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CAFFEINE SYNTHASE AND RELATED METHYLTRANSFERASES IN PLANTS

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1. ABSTRACT

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid present in high concentrations in tea and coffee and it is also found in a number of beverages such as coca cola. It is necessary to elucidate the caffeine biosynthetic pathway and to clone the genes related to the production of caffeine not only to determine the metabolism of the purine alkaloid but also to control the content of caffeine in tea and coffee. The available data support the operation of a xanthosine → 7-methylxanthosine→7-methylxanthine → theobromine → caffeine pathway as the major route to caffeine. Since the caffeine biosynthetic pathway contains three *S*-adenosyl-L-methionine (SAM) dependent methylation steps, *N*-methyltransferases play important roles. This review focuses on the enzymes and genes involved in the methylation of purine ring. Caffeine synthase, the SAM-dependent methyltransferase involved in the last two steps of caffeine biosynthesis, was originally purified from young tea leaves (*Camellia sinensis*). The isolated cDNA, termed TCS1, consists of 1,483 base pairs and encodes a protein of 369 amino acids. Subsequently, the homologous genes that encode caffeine biosynthetic enzymes from coffee (*Coffea arabica*) were isolated. The recombinant proteins are classified into the three types on the basis of their substrate specificity i.e. 7-methylxanthosine synthase, theobromine synthase and caffeine synthase. The predicted amino acid sequences of caffeine biosynthetic enzymes derived from *C. arabica* exhibit more than 80% homology with those of the clones and but show only 40% homology with TCS1 derived from *C. sinensis*. In addition, they share 40% homology with the amino acid sequences of salicylic carboxyl methyltransferase, benzoic acid carboxyl methyltransferase and jasmonic acid carboxyl methyltransferase which belong to a family of motif B' methyltransferases which are novel plant methyltransferases with motif B' instead of motif B as the conserved region.

2. INTRODUCTION

Extensive metabolic studies with purine alkaloids including caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) have elucidated the caffeine biosynthetic pathway in some detail in leaves of tea (*Camellia sinensis*) and coffee (*Coffea arabica*) (1-3). The available data support the operation of a xanthosine →7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway as the major route to caffeine. In addition, a 7-methylxanthine → paraxanthine → caffeine pathway is one of a number of minor pathways operating in tea leaves (4). These pathways are illustrated in Figure 1. There is one report of an alternative entry in the caffeine biosynthesis pathway in coffee that involves conversion of xanthosine 5'-monophosphate (XMP) →7-methyl XMP → 7-methylxanthosine (5).

Three methylation steps occur in caffeine biosynthesis. *S*-adenosyl-L-methionine (SAM), which is widely distributed *in vivo*, is the purine base methyl donor (6). The methylation steps are catalyzed by SAM-dependent *N*-methyltransferases. This article will review what is currently known about the specific *N*-methyltransferases involved in caffeine biosynthesis.

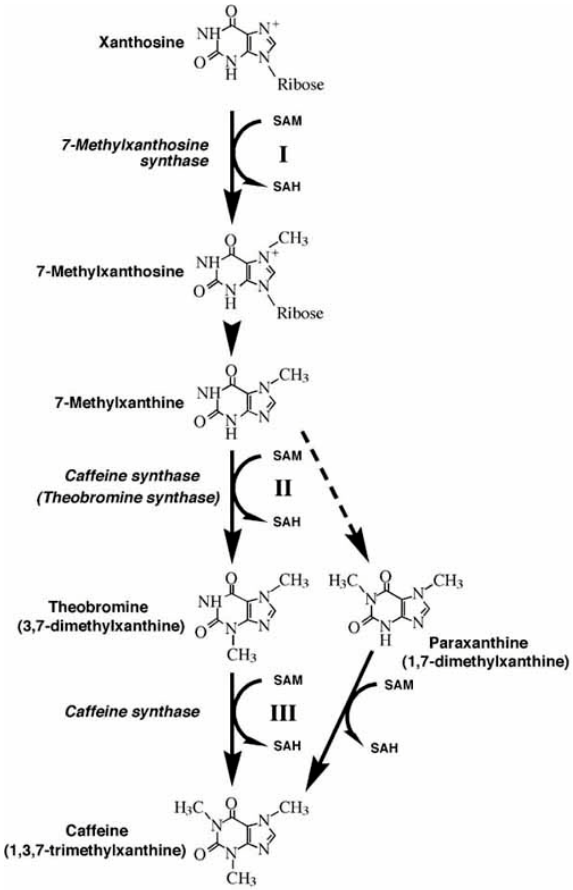


Figure 1. Pathways for the biosynthesis of caffeine. 7-Methylxanthosine synthase and theobromine synthase have closely substrate specificity and only catalyze the conversion of xanthosine to 7-methylxanthosine (reaction I) and of 7-methylxanthine to theobromine (reaction II), respectively. Caffeine synthase has broad substrate specificity (reactions II and III). Abbreviations: SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine. Three methylation reactions were indicated by I, II, and III.

