Essential Region for 3-*N* Methylation in *N*-Methyltransferases Involved in Caffeine Biosynthesis

Kouichi Mizuno[a,*], Shin-ichi Kurosawa[a], Yuko Yoshizawa[a], and Misako Kato[b]

[a] Faculty of Bioresource Sciences, Akita Prefectural University, Akita City, Akita 010–0195, Japan. Fax: + 81-18-872-1676. E-mail: koumno@akita-pu.ac.jp
[b] Graduate School of Humanities and Sciences, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112–8610, Japan

* Author for correspondence and reprint requests

Z. Naturforsch. **65c**, 257–265 (2010); received December 2, 2009/January 8, 2010

The caffeine biosynthetic pathway is composed of three methylation steps, and *N*-methyltransferase catalyzing each step has high substrate specificity. Since the amino acid sequences among coffee 7-methylxanthosine synthase (*CmXRS1*), theobromine synthase, and caffeine synthase are highly homologous to each other, these substrate specificities seem to be determined in a very restricted region. The analysis of site-directed mutants for CmXRS1 that naturally acts at the initial step, *i.e.* 7-*N* methylation of xanthosine, revealed that the activity of 3-*N* methylation needs a histidine residue at corresponding position 161 in the CmXRS1 sequence. We succeeded in producing the mutant enzyme which can catalyze the first and second methylation steps in caffeine biosynthesis.

Key words: Coffee, Caffeine, *N*-Methyltransferase

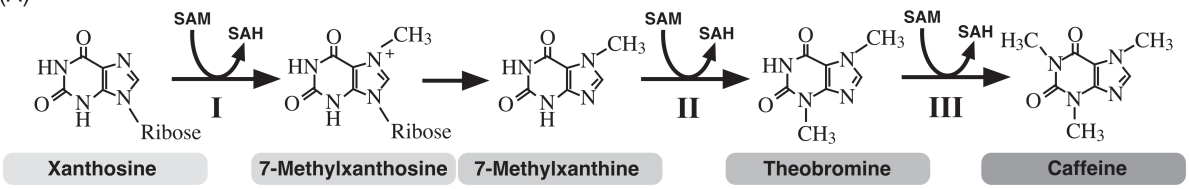
Introduction

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid present in high concentration in tea and coffee. Extensive metabolic studies with purine alkaloids have elucidated the caffeine biosynthetic pathway in some detail in tea and coffee. Caffeine is synthesized from xanthosine via a pathway that has three *S*-adenosyl-L-methionine (SAM)-dependent methylation steps (Fig. 1). Three methylations of the purine base occur in order of 7-*N*, 3-*N* and 1-*N* (Suzuki *et al.*, 1992; Ashihara *et al.*, 1997; Kato *et al.*, 1996). In addition, 7-methylxanthine is converted to paraxanthine by 1-*N* methylation and paraxanthine is converted to caffeine by 3-*N* methylation by a minor pathway operating tea leaves (Kato *et al.*, 1999). We identified and characterized the genes encoding enzymes for these three methylation steps of caffeine biosynthesis (Mizuno *et al.*, 2001, 2003a, b; Kato and Mizuno, 2004). Although these genes, named coffee 7-methylxanthosine synthase (*CmXRS1*), coffee theobromine synthase (*CTS1* and *CTS2*), and coffee caffeine synthase (*CCS1*), are highly homologous to each other (80–85% identities), the substrate specificities of these gene products are strictly divided. Caffeine biosynthesis is presumed to begin with CmXRS1 converting xanthosine to 7-methylxanthosine. CmXRS1 is 7-methylxanthosine synthase which can catalyze only the first methylation step (Mizuno *et al.*, 2003b). CTS1 and CTS2 are theobromine synthases which catalyze only the second methylation step. The substrate of theobromine synthase *in vivo* is assumed to be only 7-methylxanthine. CCS1, like tea TCS1 (Kato *et al.*, 2000), is caffeine synthase which can catalyze the second and third methylation steps (Mizuno *et al.*, 2003a). 7-Methylxanthosine synthase shows the most critical substrate specificity of the three types of *N*-methyltransferases. Theobromine synthase is 3-*N*-methyltransferase and so 7-methylxanthine is the best substrate (Mizuno *et al.*, 2001). However, minor activity against 3-*N* methylation of paraxanthine is present in theobromine synthase. On the other hand, paraxanthine is the best methyl acceptor for caffeine synthase. The order of activity of the purine base with caffeine synthase is *N*-3 > *N*-1 >> *N*-7. However, the relative activity of theobromine in CCS is higher than that in TCS (Mizuno *et al.*, 2003a; Uefuji *et al.*, 2003).

