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Conversion of nicotinic acid to trigonelline is catalyzed by *N*-methyltransferase belonged to motif B′ methyltransferase family in *Coffea arabica*

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ABSTRACT

Trigonelline (*N*-methylnicotinate), a member of the pyridine alkaloids, accumulates in coffee beans along with caffeine. The biosynthetic pathway of trigonelline is not fully elucidated. While it is quite likely that the production of trigonelline from nicotinate is catalyzed by *N*-methyltransferase, as is caffeine synthase (CS), the enzyme(s) and gene(s) involved in *N*-methylation have not yet been characterized. It should be noted that, similar to caffeine, trigonelline accumulation is initiated during the development of coffee fruits. Interestingly, the expression profiles for two genes homologous to caffeine synthases were similar to the accumulation profile of trigonelline. We presumed that these two CS-homologous genes encoded trigonelline synthases. These genes were then expressed in *Escherichia coli*, and the resulting recombinant enzymes that were obtained were characterized. Consequently, using the *N*-methyltransferase assay with *S*-adenosyl[methyl-^{14}C]methionine, it was confirmed that these recombinant enzymes catalyzed the conversion of nicotinate to trigonelline, coffee trigonelline synthases (termed CTgS1 and CTgS2) were highly identical (over 95% identity) to each other. The sequence homology between the CTgSs and coffee CCS1 was 82%. The pH-dependent activity curve of CTgS1 and CTgS2 revealed optimum activity at pH 7.5. Nicotinate was the specific methyl acceptor for CTgSs, and no activity was detected with any other nicotinate derivatives, or with any of the typical substrates of B′-MTs. It was concluded that CTgSs have strict substrate specificity. The K_m values of CTgS1 and CTgS2 were 121 and 184 μM with nicotinic acid as a substrate, and 68 and 120 μM with *S*-adenosyl-L-methionine as a substrate, respectively.

1. Introduction

Trigonelline, *N*-methylnicotinic acid, was first isolated from the seeds of *Trigonella foenum-graceum* and has been found in many plant species [1,2]. It is known that coffee plants contain trigonelline and, in particular, similar to caffeine, there is a large amount of trigonelline (approximately 1–2% DW) in the coffee seed [3]. Following salt-stress, alfalfa plants have demonstrated a 5-fold increase in trigonelline, suggesting that trigonelline may play a role as an osmoregulator in salt-stressed plants [4]. Evans and Tramontano previously reported that trigonelline in pea cotyledons promoted G2 arrest in root and shoot meristems, with an effective concentration of trigonelline as low as 10^{-7} M [5]. Trigonelline may also act as a cell cycle regulator by preventing the ligation of replicons during the S-phase of the cell cycle [6]. Additionally, because it is reported that trigonelline contributes to the elongation of nerve fibers, it is expected that this reagent will be a useful compound in the treatment of Alzheimer's disease [7]. Therefore, there is now great interest in the use of this classic compound, trigonelline [8].

Trigonelline is the *N*-methyl conjugate of pyridine alkaloid, synthesized from nicotinic acid by *S*-adenosyl-L-methionine (SAM)-dependent nicotinic acid *N*-methyltransferase (trigonelline synthase, EC 2.1.1.7) (Fig. 1). The enzyme activity was first detected in crude extracts from peas [9] and has been partially purified from cell suspension cultures and leaves of *Glycine max* [10,11]. Ashihara and collaborators elucidated the biosynthesis of trigonelline and the pathways of pyridine salvage [12]. However, little is known about the molecular studied of *N*-methyltransferases involved in trigonelline biosynthesis has not been demonstrated yet. Several studies have been made on methyltransferases involved in the

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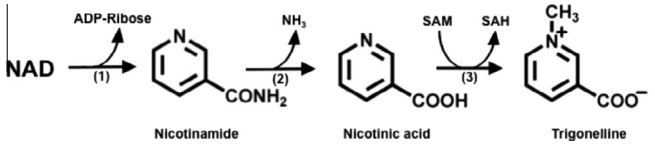

Fig. 1. Possible pathway for biosynthesis of trigonelline. (1) NAD nucleosidase (EC 3.2.2.5); (2) nicotinamidase (EC 3.5.1.19); nicotinate *N*-methyltransferase (trigonelline synthase) (EC 2.1.1.7). NAD, Nicotine adenine dinucleotide; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine.