3. *N*-METHYLTRANSFERASES INVOLVED IN CAFFEINE BIOSYNTHESIS

3.1. *N*-methyltransferase activity in cell free preparations

The activities of 7-methylxanthine *N*-methyltransferase and theobromine *N*-methyltransferase, which catalyze the second and the third methylation steps in the main pathway, were first demonstrated in crude extracts from tea leaves by Suzuki and Takahashi (7). This study shows that the two enzymes have identical pH optima and have similar metal ions and inhibitors. Since then, *N*-methyltransferase activities involved in purine alkaloid biosynthesis have been detected in cell-free extracts prepared from immature fruits (8), callus (9) and cell-suspension cultures of coffee (10). The presence of the first methylation enzyme, xanthosine *N*-methyltransferase, which catalyzes the formation of 7-methylxanthosine from xanthosine, was first demonstrated *in vitro* with tea leaf extracts (11). XMP *N*-methyltransferase activity was detected in coffee cell-free extracts by Schulthess *et al*. (12) who also reported that both xanthosine and XMP were utilized as a methyl acceptor of SAM and that xanthosine was a better methyl acceptor than XMP (12). Fujimori *et al*. confirmed the presence of activities of the three *N*-methyltransferases in tea-leaf extracts and found that they were present at high levels in very young developing leaves but were absent in fully developed leaves (13).

Several investigators attempted the purification of the *N*-methyltransferase(s) involved in caffeine biosynthesis. Mazzafera *et al*. (14) first reported the purification of an *N*-methyltransferase from the endosperm and leaves of coffee and showed the presence of 7-methylxanthine and theobromine *N*-methyltransferase activity. However, these activities were extremely labile and the specific activity of the enzyme diminished markedly with purification. Gillies *et al*. (15) partially purified *N*-methyltransferase from liquid endosperm of coffee by ion-exchange chromatography. The final specific activity of this preparation was 420 fkat mg^{-1}. Kato *et al*. (4) partially purified *N*-methyltransferase from tea leaves by ion-exchange and gel-filtration chromatography. The final specific activity of this preparation was 100 pkat mg^{-1} with paraxanthine. MösliWaldhauser *et al*. (16) partially purified *N*-methyltransferases from coffee leaves using ion-exchange chromatography and chromatofocusing, showing that XMP *N*-methyltransferase was different from the other two *N*-methyltransferases.

3.2. Purification of caffeine synthase from tea leaves

N-methyltransferase activities involved in caffeine biosynthesis are extremely labile in cell-free preparations. It is for this reason that obtaining a purified preparation with the high specific activity proved elusive. Kato *et al*. (17) first reported the isolation of the SAM-dependent *N*-methyltransferase protein for caffeine biosynthesis with high specific activity. A *N*-methyltransferase from young tea leaves (*Camellia sinensis*) was purified 520-fold to apparent homogeneity and a final specific activity of 5.7 nkat mg^{-} protein by ammonium sulphate fractionation and hydroxyapatite, anion-exchange, adenosine-agarose, and gel-filtration chromatography. The key step was affinity chromatography on adenosine-agarose, which was previously used with success to purify an *S*-methyltransferase of plant origin (18). This single procedure yielded 11.4-fold purification. The estimated molecular mass of the native enzyme was 61 kD by gel-filtration chromatography and 41kD by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The final preparation exhibited 3- and 1-*N*-methyltransferase activity with broad substrate specificity, showing high activity toward paraxanthine, 7-methylxanthine, and theobromine and low activity with 3-methylxanthine and 1-methylxanthine. However, the enzyme had no 7-methyltransferase activity toward xanthosine and XMP. Therefore, the single *N*-methyltransferase obtained was referred to as caffeine synthase (CS). It is likely that xanthosine *N*-methyltransferase protein is different to caffeine synthase, and that at least two different enzymes catalyze the three methylation steps in the caffeine

Table 1. Substrate specificity of recombinant and native caffeine synthase

Substrate	Methylated product	*N*-methylation position	Recombinant TCS1	Native CS
Monomethylxanthines				
7-Meythylxanthine	Theobromine	3	100	100
3-Meythylxanthine	Theobromine	1	1.0	17.6
1-Meythylxanthine	Theobromine	3	12.3	4.2
Dimethylxanthines				
Theobromine	Caffeine	1	18.5	26.8
Theobromine	Caffeine	7	<0.1	<0.1
Paraxanthine	Caffeine	3	230	210

Caffeine synthase activity is expressed as a percentage of the activity on 7-methylxanthine. Data from Kato *et al.* (20)