Abbreviations: CCS, coffee caffeine synthase; CtCS, coffee tentative caffeine synthase; CTS, coffee theobromine synthase; CmXRS, coffee 7-methylxanthosine synthase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosyl-L-methionine; TCS, tea caffeine synthase.

0939–5075/2010/0300–0257 $ 06.00

(A)



(B)

	I 7-Methylxanthosine synthase	II Caffeine synthase (Theobromine synthase)	III Caffeine synthase
Coffee	CmXRS1	CTS1, CTS2, CCS1	CCS1
Tea	unidentified	TCS1	TCS1

Fig. 1. Pathways for the biosynthesis of caffeine. Numbers (I, II and III) in (B) correspond to the reactions I, II and III in (A). TCS1 and CCS1 have broad substrate specificities and catalyze the conversion of 7-methylxanthine to caffeine via theobromine [reactions II and III in (A)].

The substrate specificity in theobromine synthase and caffeine synthase is dependent on a single amino acid in *Camellia* plants (Yoneyama *et al.*, 2005). 7-Methylxanthosine synthase has not been identified in *Camellia* plants; therefore, there is no evidence that 7-*N* methylation of the purine base depends on a single amino acid as is the case in *Camellia*. It is quite likely that the specific sequence of 7-methylxanthosine synthase contributes to the selection of 7-methylxanthosine since there is an extra sequence close to the carboxylterminal region only in CmXRS1. Moreover, xanthosine, which is the only substrate for 7-methylxanthosine synthase, is a nucleoside. 7-Methylxanthosine synthase cannot convert free xanthine to 7-methylxanthine. It seems probable that 7-*N* methylation is due to the three-dimensional structure of the substrate and 7-*N* methylation is distinct from 3-*N*/1-*N* methylations.

Recently, the three-dimentional structure of *Clarkia breweri* salicylic acid carboxyl methyltransferase (CbSAMT) that belongs to the motif B' methyltransferase family was elucidated by Zubieta *et al.* (2003). The sequence of caffeine synthase shares approx. 40% amino acid homology with CbSAMT, and the sequence of the SAM-binding region that composes the active site is highly conserved among these enzymes. The structure of the salicylic acid binding site of CbSAMT that corresponds to the 7-methylxanthine or paraxanthine binding site of caffeine synthase has also been identified in *Camellia* plants (Yoneyama *et al.*, 2005). The aim of the present study was to prove the origin of the substrate specificity in 7-methylxanthosine synthase in coffee and to discuss the variation in *N*-methyltransferases involved in caffeine biosynthesis.

Material and Methods

Materials

Oligonucleotide primers for PCR mutagenesis were purchased from Sigma-Aldrich (www.genosys.jp). We used Taq-polymerase *ExTaq* (TaKaRa Bio Inc., Otsu, Japan). [Methyl-^{14}C]SAM (2.00 GBq/mmol) was purchased from GE Healthcare Bio-Science Corp. (Piscataway, NJ, USA). All other reagents were of the highest purity available.

Construction of expression plasmids

Plasmids for expressing *CmXRS1*, *CCS1* and these mutants in *Escherichia coli* were constructed in the pET23d vector (Merck, Darmstadt, Germany) (Mizuno *et al.*, 2001, 2003a, b). As the pET23d vector carries an optimal C-terminal His-Tag sequence, the 3'-termination sites of CmXRS1- and CCS1-cDNA were replaced by an *Xho*I restriction site using polymerase chain

reaction (PCR)-directed mutagenesis, and the expression plasmids for *CmXRS1* and *CCS1* carrying the C-terminal His-Tag (named pET23d-CmXRS1 and pET23d-CCS1, respectively) were constructed.

