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S-Adenosyl-L-methionine-dependent cinnamic acid carboxyl methyltransferase from the basidiomycete *Tricholoma matsutake*, which has low identity but similar overall structure to plant SABATH-family enzymes

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Abstract

We have cloned cDNA encoding an S-adenosyl-L-methionine (SAM)-dependent cinnamic acid carboxyl methyltransferase from the basidiomycete *Tricholoma matsutake*. For cloning, we focused on four conserved motifs in SABATH (Salicylic Acid, Benzoic Acid and Theobromine)-family proteins, including the cinnamic acid carboxyl methyltransferases from *Ocimum basilicum* (ObCCMT1) and *Conocephalum salebrosum* (CsCAMT), not their full amino acid sequences. On the basis of the homology of the motifs between ObCCMT1 and putative SAM-dependent methyltransferases predicted in RNA-Seq data for *T. matsutake*, we selected and cloned three *T. matsutake* cDNAs, named 1391973, 1389993, and 1330427, respectively. Recombinant 1330427 protein showed the target activity, and hence was renamed TmCAMT1 (*T. matsutake* Cinnamic Acid carboxyl MethylTransferase). The identities between TmCAMT1 and ObCCMT1 and CsCAMT were just 17% and 19%, respectively. TmCAMT1 was categorized into Pfam database family Methyltransf_31, not Methyltransf_7 to which ObCCMT1 and CsCAMT belong. However, structural prediction using AlphaFold2 and comparison with the crystal structure of *Clarkia breweri* SAM-dependent salicylic acid carboxyl methyltransferase (CbSAMT; PDB ID: 1M6E) indicated that TmCAMT1, ObCCMT1, CsCAMT and CbSAMT are similar in overall structure and amino acid residues in the active site. Meanwhile, during the course of this study, MnMT2, a cinnamic acid carboxyl methyltransferase from mycobacteria, was reported. TmCAMT1 shows slightly greater identity, 21%, with MnMT2, although MnMT2 belongs to the leucine carboxyl methyltransferase family, not the SABATH family, and AlphaFold2 predicted that TmCAMT1 and MnMT2 have limited overall structural similarity. In conclusion, our cloning strategy successfully obtained TmCAMT1, the first characterized SAM-dependent cinnamic acid carboxyl methyltransferase that is similar to SABATH family proteins in overall structure and contains a few motifs of this family, but it comes from a non-plant species. However, the low *k_{cat}* value suggests that further research is required to elucidate whether TmCAMT1 plays a predominant role for (*E*)-methyl cinnamate biosynthesis in *T. matsutake*.

Keywords Carboxyl methyltransferase, (*E*)-Methyl cinnamate, *Tricholoma matsutake*, SABATH family, Methyltransf_31, S-Adenosyl-L-methionine

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Introduction

Tricholoma matsutake is an edible mushroom that is prized in Japanese cuisine because of its distinct odor. However, *T. matsutake* has been listed as vulnerable in the International Union for Conservation of Nature Red List since 2019 [[Tricholoma matsutake \(Matsutake\) \(iucnredlist.org\)](#)]. Therefore, conservation methods should be developed for *T. matsutake*. (*E*)-Methyl cinnamate [(*E*)-MC] is one of the major odorous components of fruiting bodies of this basidiomycete [1–3], and is found in the lamellae and spores in the pileus of the fungus [2]. The (*E*)-MC content increases with growth of the *T. matsutake* fruiting body [2, 4]. In nature, (*E*)-MC together with the (*R*)-(-)-1-octen-3-ol in *T. matsutake* repel the mycophagous arthropod *Proisotoma minuta* [5]. Elucidation of the biosynthesis of (*E*)-MC in *T. matsutake* will bring insights into other possible roles of (*E*)-MC in the lifecycle of this fungus. We have reported from a tracer experiment that (*E*)-MC is biosynthesized from L-phenylalanine via cinnamic acid [6]. Accumulation of (*E*)-MC is induced by the addition of L-phenylalanine to mycelial culture of *T. matsutake* [7]. However, the molecular mechanism of formation of (*E*)-MC from cinnamic acid in *T. matsutake* has not yet been determined. S-Adenosyl-L-methionine (SAM)-dependent transmethylation activity for production of (*E*)-MC from cinnamic acid was detected in cell-free extracts of another basidiomycete, *Neolentinus lepideus* [8].

This study aimed to obtain a cDNA encoding a SAM-dependent cinnamic acid carboxyl methyltransferase from *T. matsutake* that forms (*E*)-MC and to characterize the recombinant enzyme. cDNAs encoding a SAM-dependent cinnamate/*p*-coumarate carboxyl methyltransferase from basil (*Ocimum basillicum*; ObCCMT1) [9] and a cinnamic acid carboxyl methyltransferase from liverwort (*Conocephalum salebrosum*; CsCAMT) [10] have been reported and the recombinant enzymes have been characterized. ObCCMT1 and CsCAMT belong to the SABATH (Salicylic Acid, Benzoic Acid and Theobromine) family of proteins, which is based on homology to the amino acid sequences [11] of methyltransferases to the carboxyl group of salicylic acid [12] and benzoic acid [13] and the ring nitrogen of theobromine [14]. SABATH family proteins are from the plant kingdom [11] and several sequence motifs are conserved among them [15]. Therefore, we hypothesized that there may be significant homology, at least in the motifs, between a SAM-dependent cinnamic carboxyl methyltransferase in *T. matsutake* and ObCCMT1 and CsCAMT.

This paper describes how we achieved the cloning of cDNA *TmCAMT1* (encoding *T. matsutake* Cinnamic Acid carboxyl MethylTransferase), focusing not

on the whole sequence of ObCCMT1 but on four conserved motifs within that protein. In addition, during the course of this study, MnMT2, a cinnamic acid carboxyl methyltransferase from *Mycobacterium neoaurum*, has been reported [16]. Therefore, our results are discussed in terms of the differences and similarities between the SAM-dependent cinnamic acid carboxyl methyltransferases ObCCMT1 and CsCAMT from the plant kingdom, MnMT2 from bacteria, and TmCAMT1 from a basidiomycetous fungus.

Materials and methods

RNA-Seq

Mycelia of *T. matsutake* were cultivated with and without addition of L-phenylalanine for induction and not induction of (*E*)-MC production, respectively [6], then total RNA prepared from cultivated mycelia was used for preparation of a sequencing library. Sequences were assembled based on 100-bp paired-end sequencing data obtained using HiSeq apparatus (Illumina, San Diego, CA, USA) by Hokkaido System Science Co., Ltd. (Sapporo, Japan).

