Caffeine is synthesized through sequential three-step methylation of xanthine derivatives at positions 7-N, 3-N, and 1-N. However, controversy exists as to the number and properties of the methyltransferases involved. Using primers designed on the basis of conserved amino acid regions of tea caffeine synthase and Arabidopsis hypothetical proteins, a particular DNA fragment was amplified from an mRNA population of coffee plants. Subsequently, this fragment was used as a probe, and four independent clones were isolated from a cDNA library derived from coffee young leaves. Upon expression in Escherichia coli, one of them was found to encode a protein possessing 7-methylxanthine methyltransferase activity and was designated as CaMXMT. It consists of 378 amino acids with a relative molecular mass of 42.7 kDa and shows similarity to tea caffeine synthase (35.8%) and salicylic acid methyltransferase (34.1%). The bacterially expressed protein exhibited caffeine synthase (35.8%) and salicylic acid methyltransferase (34.1%) activities toward substrates such as 7mX, theobromine, and paraxanthine. Indeed, it is not clear yet whether the activities are catalyzed by independent or multifunctional proteins (2, 12). Despite such difficulties, a number of studies describing their purification and characterization in cytoplasmic fractions. The results suggest that, in coffee plants, caffeine is synthesized through three independent methylation steps from xanthosine, in which CaMXMT catalyzes the second step to produce theobromine.

Among more than 50,000 secondary metabolites of plants, 12,000 are alkaloids. Their physiological roles are considered to be chemical defense against invertebrate herbivores. Caffeine, a typical purine alkaloid, is found in seeds and leaves of coffee (Coffeea arabica), cola (Cola nitida), maté (Ilex paraguariensis), and tea (Camellia sinensis) at concentrations up to 1 mg/g dry weight (1, 2). It exhibits a lethal effect on tobacco horn worm (Manduca sexta) by inhibiting phosphodiesterase activity, which hydrolyzes cAMP (3).

The biosynthetic pathway of caffeine has been intensively studied, and it is now established that it is successively synthesized from adenine nucleotides through multiple steps catalyzed by several enzymes (4–6). The final series of steps involves methylation of xanthosine by N-methyltransferase, yielding 7-methylxanthosine, whose ribose residue is removed by 7-methylxanthosine nucleosidase. The resulting 7-methylxanthine (7mX) is methylated at the 3-N-position by N-methyltransferase, producing 3,7-dimethylxanthine (theobromine), which is again methylated at the 1-N-position to give 1,3,7-trimethylxanthine (caffeine) (Fig. 1). All reactions require S-adenosyl-L-methionine (AdoMet) as a methyl donor. Some bypass pathways, for example featuring paraxanthine, have also been suggested, but in coffee and tea plants, it was confirmed that the major pathway is through theobromine (5, 6).

At least three N-methyltransferases are considered to contribute to this pathway; these catalyze methylation of xanthosine (the first), methylation of 7mX (the second), and methylation of theobromine (the third). Their isolation and characterization have attracted a good deal of attention, and enzymes catalyzing the second and the third steps were first identified in crude extract of tea leaves (7). Since then a dozen surveys describing their purification and characterization in coffee and tea plants have been published (2, 8–13). However, it was found that the enzymes are extremely labile, making it difficult even to distinguish each activity. Indeed, it is not clear yet whether the activities are catalyzed by independent or multifunctional proteins (2, 12). Despite such difficulties, a caffeine synthase (CS) was recently isolated successfully from tea leaves (14). The enzyme has a native molecular mass of 61 kDa and exhibits 3- and 1-N-methyltransferase activities toward substrates such as 7mX, theobromine, and paraxanthine (14). It was thus concluded that, at least in tea leaves, a single enzyme has dual functions in caffeine synthesis. Subsequently, the gene encoding this CS was isolated (TCS1), and the predicted amino acid sequence was found to show considerable similarity with salicylic acid O-methyltransferase (15). Whether or not a similar enzyme(s) functions in coffee plants has not been hitherto determined. Although a coffee gene encoding xanthosine methyltransferase (XMT), was reported in a patent (16), the details remain to be clarified.

In this work, we document isolation of a gene encoding an enzyme that catalyzes methylation of 7mX from coffee plants. In contrast to tea CS, the enzyme features strict substrate specificity toward methylation only at the 3-N-position of the

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession numbers AB039725 (CaMTL1), AB048792 (CaMTL2), AB048793 (CaMTL3), and AB048794 (CaMXMT).

† To whom correspondence should be addressed. Tel.: 81-743-72-5650; Fax: 81-743-72-5659; E-mail: sano@bs.aist-nara.ac.jp.