Table 2. Caffeine synthase family from coffee

Function	Name	Accession No.	Origin	Method
7-Methylxanthosine synthase	CmXRS1	AB034699	Leaf cDNA library	Screening
	CaXMT1 [a]	AB048793	Immature fruit cDNA	RACE
Theobromine synthase	CTS1	AB034700	Leaf cDNA library	Screening
	CTS2	AB054841	Leaf cDNA library	Screening
	CaMXMT1 [b]	AB048794	Leaf cDNA	RACE
	CaMXMT2	AB084126	Immature fruit cDNA	RACE
Caffeine synthse	CCS1	AB086414	Liquid endosperm cDNA	RACE
	CtCS7	AB086415	Liquid endosperm cDNA	RACE
	CaDXMT1	AB084125	Immature fruit cDNA	RACE
Unknown	CtCS3	AB054842	Leaf cDNA library	Screening
	CtCS4	AB054843	Leaf cDNA library	Screening
	CaMTL1 [c]	AB039725	Leaf cDNA	RACE
	CaMTL2	AB048792	Leaf cDNA	RACE

[a] The amino acid sequence is same as that of CmXRS1, [b] The amino acid sequence has only one substitution of that of CTS1, [c] The amino acid sequence has only one substitution of that of CtCS4

biosynthetic pathway. The Km values of CS for paraxanthine, theobromine, 7-methylxanthine, and SAM were 24, 186, 344 and 21μM, respectively. The K_m value of paraxanthine is the lowest, and the V_{max} for this substrate is the highest, of the substrate tested; hence, paraxanthine is the best substrate for CS. However, as there is limited synthesis of paraxanthine from 7-methylxanthine, it is not an important methyl acceptor *in vivo* (4). The K_m value for theobromine is high, which may explain the transient accumulation of theobromine in young tea leaves (19). The effects of the concentration of SAM and several methyl acceptors on the activity of CS show typical Michaelis-Menten type kinetics, and there is no feedback inhibition by caffeine. It is unlikely that allosteric control of the CS activity is operating in tea leaves. The K_m of CS for SAM (21 μM) in the presence of paraxanthine was similar to the values for 7-methylxanthine and theobromine (25μM) obtained with crude tea enzyme preparations (7).

3.3. Molecular cloning of caffeine synthase gene from tea leaves

The determination of the amino-terminal sequence of CS led to the molecular cloning of the gene encoding CS (17). Kato *et al*. (20) used the RACE (rapid amplification of complementary DNA ends) technique with degenerate gene-specific primers based on the amino-terminal sequence of CS to obtain a 1.31-kilobase sequence of cDNA. The 5' untranslated sequence of the cDNA fragment was isolated by 5'RACE. The isolated cDNA, termed *TCS1* (GenBank accession no. AB031280), consists of 1,438 base pairs and encoded a protein of 369 amino acids. To determine whether *TCS1* encoded an active caffeine synthase enzyme, the full-length coding region for tea CS protein was ligated into pET23d plasmid and the resultant expression vector introduced into *Escherichia coli* (BL21) for the expression of TCS1. The bacterial lysates containing the recombinant CS protein demonstrated CS activity *in vitro*. The substrate specificity of the recombinant CS was very similar to that of the native enzyme purified from young tea leaves. The recombinant enzyme mainly catalyzed 3-*N*-methylation and 1-*N*-methylation of the purine ring of mono- and dimethylxanthines. Moreover, the [^{14}C] caffeine as the reaction product was detected in the reaction mixture following incubation with [methyl-^{14}C] SAM. These results indicated that *TCS1* encoded CS. (Table 1)

High levels of transcripts of *TCS1* were detected in developing leaves and much lower amounts were present in old leaves (21). These expression patterns mirror the distribution of caffeine synthase activity in tea leaves (13).

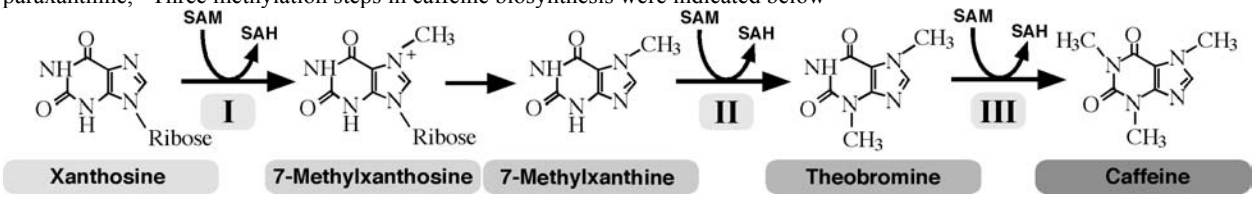
3.4. The orthologous genes in coffee

Many orthologous genes to caffeine synthase from coffee were reported and the obtained results were summarized in Table 2. Using primers designed on the basis of conserved amino acid regions of tea caffeine synthase, *O*-methyltransferases derived from plant origin and *Arabidopsis* hypothetical proteins, a particular DNA fragment was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and RACE technique. Subsequently, the resulting PCR products were used as a probe

Table 3. Comparison of the substrate specificity of recombinant and native caffeine synthases