Selection of cDNAs encoding candidate proteins for SAM-dependent cinnamic acid carboxyl methyltransferase activity

BLASTx analysis with a query of each cDNA encoding a putative SAM-dependent methyltransferase in the RNA-Seq data was conducted against the *Tricholoma matsutake* 945 database (*Tricholoma matsutake* 945 v3.0, JGI MycoCosm web portal, <https://mycocosm.jgi.doe.gov/Trima3/Trima3.home.html>) to identify possible complete amino acid sequences for them. Each of the coding regions thus predicted was aligned with ObCCMT1 using Clustal Omega software (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) [17]. The homology was estimated based on identity and similarity of conserved motifs in SABATH family proteins—ADLGCS in motif 3, NDFN in motif 5, GSFY in motif 1, and SLHWL in motif 1 [15]—as follows, in order: (1) identical, similar, and weakly similar amino acids were scored 3, 2, and 1, respectively; (2) when the alignment of a motif was divided, 2 was subtracted for that motif; (3) for each candidate protein, the sum of the scores was calculated to determine an expedient homology value. To determine a suitable threshold expedient value, we first conducted a keyword search with “Methyltransf_7” as the query against proteins in Basidiomycota [JGI database Basidiomycota (doe.gov)] including *T. matsutake*, because the SABATH family proteins ObCCMT1 and CsCAMT belong to family Methyltransf_7 (PF03492) [10] in the Pfam database [18]. We found that uncharacterized protein UHOR_01838 in the basidiomycetous fungus *Ustilago hordei* is classified as a

SAM-dependent carboxyl methyltransferase belonging to the Methyltransf_7 family. In addition, UHOR_01838 was previously treated like a SAM-dependent carboxyl methyltransferase (based on homology but not experimental data) [9]. Therefore, UHOR_01838 was used as a marker for the threshold criterion. The expedient homology value for UHOR_01838 was determined in the same way as described above by alignment with ObCCMT1. The cDNAs encoding putative SAM-dependent methyltransferases in *T. matsutake* for which the expedient values were greater than or equal to that for UHOR_01838 were named 1391973, 1389993, and 1330427.

Cloning of cDNAs 1391973, 1389993, and 1330427

First-strand cDNA synthesis was performed using Superscript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and an oligo (dT)₂₀ primer. The cDNAs 1391973, 1389993, and 1330427 were individually amplified by PCR with KOD Plus Neo DNA polymerase (TOYOBO, Osaka, Japan) in a 25- μ L reaction mixture containing 0.5 U of polymerase, 0.3 μ M of each primer, and cDNAs as the template. The primers were designed to amplify the putative coding regions of the candidate proteins 1391973, 1389993, and 1330427. Primer sequences: 1391973, 5'-GAAGGAGATATACATATGGCGACCTTCGCAAAAT-3' and 5'-TGCTCGAGTGC GGCGCCGCGCTTAAACAAGCAACA-3'; 1389993, 5'-GAAGGAGATATACATATGACTGACGCACTGAGGA-3' and 5'-TGCTCGAGTGC GGCGCCGCTGCAGCCGAGTATACCAC-3'; 1330427, 5'-GAA GGAGATATACATATGTCCCCGTCTACTAGCAA-3' and 5'-TGCTCGAGTGC GGCGCCGCGCCACCAAGATCGCGAAAATC-3'.

The PCR protocol was 94 °C for 2 min, and 35 cycles of 98 °C for 10 s and 68 °C for 90 s. The amplified cDNA fragments, the sizes of which in electrophoresis were almost identical to those of the cDNAs 1391973, 1389993, and 1330427, were purified and sequenced. By In-Fusion cloning (Takara Bio USA, Inc., San Jose, CA, USA), the obtained cDNAs were cloned into vector pET-23a(+) (Merck, Darmstadt, Germany) digested with *Nde*I and *Not*I. The vectors thus prepared were named 1391973-pET-23a(+), 1389993-pET-23a(+), and 1334427-pET-23a(+).

Preparation of recombinant enzymes

Escherichia coli BL21 (DE3) was transformed with 1391973-pET-23a(+), 1389993-pET-23a(+), and 1334427-pET-23a(+), respectively. Cells were cultured in 900 mL of Luria-Bertani medium supplemented with ampicillin (50 μ g/mL). When OD₆₀₀ of the culture reached around 1.0, protein expression was induced with

0.4 mM isopropyl β -D-1-thiogalactopyranoside for 18 h at 20 °C with shaking at 250 rpm. The *E. coli* cells thus cultivated were pelleted by centrifugation at 6800 $\times$ g for 3 min at 4 °C. The pellet was suspended in 30 mL of lysis buffer [50 mM Tris-HCl (pH 7.5) containing 1 mM phenylmethylsulfonyl chloride]. The cell suspension was sonicated at amplitude 80% for 5 s (twice) using a Sonicator S-4000 (QSonica, Newtown, CT, USA), and centrifuged at 10,000 $\times$ g at 4 °C for 20 min. The supernatant was kept on ice overnight. Meanwhile, the sonication debris pellet was resuspended in 4 mL of lysis buffer with 1% Triton X-100 (v/v), then the suspension was further homogenized by five strokes of a Potter-Elvehjem homogenizer. The suspension was stirred on ice for 1 h and centrifuged at 10,000 $\times$ g at 4 °C for 10 min. The pellet was resuspended in lysis buffer without Triton X-100 and the suspension was centrifuged at 10,000 $\times$ g at 4 °C for 10 min. The pellet was resuspended in lysis buffer without Triton X-100 and the suspension was stirred on ice for 3 h, then further stirred at 7 °C for 15 h. The suspension was centrifuged at 17,900 $\times$ g for 60 min. The supernatant was mixed with the supernatant obtained from sonication and the mixed supernatant was loaded onto four His-Bind resin columns (1-mL bed volume each; Merck, Billerica, MA, USA) equilibrated with 1 $\times$ binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) according to the manufacturer's protocol. Each column was washed under gravity with 6 mL of 1 $\times$ binding buffer and 10 mL of wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 75 mM imidazole, pH 7.9), successively. The target protein was eluted with 4 mL of elution buffer (0.1 M NaCl, 4 mM Tris-HCl, 200 mM imidazole, pH 7.9) with pressure. The eluate was concentrated and washed five times with 50 mM Tris-HCl (pH 7.5) using an Amicon Ultra centrifugal filter (0.5 mL, 10 kDa) at 14,000 $\times$ g at 4 °C for 6 min each time. The purified recombinant proteins 1391973, 1389993, and 1330427 were used for assay immediately.

Standard enzyme assay for recombinant 1391973, 1389993, and 1330427

Enzyme reaction mixture (400 μ L), containing 50 mM Tris-HCl (pH 8.0), 2 mM cinnamic acid, 0.25 mM SAM, and purified enzyme solution, was incubated at 37 °C for 3 h ($n=3$, biological triplicates). After the incubation, 12.5 ng of (*E*)-[2, 3, 4, 5, 6-²H₅]methyl cinnamate [6, 19] was added to each reaction mixture as an internal standard, then the reaction product was extracted with 800 μ L of ethyl acetate by mixing vigorously with a vortex mixer for 1 min. The mixture was centrifuged at 17,900 $\times$ g for 3 min at room temperature and the supernatant (750 μ L) was transferred to a fresh 1.5-mL microcentrifuge tube. To remove water from the ethyl acetate layer, saturated

aqueous NaCl solution (100 μ L) was added, and then the solution was vortexed in the same way. After centrifugation at 17,900 $\times$ g for 3 min, the ethyl acetate layer (600 μ L) was transferred to a fresh 1.5-mL microcentrifuge tube, then dried by using a SpeedVac (Thermo Fisher Scientific) for 13 min at 35 °C. The dried sample was subjected to liquid-chromatography–tandem mass spectrometry (LC–MS/MS) analysis for quantitative analysis of (*E*)-MC.