Preparation of mutant enzymes

For the construction of the CCS1-deletion mutant, pET23d-CCS1 was amplified with CCS-N1/CS1delR (for 5' region) and CS1delF/CS1-CT (for 3' region) as primers. The mutagenetic primers are shown in Table I. The PCR was conducted in a thermal cycler for 30 cycles (94 ºC for 60 s, 55 ºC for 60 s, and 72 ºC for 90 s). To obtain the full length of the deletion mutant, we used CCS-N1 and CS1-CT as primers and the 5' region and the 3' region, which were purified by PAGE, as templates. The reaction program consisted of 30 cycles of 94 ºC for 60 s, 55 ºC for 60 s, and 72 ºC for 120 s. The resulting fragment was introduced into pGEM-T easy vector (Promega Corp., Madison, WI, USA). The subclone of the PCR product was digested with *Hin*dIII and *Xho*I, and then the *Hin*dIII/*Xho*I fragment was replaced into pET23d-CCS1 at the *Hin*dIII and *Xho*I sites. For construction of *CmXRS1* mutants, site-directed mutagenesis of the specific amino acid was carried out using the PCR method (Mizuno *et al.*, 2003b). External primers for five *CmXRS1* mutants, which had a single amino acid replacement, were CtCS-N1 (5' end) and CtCS-CT (3' end), and pET23d-CmXRS1 was used as a template. The mutagenetic primers for each mutation were designed in complemental pairs as follows: CmXRS1-P104Qf/CmXRS1-P104Qr (P^{104} to Q, named m1), CmXRS1-Q161Hf/CmXRS1-Q161Hr (Q^{161} to H, named m2), CmXRS1-L191Pf/CmXRS1-L191Pr (L^{191} to P, named m3), and CmXRS1-H219Rf/CmXRS1-H219Rr (H^{219} to R, named m5). Except for CmXRS1-A23Sf (A^{23} to S, named m4), as the oligonucleotide contains the internal *Eco*RI site, we used the *Eco*RI site for reconstruction of the mutated plasmid. For example, for construction of the m1 mutant, the pairs of primers were as follows: CtCS-N1/CmXRS1-P104Qr and CmXRS1-P104Qf/CtCS-CT for the 5' region and the 3' region of m1, respectively. To obtain the full length of the site-directed mutant, we used CtCS-N1 and CtCS-CT as primers and the 5' region of m1 and the 3' region, which were purified by PAGE, as templates. PCR was conducted under the same

Table I. Oligonucleotides for site-directed mutagenesis.

Name	Sequence (5' to 3' direction)	Sense/antisense	Method	Source
CCS-N1	CCATGGAGCTCCAAGAAGTCC	Sense	Construction of expression plasmid	CCS1
CS1delR	TGTTCATCATCAATAGAGAAGCC	Antisense	Mutagenesis for deletion	CCS1
CS1delF	TTGATGATGAACATGCTAGAGCAGC	Sense	Mutagenesis for deletion	CCS1
CS1-CT	GCTCGAGCATGTCTGMCTTCTCYGG	Antisense	Construction of expression plasmid	CCS1
CtCS-N1	CCATGGAGCTCCAAGCAGTCC	Sense	Construction of expression plasmid	CmXRS1
CtCS-CT	GCTCGAGCACGTCTGACTTCTCTGG	Antisense	Construction of expression plasmid	CmXRS1
CmXRS1-P104Qf	TGATCTTTTCCAGAATGATTTC	Sense	Site-directed mutagenesis	CmXRS1
CmXRS1-P104Qr	TTGAAATCATTCTGGAAAAGATC	Antisense	Site-directed mutagenesis	CmXRS1
CmXRS1-Q161Hf	CTGTCTTCATTGGTTATCTC	Sense	Site-directed mutagenesis	CmXRS1
CmXRS1-Q161Hr	GAGATAACCAATGAAGACAG	Antisense	Site-directed mutagenesis	CmXRS1
CmXRS1-L191Pf	GTCGACCACCCGTCCAGAAGGC	Sense	Site-directed mutagenesis	CmXRS1
CmXRS1-L191Pr	CGGGTGGTCGACTTGCTTTG	Antisense	Site-directed mutagenesis	CmXRS1
CmXRS1-A23Sf	AGAATTCATCCTACAATCAAC	Sense	Site-directed mutagenesis	CmXRS1
CmXRS1-H219Rf	GTTGTTTTCACGTGGCCGAATG	Sense	Site-directed mutagenesis	CmXRS1
CmXRS1-H219Rr	CATTCGGCCACGTGAAAACAAC	Antisense	Site-directed mutagenesis	CmXRS1