Plant	Substrate[a]/methylation position									Steps in the pathway[b]	References
Proteins	**XR/7*N***	**XMP/7*N***	**X/7*N***	**7-mX/3*N***	**3-mX/1*N***	**1-mX/3*N***	**Tb/1*N***	**Px/3*N***	**Tp/7*N***		
Coffee											
Recombinant CmXRS1 (native form)	100	nd	nd	nd	nd	nd	nd	nd	nd	I	Mizuno *et al.* (23)
Recombinant CaXMT1 (GST fusion)	100	nd	nd	nd	nd	nd	nd	nd	nd	I	Uefuji *et al.* (27)
Recombinant CTS1 (native form)	nd	nd	nd	100	nd	nd	nd	1.4	nd	II	Mizuno *et al.* (25)
Recombinant CaMXMT1 (GST fusion)	nd	nd	nd	100	nd	nd	nd	5.0	nd	II	Uefuji *et al.* (27)
Recombinant CTS2 (native form)	nd	nd	nd	100	nd	nd	nd	1.1	nd	II	Mizuno *et al.* (25)
Recombinant CaMXMT2 (GST fusion)	nd	nd	nd	100	nd	nd	nd	5.3	nd	II	Uefuji *et al.* (27)
Recombinant CCS1 (native form)	nd	nd	nd	24	0.82	0.53	25	100	nd	II and III	Mizuno *et al.* (24)
Recombinant CCS1 (Trx fusion)	nd	nd	nd	16	0.56	0.43	23	100	nd	II and III	Mizuno *et al.* (24)
Recombinant CtCS7 (Trx fusion)	-	-	-	-	-	-	-	detected	-	II and III	Mizuno *et al.* (24)
Recombinant CaDXMT1 (GST fusion)	nd	nd	nd	1.0	nd	nd	3.8	100	nd	II and III	Uefuji *et al.* (27)
Endosperm (partially purified)	-	-	-	100	-	-	185	-	-		Mazzafera *et al.* (14)
Fruits (crude)	-	-	-	100	-	5.7	127	175	4.6		Roberts and Waller (8)
Leaves (crude)	-	83	-	100	-	-	-	-	-		Schulthess *et al.* (12)
Tea											
Recombinant TCS1 (native form)	-	-	-	100	1	12.3	18.5	230	nd	II and III	Kato *et al.* (20)
Leaves (purified)	-	-	-	100	17.6	4.2	26.8	210	nd	II and III	Kato *et al.* (17)
Methylated product	7mXR	7mXMP	7mX	Tb	Tp	Tp	caffeine	caffeine	caffeine		

The relative activity is indicated as the percentage of the activity with the most preferred substrate. Abbreviations as follows: nd, not detected; -, not determined, [a] XR, xanthosine; 7mXR, 7-methylxanthosine; XMP, xanthosine 5'-monophosphate; 7mXMP, 7-methyl xanthosine 5'-monophosphate; X, xhanthine; mX, methylxanthine; Tb, theobromine; Tp, theophylline; Px, paraxanthine, [b] Three methylation steps in caffeine biosynthesis were indicated below



independent clones were isolated from a cDNA library derived from coffee young leaves. Three types of *N*-methyltransferase, which were classified by the substrate specificity of the recombinant proteins, were identified (Table 3).

Caffeine biosynthesis in coffee is presumed to begin with a 7-*N*-methyltransferase converting xanthosine to 7-methylxanthosine or XMP to 7-methyl XMP. Previously, Moisyadi *et al*. (22) reported cloning of a cDNA encoding xanthosine 7-*N*-methyltransferase. The cDNA sequence, which encoded a 371 amino acids protein, has been shown to be similar to that for a lipase. Mizuno *et al*. (23) first reported the identification of 7-methylxanthosine synthase gene (*CmXRS1*). They have obtained seven homologous genes to *TCS1* from coffee and referred them to CtCS (Coffee tentative caffeine synthases) series. They demonstrated that CtCS1 catalyzed the 7-*N*-methylation of xanthosine. The full-length of cDNA of CmXRS1 had 1,307 nucleotides and encoded 372 amino acids. The recombinant CmXRS1 was specific for xanthosine and XMP could not be used as a substrate. This supports the findings Negishi (11) that xanthosine is the first committed precursor of the caffeine biosynthetic pathway. Other group also isolated the gene of 7-*N*-methylxanthosine synthase (24). They referred it *CaXMT1* and it was identical to *CmXRS1*.

The second methylation step, which was the conversion of 7-methylxanthine to theobromine, is catalyzed by theobromine synthase. Theobromine synthase only catalyzed 3-*N*-methylation of 7-methylxanthine and did not have 1-*N*-methylation activity. 7-Methylxanthine was the specific methyl acceptor, while 3-*N*-methylation activity of paraxanthine was very low. The genes encoding theobromine synthase from coffee were reported as *CTS1*, *CTS2* (25) and *CaMXMT1* (26).