Characterization of 1330427

To elucidate the optimum pH for the enzymatic reaction of 1330427 the standard enzyme assay was performed in the presence of 50 mM potassium phosphate (pH 6.0, 7.0 or 8.0), or Tris–HCl (pH 7.0, 8.0 or 9.0). To elucidate the optimum reaction temperature, the standard enzyme assay was performed at 7, 17, 27, 37, 47, and 57 °C. To determine the K_m value for cinnamic acid, the standard enzyme assay was performed in the presence of 0.25, 0.5, 0.67, 1.0, 2.0, and 4.0 mM cinnamic acid. To elucidate any effect of metals on the enzymatic reaction, the reaction was performed in the presence or absence of 5 mM Mn^{2+} , Mg^{2+} , or Zn^{2+} .

Quantification of (*E*)-MC by LC–MS/MS

The LC–MS/MS system consisted of a Shimadzu Nexera X2 HPLC series liquid chromatograph and a Shimadzu LCMS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Shimadzu LabSolutions LCMS software was used for data acquisition and processing. LC separation was achieved using an XBridge C18 column (150 $\times$ 2.1 mm, 3.5 μ m; Waters, Milford, MA, USA) maintained at 40 °C. The mobile phase was delivered at a flow-rate of 0.19 mL/min and consisted of 0.1% formic acid (w/w) in methanol or H_2O . The methanol concentration (%) applied to the column was: 0–16.5 min, 50%; 16.5–17 min, linear gradient to 100%; 17–25 min, 100%; 25–30 min, 50%. Atmospheric pressure chemical ionization was performed with parameters: desolvation line temperature, 250 °C; block temperature, 400 °C; and interface voltage, 3.5 kV. Nitrogen served as the nebulizer gas and drying gas, at flow-rates of 2 and 15 L/min, respectively, and argon was the collision gas, at 230 kPa.

The chemical structures of the reaction products were confirmed by retention time with authentic non-labeled (*E*)-MC as a standard, and by multiple reaction monitoring (MRM) transitions (Table 1). For quantitative analysis, (*E*)-[2,3,4,5,6- 2H_5]methyl cinnamate was used as an internal standard to prepare a calibration curve to determine amounts of enzymatic products based on ratios of ions in MRM (Table 1).

Table 1 MRM transitions for compounds in LC–MS/MS analysis

Compounds	MRM transitions ^a
(<i>E</i>)-Methyl cinnamate	163.15 $\rightarrow$ 103.15, 163.15 $\rightarrow$ 77.15
(<i>E</i>)-Methyl <i>p</i> -coumarate	179.10 $\rightarrow$ 147.10, 179.10 $\rightarrow$ 91.15
(<i>E</i>)-Methyl ferulate	209.05 $\rightarrow$ 177.05, 209.05 $\rightarrow$ 117.00
(<i>E</i>)-Methyl sinapate	239.05 $\rightarrow$ 207.05, 239.05 $\rightarrow$ 175.00
(<i>E</i>)-[2,3,4,5,6- 2H_5]methyl cinnamate	168.10 $\rightarrow$ 135.10

^a The first MRM transitions for each of 5 compounds were used for preparation of calibration curve

Modeling of protein structures

Protein structure modeling of TmCAMT1, ObCCMT1, CsCAMT, and MnMT2 was performed using AlphaFold2 [20]. Molecular graphics figs. were created using PyMOL (<http://www.pymol.org/>).

Statistical analyses

Analyses were conducted by analysis of variance followed by the Tukey test, using SPSS Statistics software v27.

Results and discussion

Selection of cDNAs encoding candidate proteins for SAM-dependent cinnamic acid carboxyl methyltransferase activity

tBLASTn analysis with the full amino acid sequences of ObCCMT1 and CsCAMT as queries against the RNA-Seq database prepared for *T. matsutake* gave nonsignificant lowest e-values for homologous proteins, i.e., 6.4 and 1.9 for ObCCMT1 and CsCAMT, respectively, and thus no candidate proteins could be selected by this method. A keyword search using “Methyltransf_7” as the query for proteins in the genome of *T. matsutake* 945 via the JGI MycoCosm web portal gave no hits. Therefore, we evaluated homology between ObCCMT1 and putative SAM-dependent carboxyl methyltransferases predicted in RNA-Seq data for *T. matsutake* by focusing on just four motifs found in SABATH family proteins [15], including ObCCMT1 and CsCAMT.

In this evaluation, the expedient value for UHOR_01838, considered the threshold criterion, was calculated to be 29 (please see the Materials and methods section for details of determination of expedient values). We identified three candidate proteins in *T. matsutake* with expedient value $\geq$ 29; the corresponding cDNAs were named 1391973, 1389993, and 1330427, respectively. For 1391973 (expedient value 31), 1389993 (expedient value 30), and 1330427

(expedient value 29), we cloned 981-, 1332-, and 822-bp cDNAs (excluding database-predicted stop codons), encoding 327, 444, and 274 amino acids, respectively.

SAM-dependent cinnamic acid carboxyl methyltransferase activity of candidate proteins 1391973, 1389993, and 1330427

In analysis by LC–MS/MS, the product of enzymatic reaction of recombinant purified protein 1330427 gave peaks 163.15 > 103.15 (+) and 163.15 > 77.15 (+) at retention time 15.505 min (precursor ion > product ion in MRM analysis) (Table 1, Fig. 1A and B). The ratio of 163.15 > 103.15 (+) to 163.15 > 77.15 (+) was 1.360 ± 0.036 . Authentic (*E*)-MC showed the same ion peaks at the same retention time (15.508 min) with the same ratio (1.393 ± 0.008 ; $n=3$ technical triplicates) (Fig. 1C and D). No peak was observed for the negative control using heat-denatured enzyme (Fig. 1E and F). Therefore, the results indicate that enzyme 1330427 possessed carboxyl methyltransferase activity toward (*E*)-cinnamic acid to produce (*E*)-MC. On this basis, 1330427 was renamed *Tricholoma matsutake* cinnamic acid carboxyl methyltransferase 1 (TmCMT1; cDNA *TmCMT1*, GenBank accession number LC802717). In addition, cDNA *TmCMT1* was obtained from the mycelia without adding L-phenylalanine.