conditions as in the case of the CCS1-deletion mutant. The PCR product was purified by PAGE and was subcloned into pGEM-T easy vector. Four other mutants were produced similarly. Site-directed mutants m6 and m7, which had double amino acid replacement, were constructed from the m2 mutant as a template by PCR mutagenesis using primer pairs CtCS1-N1/CmXRS1-P104Qr and CmXRS1-P104Qf/CtCS-CT. Mutant m8 was constructed from mutant m1 as a template using primer pairs CtCS1-N1/CmXRS1-L191Pr and CmXRS1-L191Pf/CtCS-CT. Mutant m12 was constructed from mutant m6 as a template using primers like m8. The subclones of the PCR products were digested with *Nco*I and *Xho*I. The *Nco*I/*Xho*I fragment was introduced into pET23d at the *Nco*I and *Xho*I sites. For mutants m9, m10, m11, m13, m14 and m15, we used m1, m2, m3, m6, m8 and m12 as templates, respectively, with the same primer pairs CmXRS1-A23Sf/CmXRS1-H219Rr (for 5' region) and CmXRS1-H219f/CtCS-CT (for 3' region). To obtain the full length of the mutants, we used CmXRS1-A23Sf and CtCS-CT as primers and the fragments of the 5' and 3' region as templates. Because the primer CmXRS1-A23Sf has an *Eco*RI site, the subclones of the PCR products were digested with *Eco*RI and *Xho*I, and then the *Eco*RI/*Xho*I fragments were replaced into pET23d-CmXRS1 at the *Eco*RI and *Xho*I sites. The fidelity of the PCR reactions and the mutants were confirmed by DNA sequencing.

