application of SA, MeJA or the fungal elicitor alamethicin, and physical wounding were administered to 6-week-old non-bolting plants. Among all of the treatments examined, alamethicin was the most effective inducer of *FAMT* gene expression. At 2 h after alamethicin treatment, the *FAMT* transcripts showed a 3.5-fold increase. This induction declined slightly as the treatment proceeded for an additional 22 h. SA, MeJA and wounding treatments increased *FAMT* expression by at most twofold. However, the time dependence of these *FAMT* gene induction patterns was different among these latter treatments. SA appeared to be an effective inducer 2 h after the commencement of treatment. However, at 24 h after the application of SA, the expression of the *FAMT* gene was lower than the control experiment. MeJA had no significant effect on *FAMT* expression at early stages of the treatment, but it was effective at inducing *FAMT* gene expression at 24 h. In contrast, *FAMT* gene expression levels at 2 and 24 h following physical wounding were very similar, with the *FAMT* mRNA levels at 24 h were slightly lower than those at 2 h (Fig. 7B).

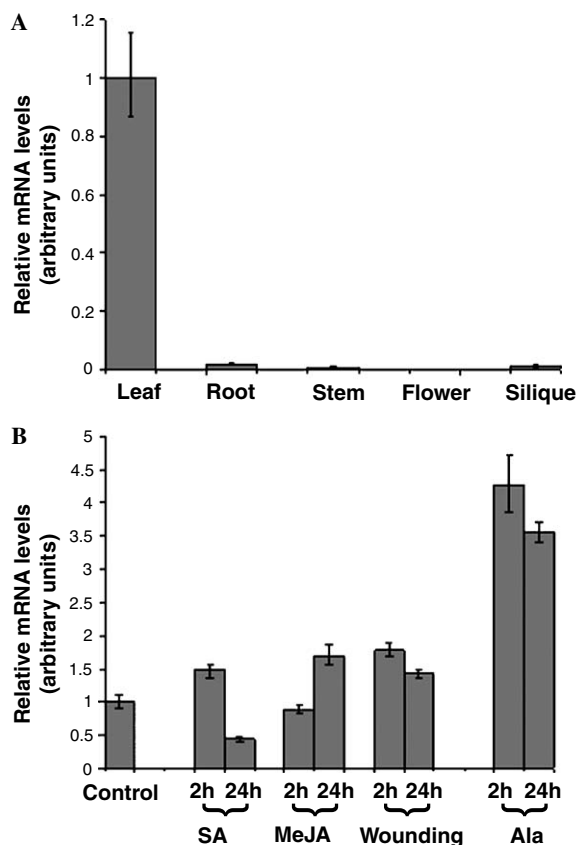


Fig. 7. Real-time quantitative RT-PCR analyses of *FAMT* gene expression. *FAMT* expression values were normalized to the levels of ubiquitin gene expression in respective samples. Data are representations of three independent experiments and are plotted as means \pm SD. (A) Quantification of *FAMT* expression in different organs of mature *Arabidopsis* plants. The level of *FAMT* expression in leaves is arbitrarily set at 1.0. (B) Induction of *FAMT* expression in leaf tissue after treatment with SA, MeJA, physical wounding and alamethicin (Ala). The level of *FAMT* expression in control leaves is arbitrarily set at 1.

Discussion

Screening of potential substrates using a genomic biochemical approach

Currently characterized SABATH family enzymes transfer a methyl group from SAM to a nitrogen or carboxyl-containing moiety of specialized (secondary) metabolites in caffeine biosynthesis as well as to important signaling molecules in plants such as jasmonic acid, SA, and indole-3-acetic acid [2,5,6]. Given the large diversity of SABATH family members within a single organism (24 in *Arabidopsis*) and within the plant kingdom in general [1], it is likely that many other small molecules are methylated by SABATH MTs. Therefore, to identify natural small molecules of plant origin that can serve as substrates for these MTs will require a relatively rapid and quantitative biochemical assay. As a test case of such an approach, we chose to examine

the catalytic properties of the SABATH family member encoded by the *Arabidopsis* gene At3g44860. We began this exploratory enzymatic screen by testing a range of diverse plant compounds each of which contains either one or more carboxyl moieties or nitrogen-containing functional groups. This list of phytochemicals included primary metabolites, specialized metabolites, and plant hormones (Table 1). Such a screening approach is limited, however, by the fact that many metabolites in *Arabidopsis* remain to be identified due to technical limitations facing current metabolic profiling techniques [15], as well as difficulties associated with testing putative substrates that are operationally insoluble, including some that are high molecular weight natural polymers.