Surprisingly, even in the negative control experiment with heat-denatured TmCMT1, in the presence of 2 mM *p*-coumaric acid, ferulic acid, and sinapic acid, respectively, methyl *p*-coumarate, methyl ferulate, and methyl sinapate were produced nonenzymatically, with 87%, 71%, and 50% yields compared with the corresponding enzymatic reactions catalyzed by TmCMT1. Thus, we did not investigate in detail if TmCMT1 possessed carboxyl methyltransferase activity toward *p*-coumaric, ferulic and sinapic acids.

Carboxyl methyltransferase activity toward cinnamic acid was not observed for recombinant 1391973 or 1389993 (data not shown).

Characterization of TmCMT1

Maximal methyltransferase activity of TmCMT1 toward cinnamic acid to produce (*E*)-MC was observed at 37 °C (Fig. 2A), and at pH 7.0 and 8.0 regardless of the kind of buffers tested (Fig. 2B). Therefore, kinetic parameters were determined based on the enzymatic reaction conducted at pH 8.0. ObCCMT1 shows its greatest activity at pH 7.5 [9], while CsCMT shows no activity at pH 8.0 and maximal activity at pH 6.5 [10].

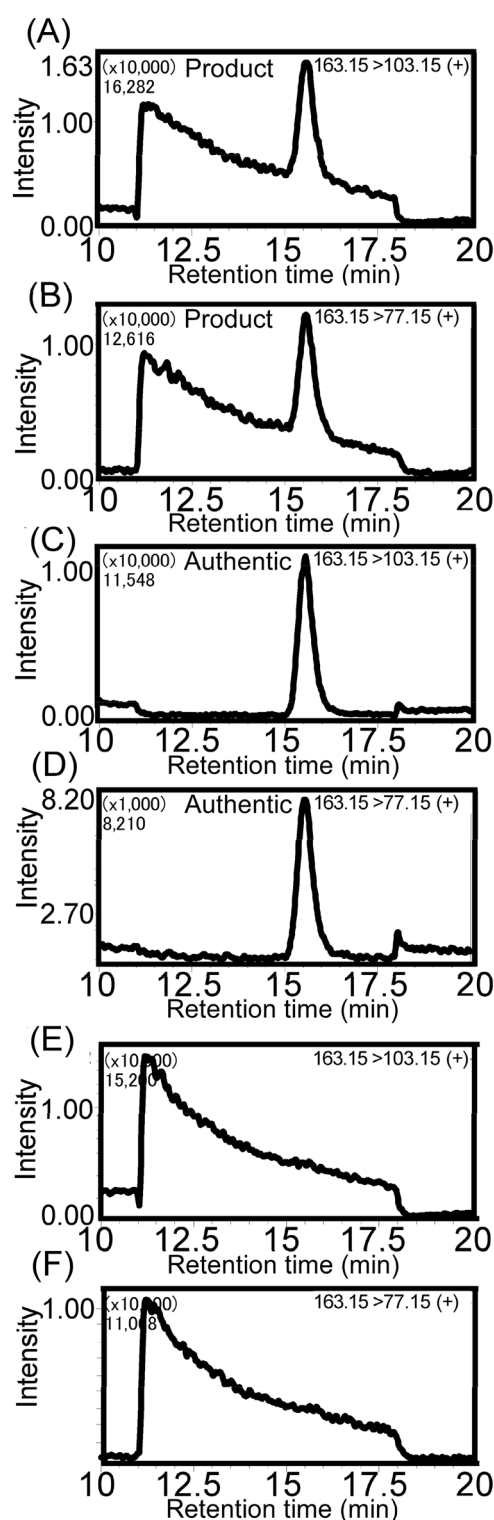


Fig. 1 Formation of (*E*)-MC in the enzymatic reaction. LC–MS/MS chromatograms for ions for product: **A** 163.15 > 103.15, **B** 163.15 > 77.15; for authentic (*E*)-MC, **C** 163.15 > 103.15, **D** 163.15 > 77.15, for isolates in negative control, **E** 163.15 > 103.15, **F** 163.15 > 77.15

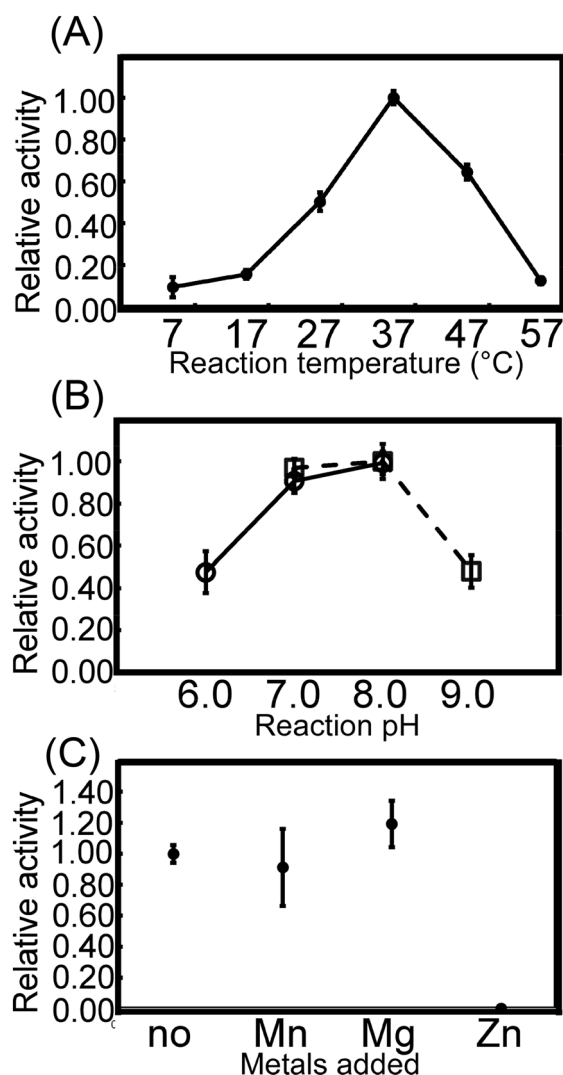


Fig. 2 Characterization of TmCAMT1 with (*E*)-cinnamic acid as a substrate. **A** Temperature optimum. Level for TmCAMT1 activity at 37 °C was arbitrarily set at 1.0. **B** pH optimum. Level for TmCAMT1 activity in the Tris-HCl buffer at pH8.0 was arbitrarily set at 1.0. The solid lines between open circles and the dashed lines between open squares show the relative activities in potassium phosphate and Tris-HCl buffers, respectively. The amounts of (*E*)-MC enzymatically produced at pH 7.0 and pH 8.0 regardless of the kind of buffers tested were statistically same ($n=3$, $p<0.05$). **C** Effects of metal ions on the enzymatic reaction. Level for TmCAMT1 activity without addition of metal ions in the buffer at pH8.0 was arbitrarily set at 1.0. The amounts of (*E*)-MC enzymatically produced without addition of metals, and with Mn^{2+} or Mg^{2+} were statically same ($n=3$, $p<0.05$).