Production of recombinant enzymes

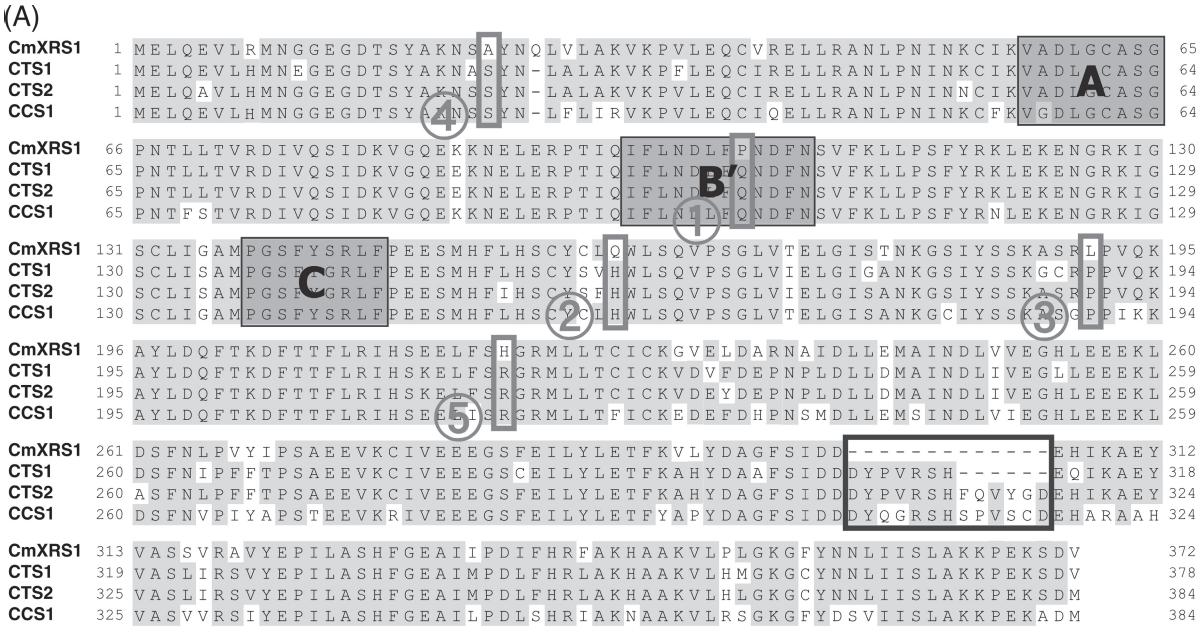
These expression plasmids were introduced into *E. coli* BL21 (DE3). A single colony of the transformants was cultured at 37 ºC overnight in 3 ml of Luria broth containing 0.2 mg/ml ampicillin (LA) with vigorous shaking. A portion (2 ml) of the bacterial culture was added to 100 ml of fresh LA, and incubated at 37 ºC for 2 h with constant shaking (100 rpm/min). To produce the recombinant protein, 300 μl of isopropyl-β-D-thiogalactopyranoside (final concentration 0.3 mM) were added, and the cells were kept at 25 ºC for 8 h. *E. coli* cells were harvested by centrifugation at 500 × *g* for 5 min, and then washed with 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 2 mM 2-mercaptoethanol (named TES + ME). Before sonication, the cell paste was suspended in 1 ml of TES + ME and frozen at –80 ºC. Then it was sonicated and subsequently centrifuged at 10,000 × *g* for 10 min at 4 ºC. The supernatant was assayed for *N*-methyltransferase.

Determination of enzyme activity

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blot analysis using an anti-CTS2 antibody showed that the recombinant proteins were produced in soluble protein fractions (Mizuno *et al.*, 2003a). The concentration of the recombinant enzyme was determined by Western blot-based densitometric analysis using ImageJ software (http://rsb.info.nih.gov/ij/). The enzymatic activity of caffeine synthase was determined using [methyl-^{14}C]SAM as described previously with a slight modification (Kato *et al.*, 1999). The final concentration of the substrate (purine derivatives) was 1 mM when mutated enzymes were used. Thin layer chromatography (TLC) was carried out as described previously (Kato *et al.*, 1996; Zheng and Ashihara, 2004), except that we used *n*-butanol/acetic acid/water (4:1:2, v/v/v) as the developing solvent. The 7-*N* methylation activity of CmXRS1 and its mutants was determined by high performance liquid chromatography (HPLC). A reaction mixture (100 μl), containing 100 mM Tris-HCl (pH 7.5), 0.2 mM $MgCl_2$, 0.2 mM SAM, 3 mM xanthosine and 50 ~ 90 ng/μl recombinant enzymes, was incubated at 27 ºC for 1 h and diluted with 900 μl of water. 20 μl of the diluted reaction mixture was applied to HPLC using an Inertsil® ODS-3 column (Ø4.6 × 150 mm, GL Sciences, Tokyo Japan) with a flow rate of 1 ml/min of methanol/acetic acid/water (20:5:75, v/v/v), and then monitored for absorbance at 272 nm.

Analytical procedure

Protein concentrations were measured by the method of Bradford (1976). Nucleotide sequencing was carried out using an ABI PRISM 3100 (Life Technologies, Carlsbad, CA, USA) genetic analyzer and was conducted at the Life Research Support Center of Akita Prefectural University. Nucleotide and protein sequences were analyzed by computer using GENETYX software (Genetyx Co., Tokyo, Japan).