methylation of low molecular weight compounds *in planta*. Joshi and Chiang [13] reported the characterization of plant *O*-methyltransferase family which catalyzed the methylation of caffeic acid and their derivatives. Then, salicylic methyltransferase from *Clarkia breweri* which was the first characterized member of motif B′ methyltransferase family and many motif B′ methyltransferases were subsequently identified [14–20]. Motif B′ methyltransferase family have the motif B′ and YFFF region in the amino acid sequence and were also known as SABATH family [21]. In the past fifteen years, majority of motif B′ methyltransferases catalyze the methylation of carboxylic acids, on the other hand, the occurrence of *N*-methyltransferase and *S*-methyltransferase were only demonstrated in purine alkaloid biosynthesis and sulfur-containing compounds, respectively [20,22]. The atoms of methyl acceptor were not seemed to be essential for the methylation reaction by motif B′ methyltransferases.

Here we show the molecular information about *N*-methyltransferase specific to trigonelline biosynthesis belonged to motif B′ methyltransferase family in *Coffea arabica*. The previously cloned CtCS3 and CtCS4 by our group were identified as trigonelline synthase 1 (CTgS1) and 2 (CTgS2), respectively, by the studies on the substrate specificity of the recombinant enzymes. This is the first report to describe the cloning of *N*-methyltransferase, the key enzyme for the biosynthesis of pyridine alkaloids from nicotinic acid metabolism, for trigonelline biosynthesis in plants.

2. Materials and methods

2.1. Materials

Oligonucleotide primers for PCR mutagenesis were purchased from Hokkaido System Science Co., Ltd. (http://www.hssnet.co.jp/). [Methyl-^{14}C]SAM (55 mCi/mmol) was purchased from GE Healthcare UK Ltd. Taq-polymerase and ExTaq were used for all PCR-based experiments (TaKaRa, Otsu, Japan). All other reagents were of the highest purity available.

2.2. Construction of expression plasmid

Plasmids for expressing CtCS3 and CtCS4 in *Escherichia coli* were constructed in the pET23d vector (Merck KGaA, Darmstadt, Germany) [23–25]. As the pET23d vector carries an optimal C-terminal His-Tag sequence, the 3′-termination sites of CtCS3- and CtCS4-cDNA were replaced by an *Xho*I restriction site using polymerase chain reaction (PCR)-directed mutagenesis. The expression plasmids for CtCS3 and CtCS4 carrying the C-terminal His-Tag (named pET23d-CtCS3 and pET23d-CtCS4, respectively) were then constructed.

2.3. Production of recombinant enzymes

The pET23d-CtCS3 and pET23d-CtCS4 expression plasmids were introduced into *E. coli* BL21 (DE3). A single colony of the transformants was cultured at 37 °C overnight in 3 ml of Luria broth containing 0.2 μg/ml ampicillin (LA) with vigorous shaking. A portion (2 ml) of the bacterial culture was added to 100 ml of fresh LA, and incubated at 37 °C for 2 h with constant shaking (100 rpm/min). To produce the recombinant protein, we added 300 μl of isopropyl-β-D-thio-galactopyranoside (final concentration is 0.3 μM), and maintained the cells at 25 °C for 8 h. *E. coli* cells were harvested by centrifugation at 500*g* for 5 min and then washed with 20 mM phosphate buffer pH 7.0 containing 0.1 M NaCl (PBS). Before the sonication, the cell paste was suspended in 1 ml of PBS and frozen at −80 °C. This cell suspension was sonicated and then centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was applied to a Ni–NTA column (1 ml) previously equilibrated with PBS containing 10 mM imidazole. After washing with PBS, proteins were eluted by PBS containing 200 mM imidazole. This purification procedure was carried out using ÄKTAprime (GE Healthcare). To remove imidazole, the eluted fractions were combined and subsequently applied to a NAP™-10 gel-filtration column (GE Healthcare) that was equilibrated with 20 mM Tris/HCl pH 7.5, containing 150 mM NaCl (TBS). The purified protein fractions were then used in the enzyme assay for trigonelline biosynthesis.