Since the coffee plant generally contains small amounts of theobromine and accumulates high concentrations of caffeine, it must have a 1-*N*-methyltransferase that converts theobromine to caffeine. The genes encoding 1-*N*-methyltransferase were isolated from immature coffee endosperm (24, 27). The recombinant proteins had both 1-*N*- and 3-*N*-methyltransferase activity, and are therefore caffeine synthases. The 1-*N* specific methyltransferase gene was not isolated. When dimethylxanthines were used as substrates, paraxanthine was the best methyl acceptor, followed by theobromine. Of three monomethylxanthines investigated,

7-methylxanthine was methylated the most readily. The order of the purine base with coffee caffeine synthase was *N*-3>*N*-1>>*N*-7, suggesting that it was same as tea caffeine synthase. However, the relative activity for theobromine in coffee caffeine synthase was higher than that in tea caffeine synthase (24, 27).

Expression of CtCS series was demonstrated in young coffee leaves, flower buds and developing fruits by semi-quantitative RT-PCR designed for specific amplification and detection of these transcripts (23, 24). The genes of *CmXRS1*, *CTS2 and CCS1* were expressed in all organs. The levels of all three transcripts were highest in the developing endosperm. Although *CTS2* was expressed strongly in flower buds, the expression of *CmXRS1* and *CCS1* was weaker. Moreover, a smaller amount of *CTS2* transcripts was detected in young leaves than those of *CmXRS1* and *CCS1*. In addition, the expression patterns of *CmXRS1* and *CCS1* were synchronized, while that of *CTS2* was not. These findings may indicate that different caffeine synthetic pathways are involved in the differential expression of *CmXRS1* and *CTS2* in the young leaves and endosperms of coffee.

3.5. The characteristic future of *N*-methyltransferase in theobromine-accumulating species

Some purine alkaloid producing species accumulate theobromine rather than caffeine. This is the case in *Theobroma cacao* (28), *Camellia irrawadiensis* (29) and *Camellia ptilophylla* (30). Caffeine synthase in these species may possess 3-*N*- but not 1-*N*-methylation activity. In keeping with this possibility, a desalted extract from *C. ptilophylla* has been shown to methylate 7-methylxanthine but not the dimethylxanthines, theobromine, theophylline and paraxanthine (30). A homologous gene (*BTS1*) to caffeine synthase was cloned from *Theobroma cacao* by RT-PCR and RACE technique (31). Expression plasmids for *BTS1* were constructed in pET23d vector and recombinant BTS1 were produced in *E. coli* (BL21). When the bacterial lysate was incubated with a variety of xanthine derivatives in the presence of [methyl-^{14}C] SAM, BTS1 catalyzed 3-*N*-methylation of 7-methylxanthine, suggesting that BTS1 was theobromine synthase. The accumulation of purine alkaloids is therefore, depend on *N*-methyltransferase substrate specificity

4. CAFFEINE SYNTHASE GENE FAMILY IN PLANTS

4.1. Comparison of the amino acid sequences of caffeine synthase family in tea and coffee

The predicted amino acid sequences of CtCS series derived from *C. arabica* are more than over 80% homologous those of the clones and but share only 40% homology with share TCS1 derived from *C. sinensis*. This indicates that the caffeine biosynthetic pathways in coffee and tea might have evolved independently, consistent with different catalytic properties of the enzymes involved. The three conserved motifs (A, B and C) of the binding site of the methyl donor SAM have been reported in most plant SAM-dependent *O*-methyltransferases (OMTs) (32). The caffeine synthase gene family shares four highly conserved regions, motif A, motif B', motif C and YFFF region, but lacks motif B. The consensus sequence of motif B' region, which is located between motif A and motif C, is 'LNDL F/P XNDFN' containing a large number of hydrophilic amino acids like as asparagine and aspartic acid. On the other hands, the consensus sequence of YFFF region, which is located downstream to motif C, is 'AYXXQFXXDFXXFL' containing bulky amino acid (24). These four regions may have important roles both in the common *N*-methylating catalytic reaction and in discriminating the position of *N*-methylation of the purine ring (Figure 2).

In CtCS series, the clones, which have similar specific activities, are highly homologous with each other. For example, the amino acid sequence of CmXRS1, which was identified as only 7-methylxanthosine synthase, was compared with those of the other CtCS series, CmXRS1 lacked 13-residue sequence at the C-terminal region (24). The site for a 13-residue sequence, lacking from CmXRS1, probably locates the substrate-binding region closely, and forms a space for binding the ribose of xanthosine as a substrate. When the amino acid sequences of CCS1 and CCS2 were compared with those of CtCS series, CCS1 and CCS2 shared a high degree of sequence identity (approximately 80%) with this family. The amino acid sequence of CTS2 had the highest sequence identity (82.6%) to those of CCS1 and CCS2. The sequence homology between CCS1 and CCS2 was 94.5%, and 21 of the 384 amino acids are substituted. Recently, 10 kinds of sequences of CtCS homologs derived from *C. arabica*, *Coffea canephora*, and *Coffea liberica* have been submitted to the DDBJ sequence database. A comparison of the amino acid sequence of CCS1 with those, these homologs showed that three sequences isolated from *C. liberica* (AF494418, AF494419 and AF494420), by Kretschmar and Baumann have high sequence homology (ca. 93%) with CCS1. It is, thus, likely that these three proteins corresponded to CCS1 or CCS2 in *C. liberica*. Since it is shown that among *Coffea* species, *C. arabica* is the only amphidiploid cultivar (2n = 44), and that *C. liberica* is diploid with 22 chromosomes (33), possibly unidentified genes that are very similar to CCS1 or CCS2 may be present in *C. arabica*.