Biochemical properties of FAMT

The protein encoded by At3g44860, produced in *E. coli*, displayed the highest specific activity with FA among all the substrates tested thus far (Fig. 3). The K_M and k_{cat} values for FAMT acting on FA are comparable to the respective values of other SABATH enzymes when assayed with their physiological substrates in vitro [2,4–6]. In addition to FA, FAMT displayed measurable but lower activity with lauric acid and the shorter chain isoprenoid geranic acid, with the latter possessing the greater specific activity of the two. Lauric acid contains an un-branched saturated hydrocarbon of the same as the length as the un-branched portion of FA (Fig. 3). Several modified lauric acid analogs that were tested, including derivatives with a hydroxyl group, a double bond, or an additional methyl group, were not methylated by FAMT. From comparative modeling of the FAMT three-dimensional structure with a docked molecule of FA, it appears that a compound with an un-branched carbon chain longer than 12 carbons will not fit into the active site due to amino acid side chain steric modulators of chain length (Fig. 6). Owing to its identical isoprenoid chain structure with one C_5 unit shorter than FA (isoprenoids such as FA and geranic acid are built up from repeating C_5 groups), geranic acid fits comfortably in the modelled FAMT active site with room to spare. This additional un-occupied space likely results in a poorer affinity of geranic acid for FAMT and would therefore be expected to cause a lower overall specific activity due to inefficient transfer of a methyl group from SAM.

These predictions are consistent with the observations that the C_{20} diterpene geranylgeranoic acid (which has one additional isoprene unit than FA) is not methylated by FAMT and neither were fatty acids with acyl chains longer than that of lauric acid. In view of the complementary fit of FA in the active site of the FAMT model, it is reasonable at this time to conclude that FA, or a chemically very similar compound, serves as the

in vivo substrate for FAMT. Further in vivo work on wild type and mutant *Arabidopsis* plants will be necessary to conclusively establish the physiological substrate for FAMT.

Possible role of FAMT in *Arabidopsis* physiology and ecology

In insects, FA is methylated on its carboxyl group and the resulting MeFA is converted to Juvenile hormone III (JH III) by an enzyme-catalyzed 10,11-epoxidation reaction [16]. In crustaceans, MeFA and FA are the only juvenoid-like compounds discovered to date, and MeFA appears to play roles in the regulation of crustacean development [17]. Recently, a silkworm enzyme that catalyzes the methylation of FA was identified [16]. The enzyme responsible for the conversion of FA to MeFA in lobster (FAOMeT) has also been characterized [17]. Neither of these enzymes possesses sequence similarity to each other or to *Arabidopsis* FAMT, suggesting there are multiple evolutionary solutions for FA methylation.

MeFA and JH III have been isolated from the Malaysian plant *Cyperus iria* (Cyperaceae; common name: grasshopper's cyperus) [18]. It was hypothesized that the high content of JH III in this plant plays a role in the plant defense against insects through its ability to interfere with insect development [18,19]. However, other physiological or defense roles for FA and MeFA in planta could not be ruled out. Our data demonstrate that *Arabidopsis* possesses an enzyme that is capable of producing MeFA or methyl esters of phytochemicals with similar structures. A role for FAMT in defense is also suggested by the observation that the transcript level of *FAMT* is induced upon alamethicin treatment, and also, to a lower extent, upon SA and MeJA treatment or wounding. Alamethicin, a fungal elicitor, is known to induce the expression of several defense genes that are also induced by SA and MeJA [4,20]. However, while SA is responsible for the induction of systemic acquired resistance (SAR) which is most effective toward pathogenic microorganisms, MeJA appears to be involved more in the induction of resistance to insect herbivory [21,22]. Furthermore, the induction of one of these defense pathways appears to have a negative effect on the ability of the plant to induce the other pathway [20]. Thus, our observation that the induction of *FAMT* by MeJA is more effective than its induction by SA lends additional support to the hypothesized role of *FAMT* in insect resistance. However, our attempts to detect insect juvenoid-like compounds such as FA or MeFA in *Arabidopsis* leaves thus far have not been successful, indicating that, if such compounds are produced, they are present at very low levels and their production may be subject to temporal and spatial regulation that remain to be determined.

Conclusions

The determination of the biochemical function of all the enzymes encoded in the *Arabidopsis* genome is a daunting task. Large-scale methods that use metabolic profiling and gene expression data have been used to identify candidate substrates for further biochemical examination [23]. Here, we show that enzyme activity screens using small pools of multiple substrates during initial phases of screening, when combined with sequence variation and structural modeling to ascertain the likely recognition principles guiding substrate selectivity in the enzyme active site, can be of enormous predictive value for the accelerated annotation of a large family of catalytically similar enzymes with diverse substrate specificities. Quantitative biochemical characterization of each enzyme must follow, and thus provides the critical starting points for in vivo substantiation of the physiological role of enzymes such as FAMT.

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