2.4. Determination of enzyme activity

The reaction mixture for standard assays contained 100 mM Tris/HCl pH 7.5, 0.2 mM $MgCl_2$, 0.2 mM SAM, 1.5 mM nicotinic acid, and 100 μl of enzyme preparation in a total volume of 300 μl. We analyzed reactants of trigonelline synthase by HPLC that was carried out using an L-2130 pump, an L-2200 autosampler, an L-2300 column oven, and an L-2450 diode array detector, all from Hitachi (Tokyo, Japan). Separation was carried out on an Inertsil ODS-3 column (ϕ 4.6 × 250 mm, 5 μm particle size) that was coupled to a cartridge guard column (Inertsil ODS-3, 4.0 × 10 mm, 5 μm particle size) from GL Science Inc. (Tokyo, Japan), with CH_3CN: CH_3COOH: H_2O = 1: 1: 98 as a solvent at a total flow rate of 0.5 ml/min. The column temperature was 40 °C and the injection volume was 20 μl for all analyses performed. Quantification was carried out using calibration curves obtained using standard solutions of pyridine derivatives, including trigonelline, at 264 nm. The fractions suspected to contain trigonelline were collected and concentrated, and the condensed product was then analyzed with ESI mass spectrometry (Exactive, Thermo Fisher Scientific, Waltham, MA, USA). Determination of trigonelline synthase activity was based on the transfer of a ^{14}C-labeled methyl group from [methyl-^{14}C]SAM to an unlabeled substrate, the methyl acceptor. To determine the kinetic parameters, the reaction conditions were as described above, except that 50 μM [methyl-^{14}C]SAM (1.8 kBq) was used and the total volume was 100 μl. The enzymatic activity of trigonelline synthase was determined by thin layer chromatography (TLC)-based densitometric analysis using ImageJ software (http://rsb.info.nih.gov/ij/). TLC was performed as previously described [26], except we used n-butanol/acetic acid/water (4:1:2, v/v) as the solvent. The K_m values were derived from Lineweaver–Burk plots analyzed with 'Enzyme Kinetics' software (Trinity Software, Campton, NH, USA).

2.5. Analysis of gene expression by semi-quantitative RT-PCR

Total cellular RNAs from *C. arabica* were extracted using a cetyltrimethylammonium bromide (CTAB) solution according to the method described by Chang et al. [27], with the exception of the use of 5% 2-mercaptoethanol. For semi-quantitative RT-PCR, total RNAs extracted from developing fruits, flower buds, and leaves of coffee trees at several stages were treated with RNase-free DNase I (TaKaRa). First strand cDNA was synthesized using a 3′-RACE core set and an oligo-dT 3 sites adaptor primer (oligo-dT 3SAP)

(TaKaRa). DNA-free total RNA (5 μg) from each tissue sample was used for first strand cDNA synthesis with a 20 μL reaction volume. The PCR reaction mixture (20 μl) contained 0.1 μl of first strand cDNA from the above reaction mixture, 2.5 units of *Ex Taq* (TaKaRa), 2 mM $MgCl_2$, 0.25 mM each of deoxyribonucleotide triphosphate and 5 μM of gene-specific primers. The gene-specific primers used were: 5′-CCCATTCCCCAGAATACA-3′ and 5′-GCTGCATTCGTCGCAAA-3′ for CTgS1, and 5′-ATTCCACAGGTTTGCGAC-3′ and 5′-ATCCTTTCCCCGTATCAG-3′ for CTgS2. A set of specific primers (5′-GCTTTCAACACCTTCTTCAG-3′ and 5′-GCTGCTCAGGGTGGAAGAG-3′) for α-tubulin (accession number AF363630) was used for the control reaction. For PCR, we used a PTC-200 thermal cycler (Bio-Rad Laboratories, Inc., CA, USA) with the following program: 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 15 s. The amplification was done for 25 cycles. The amplicons corresponding to CTgS1, CTgS2, and tubulin were 139, 167, and 133 bp in length, respectively. The reaction products were visualized by UV light on 1.2% agarose gels stained with ethidium bromide. The intensity of fluorescence was quantitated with a Macintosh computer using ImageJ software (http://rsb.info.nih.gov/ij/).

2.6. Analytical procedure

Protein concentrations were measured using methods previously described by Bradford [28]. Nucleotide sequencing was carried out using an ABI PRISM® 3100 genetic analyzer (Life Technologies Corporation, CA, USA) and was conducted with assistance from the Life Research Support Center in Akita Prefectural University. Nucleotide and protein sequences were analyzed by computer using MacVector (MacVector, Inc., NC, USA).