TCS1 and CtCS series lack typical plastid-targeting sequences at their amino terminus, suggesting that they are probably located in extra-chloroplast cytoplasm. These structures were consistent with the results obtained from transit expression of CaMXMT in onion epidermal layer (26).

4.2. Motif B' methyltransferase family in plants

The number of sequenced genes encoding SAM-dependent methyltransferases in plants is growing rapidly. Joshi and Chiang indicated the conserved amino acid motifs of plant methyltransferases were not consistent with that of animal sources (34) according to the search for 56-plant genes/cDNA (32). They proposed there were three conserved motifs (A, B and C) of the binding site of the

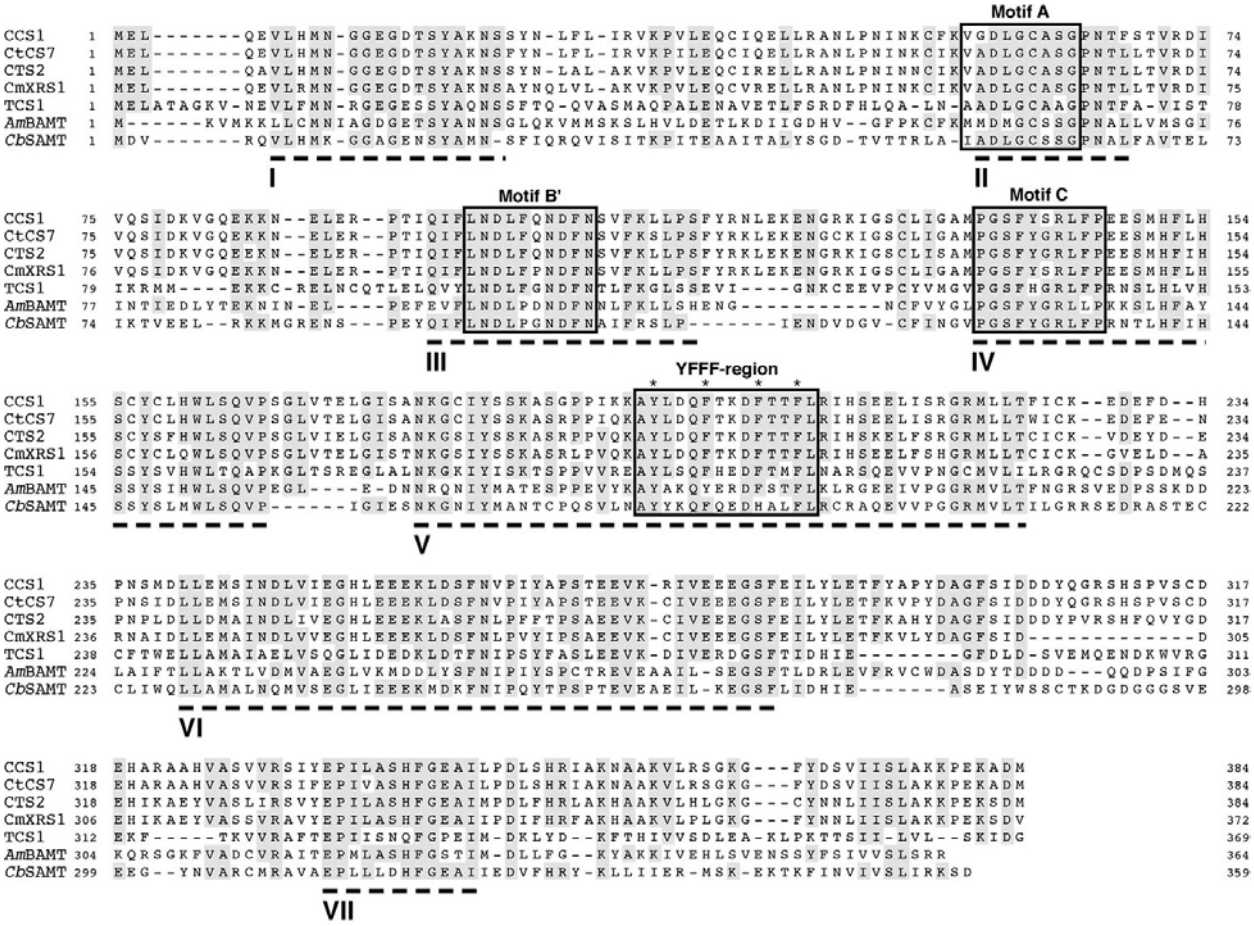


Figure 2. Comparison of the amino acid sequences of caffeine synthase family with related motif B' methyltransferases. The amino acid sequences of CCS1, CtCS7, CTS2 and CmXRS1 correspond to those in Table 2. Other gene names and accession numbers are as follows: TCS1, tea caffeine synthase 1, AB031280; AmBAMT, *Antirrhinum majus* benzoic acid carboxyl methyltransferase, AF198492; CbSAMT, *Clarkia breweri* salicylic acid crboxyl methyltransferase, 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 of nominated as "YFFF-region" are shown by open boxes. The conserved sequences in the motif B' methyltransferase family are indicated by dotted lines (I, II, III, IV, V, VI, and VII).