No statistically significant differences in the enzymatic activity of TmCAMT1 were observed in the absence or presence of Mn^{2+} or Mg^{2+} in the reaction mixture (Fig. 2C). However, no (*E*)-MC was detected in

the reaction mixture with added Zn^{2+} (5 mM). In contrast to the observations for TmCAMT1, Mn^{2+} (5 mM) inhibited the methyltransferase activity toward cinnamic acid of CsCAMT [10] and ObCCMT1 [9] by 50% and 35%, respectively. Similar to TmCAMT1, Mg^{2+} (5 mM) slightly increased the methyltransferase activity toward cinnamic acid of CsCAMT [10], but Mg^{2+} (5 mM) inhibited the methyltransferase activity toward cinnamic acid of ObCCMT1 by 35% [9]. Similar to the observation for TmCAMT1, Zn^{2+} (5 mM) completely inhibited the methyltransferase activity toward cinnamic acid of CsCAMT [10] and ObCCMT1 [9]. To our knowledge, no SABATH family enzyme, including ObCCMT1 and CsCAMT, requires a metal cofactor. The mechanisms of inhibition and stimulation by metals remain to be investigated in the SABATH family enzymes and TmCAMT1.

On the basis of a Lineweaver-Burk plot, the K_m for cinnamic acid for TmCAMT1 was determined to be 0.65 ± 0.04 mM, compared with 50.5 μ M for CsCAMT [10] and 124 μ M for ObCCMT1 [9]. The Lineweaver-Burk plot for two times of triplicate reactions at pH 8.0 and 37°C gave similar results (data not shown). The k_{cat} value was determined to be 0.003 h^{-1} .

Amino acid sequences of TmCAMT1

Sequence alignment (Fig. 3A) shows that the identities between TmCAMT1 and ObCCMT1 and CsCAMT are low, just 17% and 19%, respectively. Thus, our cloning strategy focusing only on specific sequence motifs successfully identified TmCAMT1, which has similar activity to ObCCMT1 and CsCAMT but a very different primary structure.

Amino acid residues 43–149 in TmCAMT1 were classified into Methyltransf_31 (PF13847) with e-value $6.50E-16$ by Pfam analysis, whereas amino acid residues 49–372 and 86–412 in ObCCMT1 and CsCAMT were classified as Methyltransf_7 family proteins with e-values $1.50E-97$ and $1.50E-64$, respectively. As expected, in TmCAMT1, the SAM-binding domain, amino acid residues 48–55 (IDFGCGSG), corresponding to motif A ([IV]AD[LF]GCSSG [21] and also to motif 3 in SABATH family proteins [15], was conserved with significant identity (Fig. 3A). Similarly, amino acid residues 123–127 ALHWC, corresponding to a part of motif 1 in SABATH family proteins ([SA]LHWL [15] were significantly conserved (Fig. 3A). Surprisingly, however, motif 5 (NDFN) and motif 1 (GSFY) in SABATH family proteins [15], both of which were used in screening for TmCAMT1, were not found as motifs in TmCAMT1 when TmCAMT1 was aligned with ObCCMT1 and CsCAMT (Fig. 3A). Interestingly, in BLASTp search with TmCAMT1 as the query against nonredundant proteins with expected threshold 0.05, the top 100 homologous

A

ObCCMT1	-----MARKEN-----YVVSNNMVESVLCMKGG	Motif 6	23
CsCAMT	MDMTGSSPCKFLNEHTQSMNVFKDLSSITTCMVPKQVDVEHEEPGSANSP LPTMGGG		60
TmCAMT1	-----MSPSTS-----NSFDGSG		13
	* .		*
ObCCMT1	KGEDSNNNSKMQEQHARSVLHLLMEALDGVGLSSVAAGAFVVDLGCSSGRNAINTMEF	Motif 6	83
CsCAMT	LGKNSVVRNSNGQADFMERMFPTVLKPVDEMILEKDTDVVRVVDVFGCSSGPNTIRNVET	Motif 3	120
TmCAMT1	WSASLQNKH-----ASFVYSPAF--TSAVLELLAAQPGERIIDFGCGSGEVTLEIKKR		64
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ObCCMT1	MINHLTEHYTVAAEEPEFSAFFCDLPSNDFNTLFQLLPSPDGSS-----GSYFTAGV	Motif 3	136
CsCAMT	ILRRMKRN--LSDGECPEFQAFQDQHEHETDFNTLFRFFKTVSANSNGSDDLKYFAVGA	Motif 5	178
TmCAMT1	VE-----EAPGGVVVAVDESESM-----IAQARANG---LEYAFVSD		98
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ObCCMT1	AG--SYRRLF-PAKSVDFFYSAFSLTMTLSQIPKEVMEKGSAAAYNEGRVTINGAKESTVN	Motif 1	193
CsCAMT	PG--SYGRIF-PKSSVHFAMSTFAITMTLSRIPPVIYQNDQEAAYFGAHNELVFASKATIA		235
TmCAMT1	IQALKLPNDVSPLAEKFDVFTNAAILMTCKANPTGVLESVKKVLKKGGR-FTGETGGFMN		157
	. : : : * * . * : : . : . .		
ObCCMT1	AYKKQFQSDLGVLRSRSELKP-----GGSMFLMLLGRTPSDPA	Motif 2	233
CsCAMT	AYAEQANIDLRLNFDARATEMVP-----GGVMTLVFGVRKTYYPY		275
TmCAMT1	CIG--LRSAIYQVLKSRGHDVPLHDFMYFSPDDYRKLVAASFDPVHLSLNPFRFTPLPG		215
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ObCCMT1	DQGAQILTFSTRYQDAWNLDVQEGLISSSEKRDTFNIHMYTPSLEEFKEVVERDGAFTINK	Motif 4	293
CsCAMT	SIGPT---DMTGEKVWDEMISEGIVDAKLKESFKSYMYFRYLKDVVEEVLDSSFSSMFKTE		331
TmCAMT1	GLRGWLQTRH--TVF-----		230
	. : .		
ObCCMT1	LQLFHGGSALIIDDPNDAVEISRAYSLSLCSLTGGLVDAHIGDQLGHELFSRLLSQAVDQ	Motif 7	353
CsCAMT	QR--HVSPPFTYPPGLTAREQGARFVSLQRCINSTVFEAHFGKEATNIFWERYEELVRG		389
TmCAMT1	-----ADFSDEETEEMMEEVEKICRV		252
	* : . : . .		
ObCCMT1	AK-----ELMDQFQVH-IVASLTLA-		373
CsCAMT	FSDGTITDDEENYQLILVVVATRR---		413
TmCAMT1	-----CQDENGWAIMVRLRFSAILV		274
	: : : : :		