3. Results

3.1. Comparison of coffee trigonelline synthases (CTgS1 and CTgS2) with other motif B′-methyltransferases

CTgS1 (Accession Number AB054842) and CTgS2 (Accession Number AB054843) consisted of 1373-bp and 1434-bp sequences, respectively, and encoded 386 amino acid residues. Their amino acid sequences were highly identical (over 95% identity) to each other. CTgS1 shared a high degree of sequence identity (82.3%, 80.8%, and 82.9%) with coffee caffeine synthase (CCS1), coffee theobromine synthase (CTS1), and coffee 7-methylxanthosine synthase (CmXRS1), respectively (Fig. 2A). CTgS2 had the same values of sequence identity for the specified caffeine synthetic enzymes. These values indicated that CTgS1 and CTgS2 are highly homologous to caffeine synthetic enzymes from coffee. On the other hand, CTgS1 and CTgS2 also shared approximately 40% identity with tea caffeine synthase, cacao theobromine synthase, *C. breweri* salicylate methyltransferase [14], and *Arabidopsis thaliana* jasmonate methyltransferase [17], and they have three conserved regions as SAM binding motifs. (Fig. 2A). The phylogenetic tree among the CCS family and those related enzymes, such as B′-MTs, is indicated in Fig. 2B. Thus, CTgS1 and CTgS2 are highly homologous with caffeine synthetic enzymes from coffee, and enzymes of the motif B′ methyltransferase family [22,23] (SABATH family).

3.2. Catalytic properties of CTgS1 and CTgS2

The molecular mass for both the recombinant CTgS1 and CTgS2 was 43.2 kDa. The recombinant enzymes were assayed with typical substrates for B′-MTs (salicylic acid, benzoic acid, jasmonic acid, indoleacetic acid and gibberellic acid), xanthine derivatives for substrates of caffeine synthases (xanthosine, xanthine, 7-methylxanthine, theobromine, theophylline and paraxanthine), pyridine derivatives (nicotinic acid, methylnicotinate, nicotinamide, N-methylnicotinamide, nicotinic acid hydrazide and 6-methylnicotinicacid), and three purine/pyrimidine derivatives (uracil, guanosine and adenine). The recombinant enzyme then only had methyltransferase activity that produced trigonelline from nicotinic acid used as a substrate. As shown in the HPLC profile in Fig. 3A, the product had the same retention time as trigonelline. The fractions were collected and concentrated, and then the condensed product was applied to mass spectrometer. Since the product had the same molecular weight and mass spectrum as trigonelline, it was determined that the product was trigonelline (Fig. 3B). It was concluded that, CTgS1 and CTgS2 are trigonelline synthases that can specifically catalyze *N*-methylation of nicotinic acid. The optimum temperature for both CTgS1 and CTgS2 was 25 °C. At this temperature, the optimum pH was 7.5. CTgS1 and CTgS2 displayed Michaelis–Menten kinetics toward nicotinic acid with K_m values of 121 and 184 μM, and V_{max} values of 5.20 and 23.4 nmol/min/mg of protein, respectively. The K_m values of CTgS1 and CTgS2, with SAM as a substrate were 68 and 120 μM, respectively. The specific activity of recombinant CTgS1 and CTgS2 were 0.43 and 1.3 nkat/mg proteins with nicotinic acid, respectively.

3.3. Gene expression of CTgS1 and CTgS2

As *CTgS1* and *CTgS2* are highly homologous with other coffee caffeine synthases (*CCS1*, *CTS1*, *CTS2* and *CmXRS1*) at the nucleotide level (over 80% identical), the amount of transcripts from these genes cannot be detected by Northern blot analysis. The expression of *CTgS1* and *CTgS2* was evaluated by semi-quantitative RT-PCR designed for specific amplification and detection of these transcripts (Fig. 4). Although the transcripts of *CTgS1* and *CTgS2* were detected in all organs (flower buds, closed leaves, young leaves, and mature leaves) (Fig. 4A), and in the developing stages of fruits (DAF 0 to DAF 30) used in these experiments, the gene expression in DAF 30 and the mature leaves were the strongest for those genes in the evaluated organs (Fig. 4B). The transcript accumulation of *CTgS1* was maximum in DAF 5 fruits, and then tended to decrease slowly. On the other hand, *CTgS1* in leaves was expressed in mature leaves rather than in young leaves. The signals for *CTgS2* transcripts were extremely weak in leaves.

4. Discussion

In this study, we successfully isolated and characterized the trigonelline synthase genes from coffee. The amino acid sequence for CTgS1 and CTgS2 are similar to the coffee caffeine synthases, with the sequence identity between CCS1 and CTgS1 being more than 82%. It was then assumed that the enzymes could accept xanthine derivatives as substrates, so we attempted to measure the enzymatic activity with those compounds. However, no enzymatic activity could be detected when the xanthine derivatives were used as substrates.