methyl donor SAM in plant methyltransferase (32). Recently, a novel family of methyltransferases including caffeine synthase has been identified in plants on the grounds that there is no motif B in members of this family. Most of members of this newly characterized methyltransferase family catalyze the formation of small-molecule methyl esters using SAM as methyl donor and substrates with a carboxyl group as methyl acceptors. These enzymes include SAMT (SAM: salicylic carboxyl methyltransferase) (35-37), BAMT (SAM: benzoic acid carboxyl methyltransferase) (38) and JAMT (SAM: jasmonic acid carboxyl methyltransferase) (39). Furthermore, NTR1, which was floral nectary-specific protein from *Brassica campestris* L. sp. *Pekinensis* (40), and many hypothetical proteins encoding genome sequences of *Arabidopsis thaliana* were considered as members of this family. Mizuno *et al*. pointed out the occurrence of motif B' instead of motif B in this family and referred to them as motif B' methyltransferase family (24). (Figure 3) Compared with the motif B' methyltransferases, TCS1, CtCS series, SAMT, NTR1, BAMT and JAMT share a low amino acid sequence homology (*ca.* 40%) (24) (Table 4).

Figure 4 shows the phylogenetic tree analysis of motif B' methyltransferase family and other plant *N*-methyltransferases. In particular, aspartic acid and leucine, which were present in the central part of motif B', were indicated SAM binding residues (42). The members of motif B' methyltransferases are all *O*-methyltransferases except *N*-methyltransferases involved in caffeine biosynthesis. TCS1 has only an overall 40% homology to CtCS series and this value is equivalent to other motif B' methyltransferases, suggesting that the caffeine biosynthetic pathway evolved in parallel in tea and coffee. CtCS3 and CtCS4, the activities of which are unknown, formed a branch separated from the other CtCS series. Moreover, *N*-methyltransferases involved in caffeine biosynthesis were more closely related to motif B' carboxylmethyltransferase than to plant *N*-methyltransferases involved in the biosynthesis of nicotine (41) and coclaurine (42). The evolution of *N*-methyltransferase

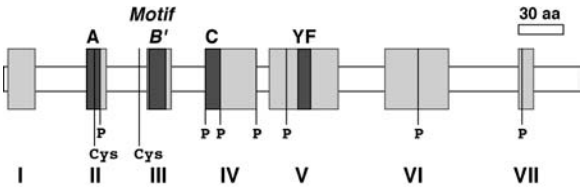


Figure 3. Schematic diagram of homologous regions of motif B' methyltransferase family. The conserved sequences in motif B' methyltransferase family are indicated by open boxes (I, II, III, IV, V, VI, and VII). The proposed SAM-binding motifs (A, B' and C) and the conserved region nominated as "YFFF-region" (YF) shown by closed-boxes. Conserved amino acid residues, proline and cysteine, are indicated by P and Cys, respectively.