B

MnMT2	MSS---LRSHDDTWDIDTSVGTAVMVAAARAAETAADPLISDPYARILVDGAGGGPWS	57
TmCAMT1	MSPSTSNFSGSGWSASLQNKHASFV--YSPAFTSAVLELLAAQPGERIIDFGCGSGEVT	58
	** . . . * . : : : : * : * : * : * : * : * : * : * : *	
MnMT2	MMLDEDFAARAEI-----DPEAAVLFAHMGNYQAVR-----TRFFDDY	96
TmCAMT1	LEIKKRVEEAPGGVVVAVDESESMIAQARANGLEYAFVSDIQALKLPNDVSPLAEKFDV	118
	: : : . : : : * : * : * : * : * : * : * : * : *	
MnMT2	FAEAGLRQVVILASGLDSRAFRLSWPAGTTVFEIDQPKVLEYKRVLAEHGVQPT-ATRV	155
TmCAMT1	FTNAA-----ITCKAN-----PTGVLESVKKVLKKGGRFTGETGGF	155
	* : * . * * . . * * : * * : * : * : *	
MnMT2	EVAIDLQDWPAAALRSAGFDDTVPTAWLAEGLLMYLPGDAQDRLEFQITALSAPGSRIAA	215
TmCAMT1	MNCIGLRSIYQVLKSRGHDVPLHDFMYFSPDDY-----RKL-----	194
	. * * . . * * * . : * . * : . * : *	
MnMT2	ETAPMQAEERRAQMKERFDRIKEFGLSDSIDVGELMYNDPDRADLAPWLAGHWDSAAV	275
TmCAMT1	-----VAASFDVPVHLSL-----NPRFTPLPGLRGWLQTRHTVFA	231
	: * * : : : * . . * *	
MnMT2	KSAD-----EMRR-----LNRWALPEGP---DIPEDDA	300
TmCAMT1	DFSDEETEEMMEEVEKICRVDCQDENGWAIMVRLRFSAILV---	274
	. : * * : : . . * : *	

Fig. 3 A and B Alignment of TmCAMT1 with ObCCMT1 from plant *Ocimum basilicum* and CsCAMT from liverwort *Conocephalum salebrosum* (A), and with MnMT2 (B). Genbank accession numbers for TmCAMT1, ObCCMT1 and CsCAMT are LC802717, EU033968 and MK673137, respectively. See supporting information for [16] for amino acid sequence of MnMT2. Squares indicate amino acid residues in the predicted active sites shown in Fig. 5A

proteins were all from basidiomycetous fungi, suggesting that homologs of TmCAMT1 have developed specifically in basidiomycetes.

Sequence alignment (Fig. 3B) shows that the identity between TmCAMT1 and MnMT2 is slightly greater, 21%, but TmCAMT1 was found to be more similar in overall structure to ObCCMT1 and CsCAMT than to MnMT2 (see below).

Modeling of TmCAMT1 structure

A structural model for TmCAMT1 was predicted by using AlphaFold2 (Fig. 4A). We found that the model for TmCAMT1 was similar to the AlphaFold2 predictions for ObCCMT1 and CsCAMT in overall structure (Fig. 4B and C). Next, we noted that other structural models for ObCCMT1 and CsCAMT have been prepared, using Modeller version 8.2 [9] and Molecular Operating Environment version 2013.08 (2015) [10], respectively, based on the crystal structure of the SAM-dependent salicylic acid carboxyl methyltransferase from *Clarkia breweri* (CbSAMT) in complex with *S*-adenosyl-L-homocysteine and salicylic acid (PDB ID: 1M6E) [22]. We found that the model for TmCAMT1 was also similar in overall structure to the experimentally determined crystal structure of CbSAMT (Fig. 4D).

During the course of this study, MnMT2, a SAM-dependent cinnamic acid carboxyl methyltransferase from *M. neoaurum* has been reported and its structure has been modeled based on that of *Mycobacterium leprae* ML2640c (PDB ID: 2UYQ) [16, 23]. The Pfam families of TmCAMT1, CbSAMT, and MnMT2 are different: Methyltransf_31, Methyltransf_7, and leucine carboxyl methyltransferase fold (IPR007213), respectively. In addition, we found that the crystal structure of CbSAMT and the AlphaFold2-predicted overall structure of TmCAMT1 had only limited overall similarity with the model of the structure of MnMT2 based on ML2640c. The carboxyl-terminal moieties were not similar. Structural similarity was partly found within residues for CbSAMT 1–154, 181–214, and 256–271, for TmCAMT1 1–156 and 181–198, and for MnMT2 1–21, 87–219, and 257–268 (Fig. 4E and F). Because of the notable predicted structural differences, we do not discuss possible common amino acid residues in the active sites of TmCAMT1 and MnMT2 in this paper.

However, the similarities in the AlphaFold2-predicted structural models for TmCAMT1, ObCCMT1, CsCAMT and the crystal structure of CbSAMT (Fig. 4), and the conservation of amino acid residues in the substrate-binding pockets of ObCCMT1, CsCAMT, and CbSAMT [9, 10], led us to predict the substrate-binding pocket of TmCAMT1 by superimposing these AlphaFold2-predicted structures with the crystal structure of CbSAMT

in complex with *S*-adenosyl-L-homocysteine (which is produced from SAM as a coproduct by CbSAMT) and salicylic acid. Structural comparison revealed that the amino acid residues around the *S*-adenosyl-L-homocysteine moiety in CbSAMT (Tyr18, Asp98, Leu99, and Phe130) are conserved in ObCCMT1 (Fig. 5B), and in CsCAMT except for Phe130 (Fig. 5C). This is consistent with previous findings obtained by different analyses [9, 10]. However, the hydrophobic residues Leu99 and Phe130 in CbSAMT are, respectively, replaced by other hydrophobic residues, Phe78 and Ile99, in TmCAMT1 (Fig. 5A). Accordingly, although some amino acid residues in TmCAMT1 were replaced with residues with similar properties, the amino acid residues surrounding the *S*-adenosyl-L-homocysteine of CbSAMT were similar overall in TmCAMT1, ObCCMT1 and CsCAMT.

As shown in Fig. 5A–C, each amino acid residue around the potential substrate salicylic acid-binding site was located at a similar position in TmCAMT1, ObCCMT1, CsCAMT and CbSAMT. This finding also suggests that the amino acid residues in the predicted binding pockets for the methyl group acceptor in TmCAMT1, ObCCMT1, and CsCAMT may be located at similar positions to those in CbSAMT even when these enzymes accept other substrates, including cinnamic acid.

Possible roles of amino acids in the predicted active site of TmCAMT1 will be reported elsewhere based on site-directed mutagenesis results of the amino acids shown in Fig. 5A. In particular, we describe the possible roles of His125 and Trp126 in the enzymatic reaction based on the following previous findings: (1) SABATH family proteins with high substrate specificity for salicylic acid frequently possess Met at the corresponding position (e.g., Met150 in CbSAMT), while SABATH proteins that preferentially accept other substrates (e.g., cinnamic acid), including ObCCMT1 and CsCAMT, possess His at the corresponding position (His160 and His 202, respectively) [22, 24]. (2) In CsCAMT, amino acid residue Trp203, which corresponds to Trp126 in TmCAMT1, was reported to interact with the carboxyl moiety of cinnamic acid [10] and it is highly conserved in not only motif 1 in members of the Methyltransf_7 family [15] but also in one of the characterized proteins classified as belonging to Methyltransf_31, a *trans*-aconitate methyltransferase (TMT1) from *Saccharomyces cerevisiae* contains amino acids His134 and Trp135 [25], which correspond to residues His125 and Trp126 in TmCAMT1 in alignment (data not shown).