It was previously speculated that trigonelline is transported to seeds after it is produced in other tissues [29]. The levels of trigonelline synthases gene expression clearly increased in fruits from 1 to 2 weeks after flowering (Fig. 4B). In the process of coffee bean maturation, the pulp and peel develop first, and at this time, the endosperm and embryo that form the bean are not yet sufficiently developed. This observation suggested that the trigonelline was transported into the developing endosperm after being produced in the pulp. However, to prove that trigonelline was transported, the trigonelline synthase expression levels in the seed, fruit pulp, and peel 30 days after flowering needed to be examined.

Previous studies have reported trigonelline biosynthesis based on biochemical analysis and characterization of enzymes purified from plant tissue. Here, we identified and characterized the gene

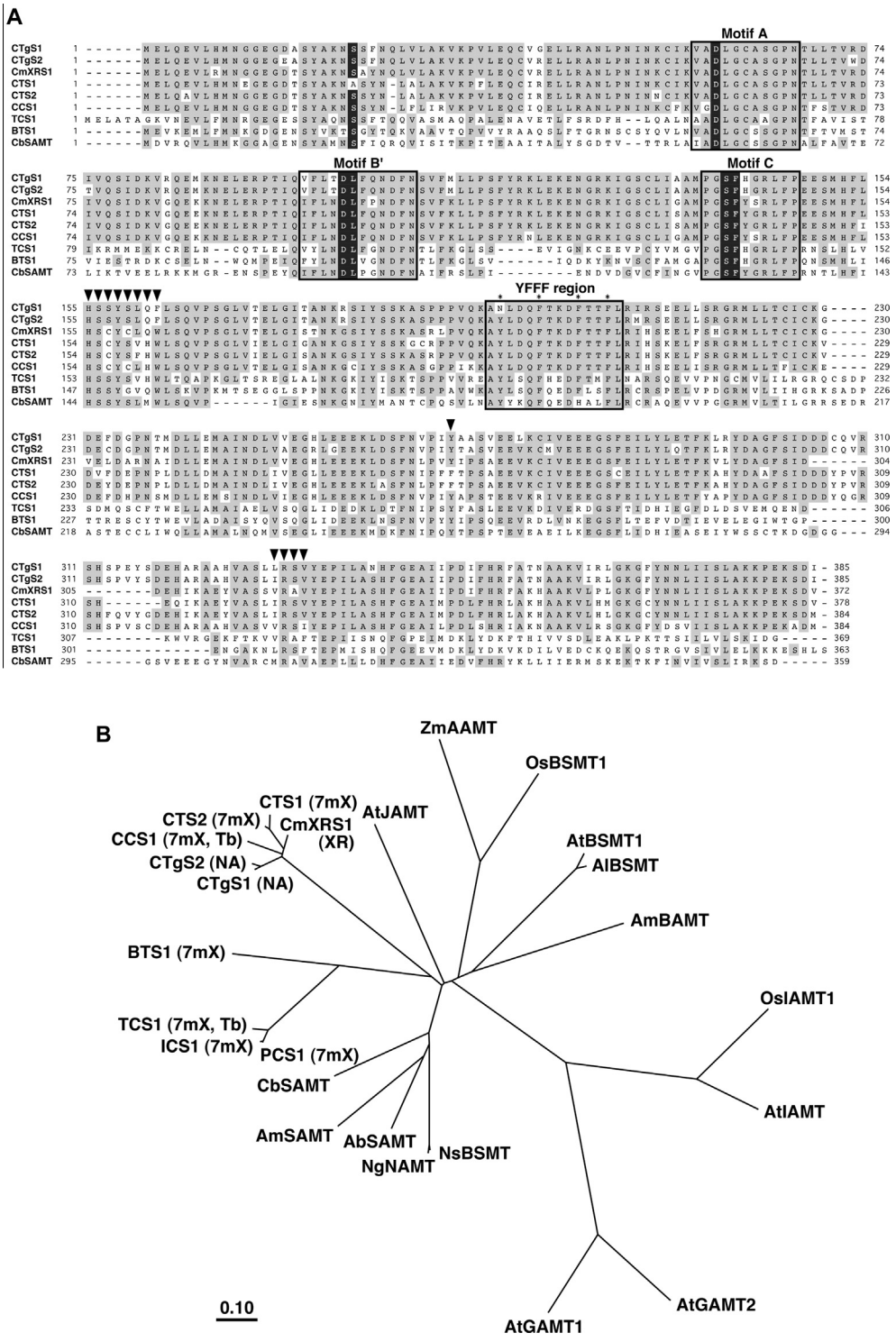

Fig. 2. Amino acid sequence comparison of coffee trigonelline synthases and related methyltransferases. (A) The following amino acid sequences (accession numbers in parentheses) were subjected to sequence alignment: CTgS1(AB054842), CTgS2(AB054843), CmXRS1(AB034699), CTS1(AB034700), CTS2(AB054841), CCS1(AB086414), TCS1(AB031280), BTS1(AB096699), and CbSAMT(AF133053). Shaded boxes represent conserved amino acid residues, and dashes represent gaps that have been inserted for optimal alignment. The proposed SAM-binding motifs (A, B′ and C) and the conserved region, designated as the "YFFF-region", are shown by open boxes. Conserved