The abbreviations used are: 7mX, 7-methylxanthine; AdoMet, S-adenosyl-L-methionine; CS, caffeine synthase; GFP, green fluorescent protein; XMT, xanthosine 7-N-methyltransferase; PCR, polymerase chain reaction; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.
purine ring. It is suggested that, in coffee plants, caffeine synthesis is mediated by three methylation steps catalyzed by distinct enzymes, including the presently identified 7mX methyltransferase.

EXPERIMENTAL PROCEDURES

Plant Materials—Coffee plants \( (C. \text{arabica} \ \text{L. var. caturra}) \) were cultivated in a greenhouse.

Preparation of the Probe for Isolating Caffeine Synthase cDNA—Two degenerated oligonucleotides, 5'G/GITGYSDSDSICACIAAYAC-3' (forward) and 5'ARIYKIYYRTRRAAISWICCIGG-3' (reverse), which correspond to the amino acid sequences of GC(A/S)(A/S)GPNT and PGSF(H/Y)(G/K)(R/N)LF, respectively, were synthesized based on conserved regions among TCS1 (Ref. 15; accession number AB031280) and two \textit{Arabidopsis} hypothetical proteins (Z99708 and AC008153). PCR was performed in 25 \( \mu l \) of reaction mixture containing \textit{C. arabica} cDNA and the pair of primers mentioned above under the conditions of 94 °C for 1 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. A 255-base pair fragment was amplified, and one of the deduced amino acid sequences from its DNA sequence showed 34% identity to that of TCS1. This fragment was used to screen the \textit{C. arabica} cDNA library.

cDNA Library Construction—Total RNA was extracted by the cetlytrimethylammonium bromide method (17) with a slight modification, and poly(A) RNA was purified using an mRNA purification kit (Am - ersham Pharmacia Biotech) according to the manufacturer's instruc- tions and converted into double-stranded cDNA using a ZAPII cDNA synthesis kit (Stratagene). The cDNA was ligated with Uni-ZAP XR vector arms and packaged using a Gigapack III kit. The titer of the library was \( 3 \times 10^{11} \) plaque-forming units.

Production of Glutathione S-Transferase (GST) Fusion Proteins—The open reading frame regions of clones 1, 6, 35, and 45 sandwiched with \textit{Sma}I and \textit{Not}I restriction sites were subcloned into the pGEX 4T-2 vector (PharmaciaPhand 

Measurement of N-Methyltransferase Activity—The enzyme activity

FIG. 1. Proposed biosynthetic pathway of caffeine in \textit{Coffea} plants.

FIG. 2. Amino acid alignment of the products deduced from cDNAs of clone 45, clone 1, clone 6, and clone 35. Dashes and colons indicate gaps and identical amino acid residues, respectively.

FIG. 3. Purification of GST fusion products and their methyltransf erase activities toward xanthosine derivatives. A, the pu- rified GST fusion proteins derived from the cDNAs of clones 6, 35, and 45 were separated by SDS-polyacrylamide gel electrophoresis and stained by Coomassie Brilliant Blue. B, thin layer chromatographic analysis of the reaction products from incubation with the recombinant proteins shown in A. Substrates used were xanthosine (X), 7mX, theobromine (Tb), paraxanthine (Px), and theophylline (Tp). Cf and Tb (right) indicate the corresponding positions of caffeine and theobro- mine, and the asterisk indicates a chloroform-extractable contaminant present in radioactive AdoMet. C, elution profiles of HPLC using a DE-613 column. Standard samples were a mixture of 7mX, theobro- mine, paraxanthine, and caffeine (Cf). The reaction mixture (see "Ex- perimental Procedures") containing 7mX and the recombinant protein of clone 45 was incubated for 2 h (Reaction) at 27 °C. The control was a sample without incubation (time 0).
was determined by an established procedure (14) with a slight modification. The reaction mixture of 100 μl containing 100 mM Tris-HCl (pH 8.3), 200 μM substrate, 4 μM S-adenosyl-l-[methyl-14C]methionine (2.15 GBq/mmol; Amersham Pharmacia Biotech), 200 μM MgCl2, and 200 ng of purified recombinant protein was incubated at 27 °C for 2 h. The reaction was terminated by the addition of 1 ml of chloroform, and the organic phase was recovered, dried at 60 °C, and dissolved in 10 μl of 50% methanol. This fraction was separated by thin layer (Silica gel 60 F254; Merck) chromatography with a solution of H2O/acetic acid/50% methanol. This fraction was separated by thin layer (Silica gel 60 F254; Merck) chromatography with a solution of H2O/acetic acid/50% methanol. This fraction was separated by thin layer (Silica gel 60 F254; Merck) chromatography with a solution of H2O/acetic acid/50% methanol. This fraction was separated by thin layer (Silica gel 60 F254; Merck) chromatography with a solution of H2O/acetic acid/50% methanol. This fraction was separated by thin layer (Silica gel 60 F254; Merck) chromatography with a solution of H2O/acetic acid/50% methanol.