TmCAMT1 is the first protein characterized at the molecular level with cinnamic acid carboxyl methyltransferase activity belonging to neither Methyltransf_7 nor the SABATH family; however, it possesses several amino acids in a few motifs conserved in SABATH

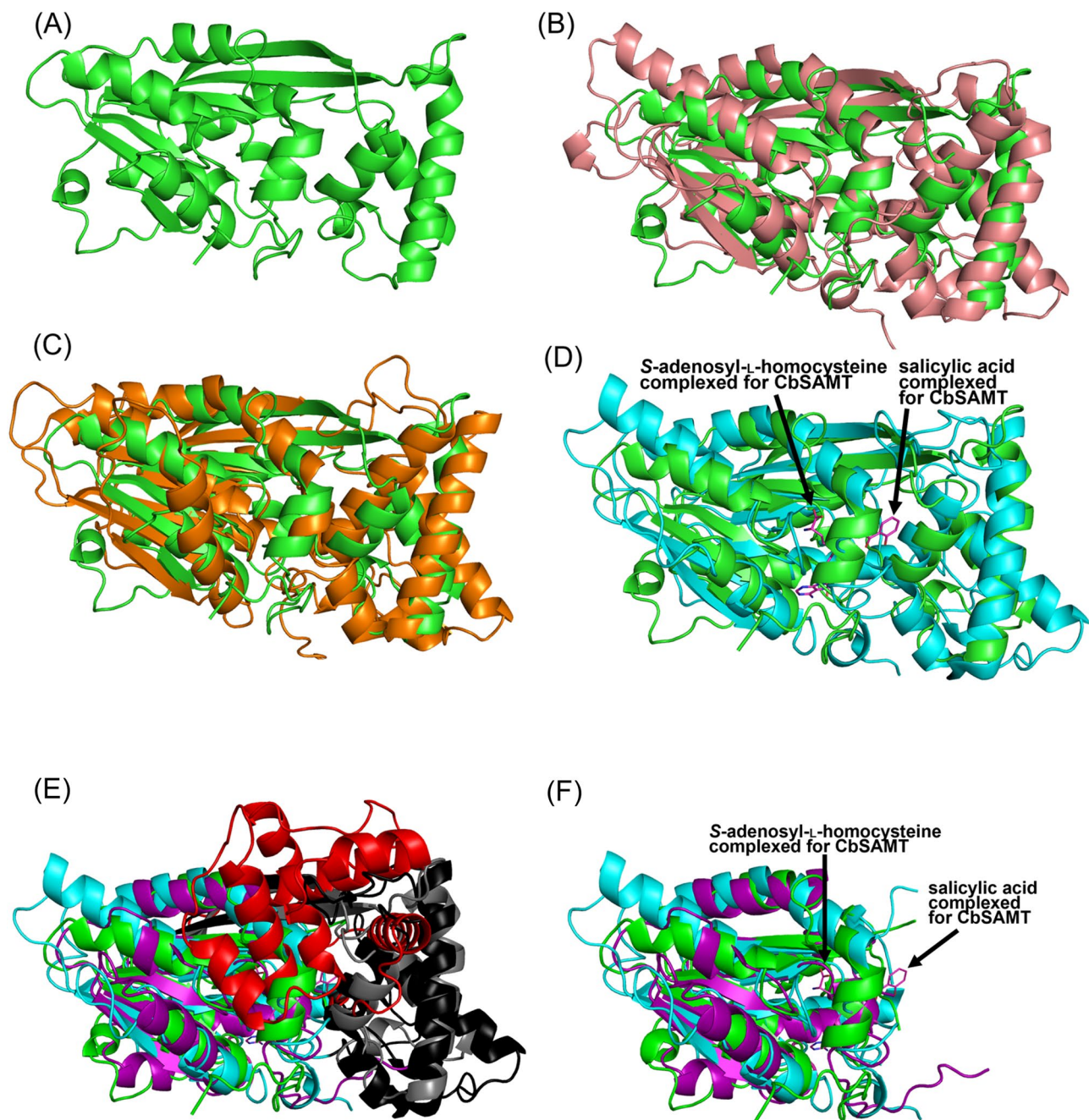


Fig. 4 The predicted structures of TmCAMT1 and its superposition with ObCCMT1, CsCAMT and CbSAMT and MnMT2. **A** The predicted structure of TmCAMT1. **B** Superposition of the predicted structures of TmCAMT1 (green) and ObCCMT1 (pink). **C** Superposition of the predicted structures of TmCAMT1 (green) and CsCAMT (orange). **D** Superposition of the predicted structures of TmCAMT1 (green) and crystal structure of CbSAMT (PDB entry 1m6e; cyan). S-Adenosyl-L-homocysteine and salicylate molecule in CbSAMT is shown in magenta. **E** Superposition of the predicted structures of TmCAMT1 (green) and MnMT2 (violet) and crystal structure of CbSAMT (PDB entry 1m6e; cyan). No similar overall structural moieties are shown in red and black. **F** An only similar overall structural moieties of TmCAMT1, CbSAMT and MnMT2 excerpted from E are shown

family proteins and is similar in overall structure and amino acid residues in the active site to SABATH family enzymes. We selected cDNA *TmCAMT1* based on the homology of motifs between plant carboxyl

methyltransferases and putative SAM-dependent methyltransferases predicted from RNA-Seq data for *T. matsutake*. This approach was taken because finding critical information from RNA-seq data for selecting