amino acids for SAM binding are indicated by black-and white inverted letters. The amino acids involved in substrate binding are indicated by arrowheads. (B) Phylogenetic analysis of the Motif B′ methyltransferase family. The unrooted tree was created with ClustalW using the neighbour joining method (http://www.genome.jp/tools/clustalw/). Substrates of the enzymes are indicated in parentheses. Abbreviations of substrates are as follows: XR, xanthosine; 7mX, 7-methylxantine; Tb, theobromine; NA, nicotinic acid. Sources of the sequences are as follows: AtJAMT(AY008434), *Arabidopsis thaliana* jasmonic acid methyltransferase; ZmAAMT(NM001195208), *Zea mays* anthranilic acid methyltransferase; OsBSMT1(XM467504), *Oryza sativa* benzoate salicylate methyltransferase; AtBSMT1(BT022049), *Arabidopsis thaliana* BSMT1; AlBSMT1(AY224596), *Arabidopsis lyrata* BSMT1; AmBAMT(AF198492), *Antirrhinum majus* benzoic acid methyltransferase; OsIAMT1(EU375746), *Oryza sativa* indole-3-acetic acid methyltransferase; AtIAMT(AK175586), *Arabidopsis thaliana* IAMT; AtGAMT1(NM118775), *A. thaliana* gibberellic acid methyltransferase1; AtGAMT2(NM125013), *A. thaliana* GAMT2; NsBSMT(AJ628349), *Nicotiana suaveolens* BSMT; NgNAMT(GU169286), *Nicotiana gossei* nicotinic acid carboxyl methyltransferase; AbSAMT(AB049752), *Atropa belladonna* salicylic acid methyltransferase; AmSAMT(AF515284), *Antirrhinum majus* SAMT; PCS1(AB207817), *Camellia ptilophylla* theobromine synthase1; ICS1(AB056108), *Camellia irrawadiensis* theobromine synthase1 and those of the other sequences are indicated in Fig. 2A.