Reverse Transcription-PCR—Total RNAs were isolated from various C. arabica fruits and roots, stems containing buds, old leaves, and young leaves of C. arabica (Fig. 6A). The level of transcripts for XMT, which catalyzes the conversion of xanthosine to 7-methylxanthosine (7mX methyltransferase-like 1, clone 1), CaMTL2 (clone 6), and CaMTL3 (clone 35) was estimated by reverse transcription-PCR together with CaMTL1 (C. arabica methyltransferase-like 1, clone 1), CaMTL2 (clone 6), and CaMTL3 (clone 35), respectively.

Catalytic Properties of CaMXMT—The optimal pH for 7mX methylation activity of CaMXMT ranged between 7 and 9, with the peak at 7.5 (Fig. 5A). The effects of 7mX and AdoMet concentrations on the reaction velocity of GST-CaMXMT protein were determined (Fig. 5B). The Km values for 7mX and AdoMet were 50 and 11.9 μM, respectively, and apparent Vmax values were estimated to be 7.14 and 7.94 pmol of theobromine/min/μg of protein upon measurement with the variable amounts of 7mX and AdoMet, respectively.

Tissue Specificity—Accumulation of CaMXMT transcripts was estimated by reverse transcription-PCR together with CaMTL1, CaMTL2, and CaMTL3 in various tissues including roots, stems containing buds, old leaves, and young leaves of C. arabica (Fig. 6A). The level of transcripts for XMT, which catalyzes the conversion of xanthosine to 7-methylxanthosine, was also tested. Transcripts of CaMXMT were detected in stems and young leaves but not in roots and old leaves, similar to the expression pattern for XMT. Transcripts of CaMTL1 and CaMTL2 were present in all tissues at high levels, whereas

| Material                  | Substrate | Sources         |
|---------------------------|-----------|-----------------|
| Recombinant CaMXMT (coffee) | 7mX | 100 | ND | ND | ND | ND | ND | ND | ND | This study |
| Crude enzyme (coffee fruits) | 7mX | 100 | 5.7 | 127 | 4.6 | 175 | ND | ND | ND | Ref. 8 |
| Native TCS1 (tea)         | 7mX | 100 | 17.6 | 4.2 | 26.8 | tr | 210 | tr | ND | ND | Ref. 14 |
| Recombinant TCS1 (tea)    | 7mX | 100 | 1.0 | 12.3 | 18.5 | <0.1 | 230 | ND | ND | Ref. 15 |

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**Substrate specificity of CaMXMT**

Relative enzyme activities of CaMXMT, a crude extract from coffee fruits, and native and recombinant caffeine synthase (TCS1) from tea were compared. Activity of each towards 7mX was set as 100, and their relative activities are shown. 7mX, 3mX, and 1mX, 7-, 3-, and 1-methylxanthine, respectively; Tb, theobromine; Tp, theophylline; Px, paraxanthine; X, xanthosine; XR, xanthine riboside; XMP, xanthosine monophosphate; ND, not detected; tr, trace.

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CaMTL3 transcripts were abundant in stems and young leaves and also in roots and old leaves at a lower level.

Subcellular Localization—To identify the cellular localization of CaMXMT, the cDNA fragment covering the entire coding region of CaMXMT was fused to pGFP2, and the resulting plasmid was introduced into the onion epidermal layer by a biolistic bombardment. Green fluorescence was detected in the cytoplasm (Fig. 6B).

DISCUSSION

This report documents isolation of a gene encoding 7mX methyltransferase from coffee plants and characterization of the bacterially expressed recombinant enzyme. Screening a coffee cDNA library with a probe constructed from a conserved amino acid region of TCS1 and similar sequences derived from Arabidopsis expressed sequence tag clones, four distinct cDNA clones were isolated. The protein encoded by one of them showed 7mX methyltransferase activity when expressed as a fusion protein with GST in E. coli and was designated as CaMXMT. Proteins encoded by other clones (CaMTL1, -2, and -3) did not show any methyltransferase activity on substrates examined for CaMXMT. The deduced amino acid sequence of CaMXMT showed rather low similarity to TCS1 (35.8%) and high similarity to CaMTLs (more than 80%) (Fig. 4B). This result indicates CaMXMT is not close in an evolutionary sense to TCS1. In other words, caffeine biosynthetic pathway in coffee and tea might have evolved independently, consistent with different catalytic properties of the enzymes involved (see CaMTL3 transcripts were abundant in stems and young leaves and also in roots and old leaves at a lower level.