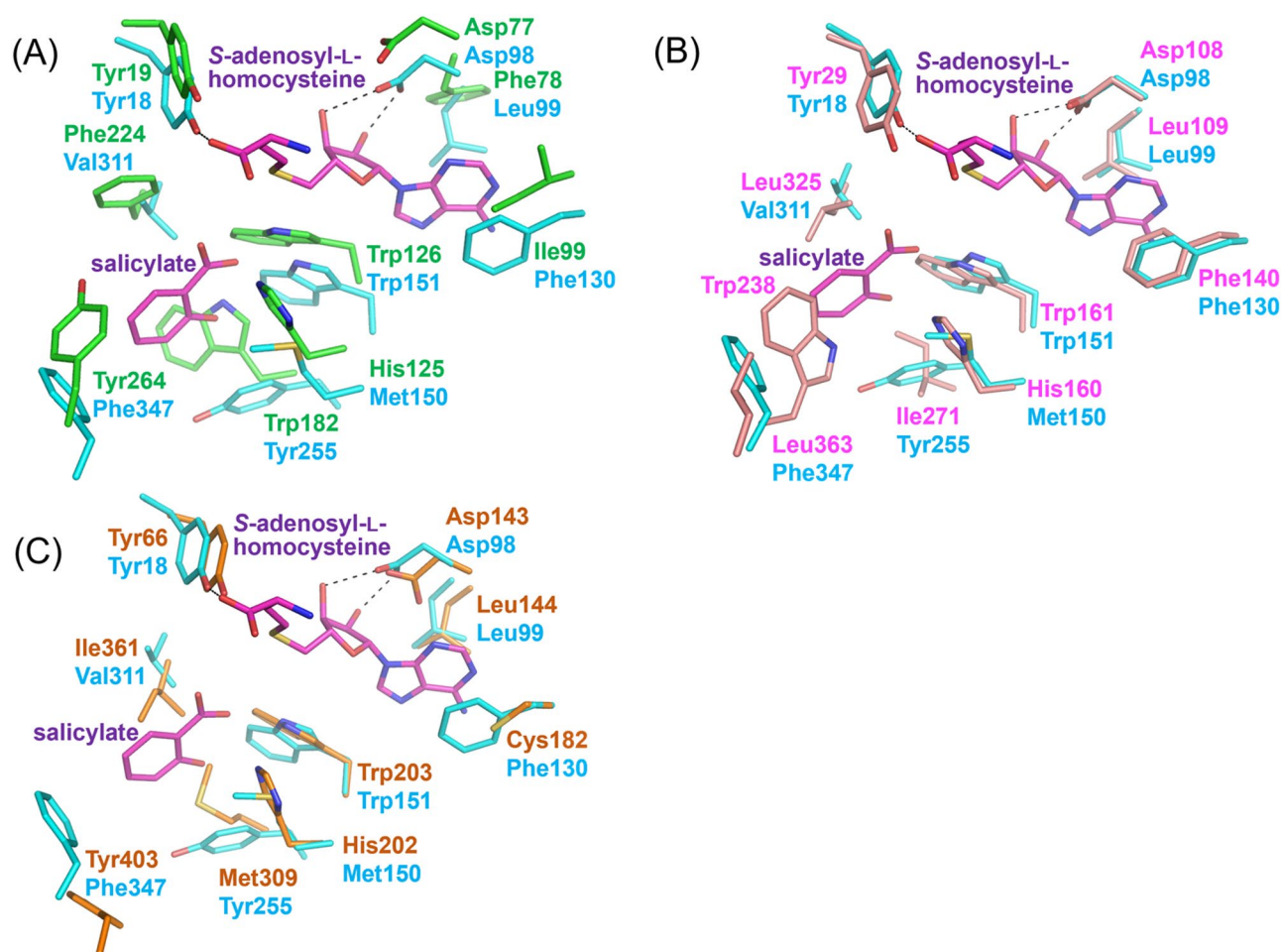


Fig. 5 Substrate-binding site structure in *C. breyeri* SAMT (CbSAMT, PDB entry 1m6e) and the predicted structures. **A** CbSAMT (residues and labels are shown in cyan) and TmCAMT1 (residues and labels are shown in green). **B** Comparison of that in CbSAMT (residues and labels are shown in cyan) and that of ObCCMT1 (residues and labels are shown in pink). **C** CbSAMT (residues and labels are shown in cyan) and CsCAMT (residues and labels are shown in orange). S-Adenosyl-L-homocysteine and salicylate molecule in CbSAMT is shown in magenta. The hydrogen bonds between S-adenosyl-L-homocysteine and CbSAMT are shown as dashed black lines. In the substrate-binding pocket for the AlphaFold2-predicted structure model for ObCCMT1, Trp238 was found to be positioned close to Leu363 (Fig. 5B). However, the possible role for Trp238 in ObCCMT1 was not discussed [9], probably because Kapteyn et al. predicted the structure model for ObCCMT1 in a different way from ours [9]

candidate carboxyl methyltransferases was unsuccessful. However, we found that the k_{cat} value for recombinant TmCAMT1 was low. This low k_{cat} may arise from TmCAMT1 accepting other substrates as the primary substrate rather than cinnamic acid. In this context, in future work, it will be important to characterize the substrate specificity of TmCAMT1 and its homologs in basidiomycetes because SABATH family proteins accept substrates, including phytohormones such as indole-3-acetic acid [26], gibberellins [27], and jasmonic acid [28], and others, including *p*-methoxybenzoic acid [29], farnesic acid [30], anthranilic acid [31], and nicotinic acid [32]. Any function of TmCAMT1 in

the production of (*E*)-MC during fruiting body formation of *T. matsutake* remains to be confirmed.

Conclusions

Our cloning strategy focusing on four conserved motifs in SABATH family proteins obtained cDNA *TmCAMT1* (*Tricholoma matsutake* Cinnamic Acid carboxyl MethylTransferase, 822 bp, GenBank accession number LC802717), encoding a SAM-dependent cinnamic acid carboxyl methyltransferase (274 amino acids) from the basidiomycete *T. matsutake*. The optimum pH and temperature for enzymatic production of (*E*)-MC from cinnamic acid were 7.0–8.0 and 37 °C. The K_m and k_{cat}

for cinnamic acid were 0.65 ± 0.04 mM and 0.003 h⁻¹. The identities between TmCMT1 and ObCCMT1 and CsCMT were just 17% and 19%, respectively. However, amino acid sequences IDFGCGSG and ALHWC, corresponding to motifs 3 and 1 in SABATH family proteins, were conserved in TmCMT1. Structural prediction using AlphaFold2 and comparison with the crystal structure of *C. breweri* SAM-dependent salicylic acid carboxyl methyltransferase (PDB ID: 1M6E) indicated that TmCMT1, ObCCMT1, CsCMT, and CbSMT are similar in overall structure and amino acid residues in the active site. However, TmCMT1 has limited overall structural similarity with MnMT2 from *M. neoaurum*. Although the findings indicate that TmCMT1 possesses a carboxyl methyltransferase activity for cinnamic acid, the low *k*_{cat} value suggests that any function of TmCMT1 in the production of (*E*)-MC during fruiting body formation of *T. matsutake* remains to be confirmed.

Abbreviations

SAM	S-Adenosyl-L-methionine
SABATH	Salicylic Acid, Benzoic Acid and Theobromine
TmCMT1	<i>Tricholoma matsutake</i> Cinnamic Acid carboxyl Methyltransferase
(<i>E</i>)-MC	(<i>E</i>)-Methyl cinnamate
LC-MS/MS	Liquid-chromatography–tandem mass spectrometry
PCR	Polymerase chain reaction
MRM	Multiple reaction monitoring

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Author contributions

Biochemical experiments concerning preparation of recombinant enzymes and their characterization were performed by TH, MK, HT, YO, KI, RK, Mariko Yoshizumi, MA and Masaomi Yamamura. Modeling of protein structures was performed by JH, TH, and Mariko Yoshizumi, and MA contributed to obtaining funding based on the ecological significance of *T. matsutake* in Tokushima Prefecture. The first draft of the manuscript was written by TH, HT, YO, KI, and JH, and it was revised by TH, Mariko Yoshizumi, Masanori Abe, RK, JH, and MY. The final manuscript was approved by all the authors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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