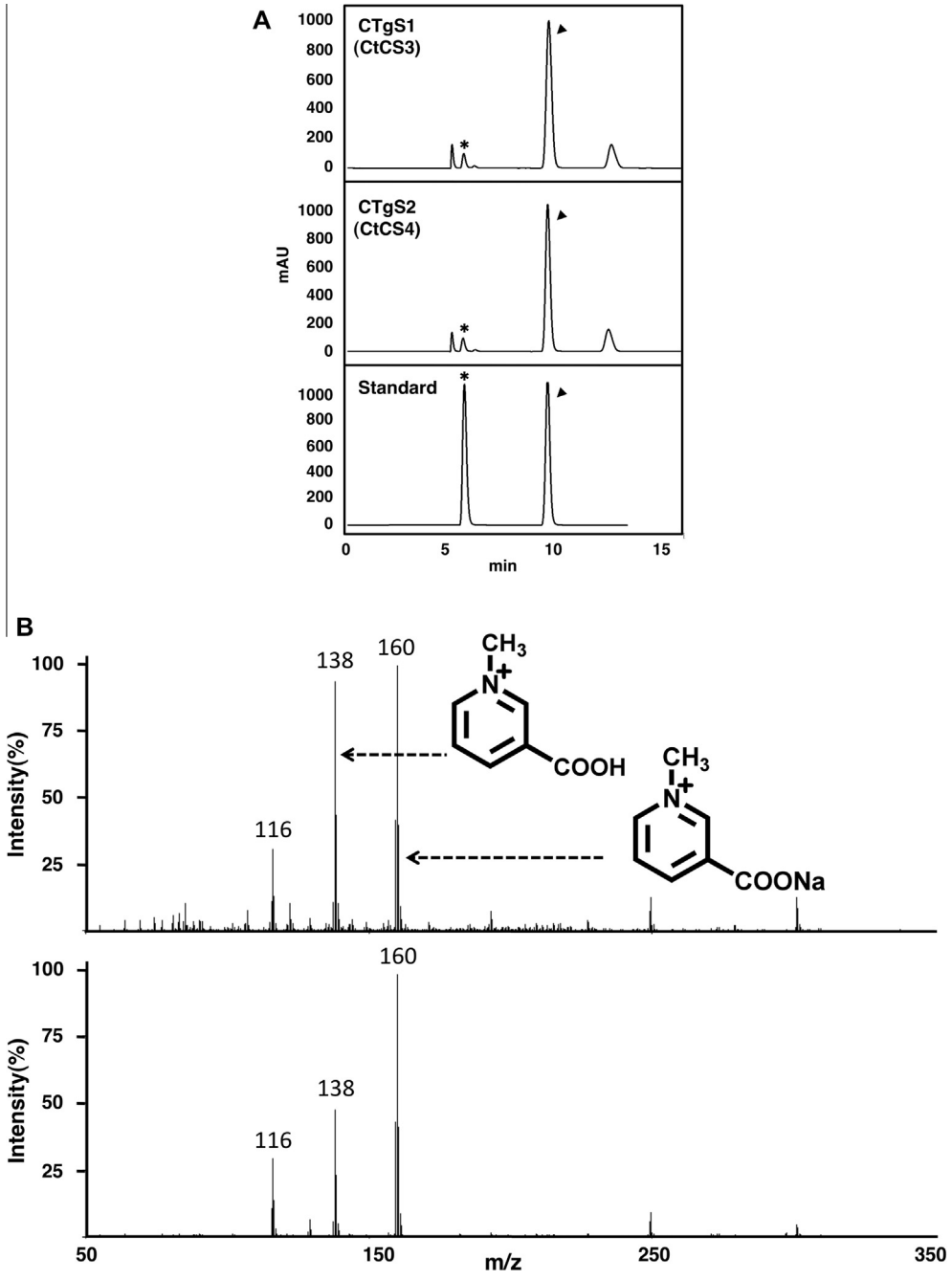

Fig. 3. HPLC and MS analysis of *in vitro* biosynthesis of trigonelline. (A) Standard samples consisted of a mixture of nicotinic acid (indicated by arrowhead) and trigonelline (indicated by asterisk). The reaction mixtures contained nicotinic acid, SAM, and the purified recombinant CTgS1 or CTgS2 and were incubated for 16 h at 25 °C. (B) Mass spectra of recombinant CTgS2 reaction product (upper) and authentic trigonelline (lower). Positive-ion ESI-MS analysis of the product corresponding to trigonelline and authentic trigonelline (*m*/*z* 138).

for trigonelline synthases, and demonstrated that they belong to the family of coffee caffeine synthases (CCS family). The substrate specificities of a member of the caffeine synthases in coffee are shown in Table 1.

Although the amino acid sequences of the CCS family enzymes were very similar to each other and belong to the same clade in the phylogenetic tree (Fig. 2B), those CCS family enzymes have respectively different substrate specificity. Thus, it was determined that

Table 1
Substrate specificities of the coffee caffeine synthase family.

	CTgS1/CTgS2[a]	CmXRS1[b]	CTS1/CTS2[b]	CCS1[b]	CCS1[b]
Substrate	Nicotinic acid	Xanthosine	7-Mehtylxanthine	7-Methylxanthine	Theobromine
Product	Trigonelline	7-Methylxanthosine	Theobromine	Theobromine	Caffeine

[a] This study.
[b] Mizuno et al. [23,24].