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CaMXMT also showed low similarity to salicylic acid O-methyltransferase from *Clarkia breweri* (21) and bezoic acid carboxyl methyltransferase isolated from snapdragon (*Antirrhinum sp.*) flowers (22). In addition, we found several related sequences in the expressed sequence tag of *Arabidopsis*. Although more than 120 methyltransferases have so far been reported from various organisms (23), methyltransferases of this type are not well characterized. Their structures appear to be unique, with little similarities to other methyltransferases, suggesting a new class. However, it has been pointed out that salicylic acid methyltransferase contains domains similar to motifs I and III found in plant O-methyltransferases (21). Those are proposed to be involved in AdoMet binding and conserved in salicylic acid methyltransferase, benzoic acid carboxyl methyltransferase, TCS, and CaMXMT (Fig. 4A), although TCS1 and CaMXMT are N-methyltransferases. The motifs are also found in CaMTLs, making it highly probable that they possess methyltransferase activity, although they do not participate in caffeine biosynthesis. The major difference in amino acid sequence between CaMXMT and CaMTLs is Val159→Ile. His160→Tyr161 (VHW), which is present in TCS1 and CaMXMT but absent in CaMTLs. It is tempting to speculate that the substrate specificity of this class is determined by a few particular amino acids, and further investigations with point-mutated proteins are needed to clarify this point.

Despite the similar pH optimum for activity, the substrate specificities of CaMXMT and TCS1 are clearly different. Whereas both native and recombinant TCS1 equally show catalytic activity toward the 1-N- and 3-N-sites of the purine ring, CaMXMT catalyzes only 3-N-methylation (Table I). In a crude extract of coffee fruits, the capacity of 1-N-methylation of theobromine to caffeine was detected (8), and we have confirmed this with crude extracts of young leaves. Since recombinant CaMXMT did not show any 1-N-methylation activity, it is obvious that, in coffee plants, 3-N- and 1-N-methylation is catalyzed by different enzymes. This is consistent with findings that the apparent $K_m$ for xanthine derivatives markedly differs among enzymes. Crude enzymes exhibit $K_m$ values for both 7-methylxanthine and theobromine ranging between 100 and 500 μM (13). This is also the case for purified tea CS, except that it has much higher affinity for paraxanthine, with a $K_m$ of 24 μM (14). Such differential $K_m$ values suggest that, despite apparent multifunctional properties, each enzyme may be able to select its correct substrate. CaMXMT methylates predominantly 7mX with a $K_m$ of 50 μM, a much higher affinity than for any other enzymes reported. The observations suggest that enzymes involved in caffeine synthesis may possess rather strict substrate preference and that this arises from diversity in a few amino acids.

The transcript accumulation profiles of CaMXMT, XMT, and CaMTLs were analyzed by reverse transcription-PCR with specific primers for each to avoid cross-hybridization between CaMXMT and CaMTLs. Transcripts of CaMXMT and XMT accumulated in young leaves and stems containing buds, suggesting that biosynthesis of caffeine occurs mainly in those tissues in coffee plants. This is consistent with the fact that theobromine and caffeine are primarily found in their buds and young leaves (5). It should be noted that the transcript accumulation profile of CaMTL3 is similar to that of CaMXMT and XMP, suggesting its involvement in the metabolism of caffeine-related compounds. Examination of the subcellular localization of CaMXMT using the fusion protein of CaMXMT and GFP demonstrated an existence predominantly in the cytoplasm of onion epidermal cells. The PSORT program with the deduced amino acid sequence also predicted a high possibility of cytoplasmic localization for CaMXMT. 2 It can thus be concluded that caffeine biosynthesis occurs in the cytoplasm of cells in buds and young leaves.

It is worthy of mention that CaMXMT may have practical applications. To cope with occasional health problems caused by caffeine, decaffeinated coffee is currently produced by chemical treatment of coffee beans. Recombinant DNA technology using CaMXMT may remove the need for this by creating caffeineless coffee plants. Furthermore, the opposite approach may also be applicable to important crops in such a way as to produce caffeine derivatives as insect repellants.

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*Fig. 6. Tissue specificity of CaMXMT expression and the intracellular localization of CaMXMT.* A, tissue-specific transcript accumulation of CaMXMT, XMT, CaMTL1, CaMTL2, and CaMTL3 was analyzed by reverse transcription-PCR. B, onion bulbs were bombarded with gold particles coated with pGFP2 and pCaMXMT-GFP (c and d) plasmids. The proteins were transiently expressed, and individual cells are observed by differential interference contrast imaging (a and c) and corresponding epifluorescence microscopy (b and d).

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