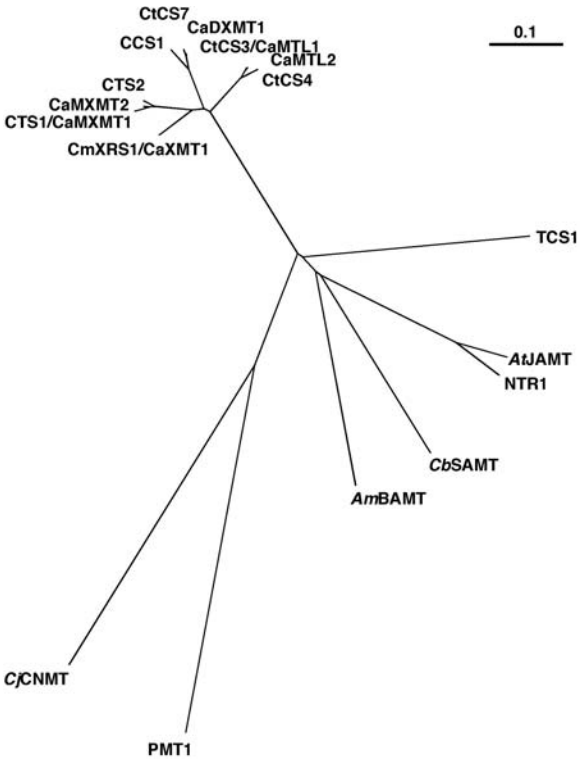


Figure 4. Unrooted phylogenetic-tree analysis of caffeine synthase family with related methyltransferases. The alignment of protein sequences and the unrooted tree were constructed with ClustalW on the web site: http://clustalw.genome.ad.jp/. Sources of the sequences of coffee caffeine synthase family (CmXRS1, CTS1, CTS2, CCS1, CtCS7, CtCS3, CtCS4, CaXMT1, CaMXMT1, CaMXMT2, CaDXMT1, CaMTL1 and CaMTL2) indicate accession numbers in Table 2. Sources of the sequences of the motif B' methyltransferases and the others are as follows: TCS1, tea caffeine synthse 1, AB031280; *At*JAMT, *Arabidopsis thaliana* jasmonic acid methyltransferase, AY008434; NTR1, Floral nectary-specific protein from *Brassica campestris* L. sp. *pekinensis*, AF179222; *Cb*SAMT, *Clarkia breweri* salicylic acid carboxyl methyltransferase, AF133053; *Am*BAMT, *Antirrhinum majus* benzoic acid carboxyl methyltransferase, AF198492; PMT1, *Nicotiana tabacum* putrescine *N*-methyltransferase, D28506; *Cj*CNMT, *Coptis japonica* coclaurine *N*-methyltransferase, AB061863.

methyltransferase and *O*-methyltransferase appear to be closely related.

4.3. Structural basis for substrate recognition in caffeine synthase gene family

The apparent molecular mass of the native caffeine synthase which was purified from young tea leaves was 61 kD, as estimated by gel-filtration chromatography, and 41 kD, as estimated by SDS-PAGE (17). The molecular mass estimated from the predicted amino acid sequence of TCS1 is 41kD and it agrees well with the size of CtCS series (20, 23-26). Most of motif B' methyltransferases were thought to function as the dimeric form *in vivo* by Zubieta *et al*. (43). They clarified the 3.0-Å crystal structure of *Clarkia breweri* SAMT in complex with the substrate salicylic acid, and the demethylated product *S*-adenosyl-L-homocysteine has a protein structure that possesses a helical active site capping domain and a unique dimerization interface and proposed the structural model of TCS1 and 7-MXMT. The positioning of the 7-methylxanthine and theobromine molecules in the modeled active sites was accomplished by preserving the hydrogen-bonding pattern that brackets the reactive carboxyl group in motif B' *O*-methyltransferases. This functional dimeric model of caffeine synthase needs to be investigated in detail.

5. FURTHER PROSPECT

The rate of caffeine biosynthesis appears to be regulated primarily by *N*-methyltransferase activity. In coffee, three types of *N*-methyltransferase have been identified, but the collaboration of theobromine synthase and caffeine synthase in overall caffeine biosynthesis was not clarified. The enzyme activities and the transcripts of *N*-methyltransferases were correlated by developmental senescence, suggesting that gene expression of them was specifically controlled. To ascertain the *cis*-element and the transcription factor of *N*-methyltransferases, the analysis of genomic clones is now in progress by our group.

Furthermore, the cloning of the caffeine synthase genes is an important advance towards the production of transgenic caffeine-deficient tea and coffee through gene silencing with antisense mRNA and RNA interference. The possible uses of genetic engineering to suppress caffeine biosynthesis are further discussed by Ashihara and Crozier (44).

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Table 4. Percentages of sequence identity among motif B' methyltransferases

	CmXRS1	**CTS1**	**CTS2**	**CCS1**	**CtCS7**	**CtCS3**	**CtCS4**	**TCS1**	***Cb*SAMT**	***Am*BAMT**	***At*JAMT**	**NTR1**
CmXRS1	100	84.4	85.2	81.0	82.1	82.9	81.6	39.5	40.6	37.4	37.8	37.7
CTS1		100	93.2	80.2	81.0	80.8	80.0	39.5	39.7	37.1	38.6	38.6
CTS2			100	82.6	83.1	82.9	82.3	38.4	39.1	37.2	37.8	39.2
CCS1				100	94.5	82.3	80.8	38.9	38.4	37.7	37.1	38.7
CtCS7					100	82.1	81.0	38.9	40.2	37.8	37.6	38.8
CtCS3						100	95.6	40.7	39.5	36.3	39.1	39.0
CtCS4							100	40.3	40.0	37.1	37.8	39.0
TCS1								100	41.8	38.5	39.1	38.4
*Cb*SAMT[a]									100	43.9	43.0	42.8
*Am*BAMT[a]										100	40.5	41.3
*At*JAMT[a]											100	85.0
NTR1[a]												100

[a] Gene names and accession numbers as follows: *Cb*SAMT, *Clarkia breweri* salicylic acid carboxyl methyltransferase, AF133053; *Am*BAMT, *Antirrhinum majus* benzoic acid methyltransferase, AF198492; *At*JAMT, *Arabidopsis thaliana* jasmonic acid carbozyl methyltransferase, AY008434; NTR1, Floral nectary-specific protein from *Brassica campestris* L. sp. *pekinensis*, AF179222

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