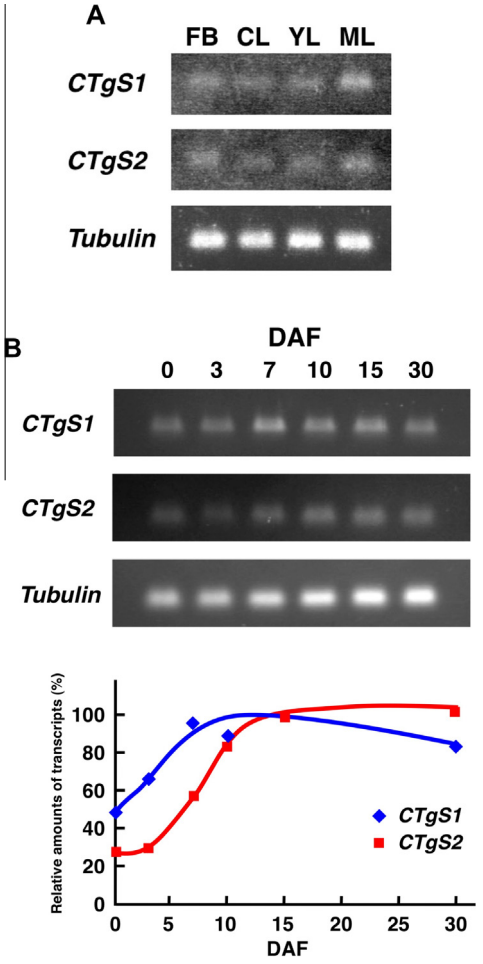

Fig. 4. Relative abundance of CTgS1 and CTgS2 transcripts in developing fruits. (A) Semi-quantitative RT-PCR of total RNAs from flower buds (FB), closed leaves (CL), young leaves (YL) and mature leaves (ML) was performed with 18–30 cycles to determine the linear range of PCR amplification. The results obtained here with 25 cycles were for all samples. (B) Semi-quantitative RT-PCR of total RNAs from developing fruits at various time periods, designated as days after flowering (DAF) was performed with 18–30 cycles to determine the linear range of PCR amplification. The results obtained here with 26 cycles were for all samples. Relative transcript levels were calculated, and the highest values (DAF15) were set as 100%.

some specific amino acid residues of those CCS family enzymes contribute directly to those substrate specificities. We revealed that the activity of 3-N methylation for the first step of caffeine biosynthesis needs a histidine residue at the corresponding position 161 in the CmXRS1 sequence [25]. The corresponding amino acid residues in the sequences of CTgS1 and CTgS2 were glutamine, the same as those for CCS1 and CTS1. Although the neighboring amino acid residue is tryptophan in the coffee caffeine synthase (CmXRS1, CTS1, CTS2, and CCS1) sequences, the two trigonelline synthases (CTgS1 and CTgS2) were shown to have phenylalanine residues at the corresponding position (Fig. 2A). We focused our attention on the correlation between the methylation acceptor molecule and the two amino acid residues at the corresponding position of those enzymes. Trigonelline synthases (CTgS1 and CTgS2) have Q161-F162 residues at the corresponding position, 7-methylxanthosine synthase (CmXRS1) corresponds to Q161-W162 residues, and theobromine synthases (CTS1 and CTS2) and caffeine synthase (CCS1) correspond to H160-W161. The substrates for those enzymes are nicotinate, xanthosine, and 7-methylxanthine or theobromine, respectively. Meanwhile, it was determined that the region consisting of 7 amino acid residues containing those two residues was a binding region for salicylic acid in *C. breweri* SAMT (CbSAMT) [18]. Homology models of those enzymes were built using the experimentally-determined structure of CbSAMT as the template. Since the Q161-F162 residues in CTgSs were comparable as the structural equivalent of the CbSAMT M150-W151 salicylate binding site, it is believed that this region was involved in the binding with the acceptor molecule of methyl groups. The Q161-F162 sequence of CTgS1 and CTgS2 is presumably involved in binding with nicotinic acid as a methyl group acceptor. Subsequently, CTgS1 and CTgS2 have a F354-A355-T356 sequence, the corresponding amino acid residues of CmXRS1 are FAK, and the corresponding amino acid residues of CTS1 and CCS1 are I/LAK. The variation of the amino acid residues corresponded to the substrate specificities respectively.

On the other hand, the amino acid sequence identity was very high (over 95%) between CTgS1 and CTgS2, with only 11 amino acid residues different from each other. It is assumed that the difference of the 11 amino acid residues is related to the intensity of the substrate affinity.

The results of semi-quantitative RT-PCR showed that the expression levels of CTgS1 and CTgS2 in immature fruits were maximum approximately 2 weeks after flowering (Fig. 4B). The expression levels were then maintained in the 30 days after flowering. Because the amounts of transcripts for CTgS1 and CTgS2 were maximized later than that of the genes for caffeine synthase [23,24], it appears that the synthesis of and the accumulation of trigonelline developed later than those for caffeine. Since organelle transportation signal sequences were not shown in the sequences of CTgSs as were other caffeine synthetic enzymes in coffee, these enzymes were determined to behave in the cytosol, and the produced trigonelline and caffeine are presumably transported to the vacuole with another transportation system. It is known that ATP-binding cassette (ABC) transporters are involved in the transport of endogenous secondary metabolites in plants. Since it is suspected that the transport of trigonelline and caffeine is conducted by a similar system, this idea is therefore the proposed focus for future research.

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