(B)

	Activity	④	①	②	③	⑤
CmXRS1	I	A	P	Q	L	H
CTS1, CTS2	II	S	Q	H	P	R
CCS1	II · III	S	Q	H	P	R

(C) **Constructed mutants**

m1:	CmXRS1:P104Q	m9:	CmXRS1:A23S:P104Q:H219R
m2:	:Q161H	m10:	:A23S:Q161H:H219R
m3:	:L191P	m11:	:A23S:L191P:H219R
m4:	:A23S	m12:	:P104Q:Q161H:L191P
m5:	:H219R	m13:	:A23S:P104Q:Q161H:H219R
m6:	:P104Q:Q161H	m14:	:A23S:Q161H:L191P:H219R
m7:	:P104Q:L191P	m15:	:A23S:P104Q:Q161H:L191P:H219R
m8:	:Q161H:L191P		

Fig. 2. Sequence alignment of caffeine synthetic enzymes from coffee and constructs generated by site-directed mutagenesis. (A) Sequence alignment of the amino acid sequences of caffeine synthetic enzymes from coffee. Shaded boxes represent conserved amino acid residues, and dashes represent gaps that have been optimal aligned. Boxes A, B' and C indicate SAM-binding regions. The CCS-CTS extended region is indicated by the large open box. The residues introduced by point mutation are shown by open boxes numbered from 1 to 5. (B) Relations among enzyme, activity and the amino acid residues. (C) Combination of point mutation and constructed mutants.

Results

Characterization of substrate preference of CCS1-deletion mutant

The amino acid sequences of CmXRS1, CTS1, and CCS1 had high homology (80–85% identities) with each other (Fig. 2A). Thus, as these substrate specificities are strictly divided when these genes are highly homologous, it was speculated that the different regions of the amino acid residues of each enzyme correlate with the substrate specificities. As shown in Fig. 2A, when the carboxylterminal region of CmXRS1 was compared with those of CCS1 and CTS1, the specific region was present in CCS1 and CTS1, and this region was deleted in CmXRS1. We prepared a mutant with deletion in the region that corresponded to the 13-amino acid residues of CCS1 and analyzed whether the deletion mutant, named CCS1-delC13, has the same 7-*N* methylation activity as CmXRS1 or not. As shown in Table II, the relative activities estimated by using [methyl-^{14}C]SAM were indicated as the percentage of the activity with 7-methylxanthine. The deletion mutant had no 7-*N* methylation activity against xanthosine. The enzyme of the deletion mutant may have higher affinity to paraxanthine (3-*N* methylation) and theobromine (1-*N* methylation) than the wild-type enzyme, since it showed higher activity with these substrates than the wild-type enzyme.

Relative activity[a] (7mX = 100%)	Substrate/methylation position[b]			
	7mX/3*N*	Px/3*N*	Tb/1*N*	XR/7*N*
CCS1	100	416	102	-
CCS1-deletion mutant	100	1458	267	-

Table II. Activities of CCS1-deletion mutant.
[a] The relative activity is indicated as the percentage of the activity with 7mX.
[b] 7mX, 7-methylxanthine; Px, paraxanthine; Tb, theobromine; XR, xanthosine.

Presumably the presence of this region has great influence on 3-*N* and 1-*N* methylation activity.

Changes in the substrate preference of CmXRS1 by site-directed mutagenesis

Since Zubieta *et al.* (2003) reported the 3.0-Å crystal structure of CbSAMT which belongs to motif B' methyltransferase in the complex with the substrate salicylic acid, and the demethylated product *S*-adenosylhomocysteine (SAH) revealed a protein structure that possesses a helical active site capping domain, we referred to the information and produced CmXRS1 site-directed mutants by replacement of five amino acid residues (Fig. 2B). We produced 15 types of mutants (m1–m15) combined with five site-directed mutations (Fig. 2C). Fig. 3 shows the specific activities detected by incubation of the recombinant enzymes from 8 mutants of CmXRS1 with paraxanthine or 7-methylxanthine in the presence of [methyl-^{14}C] SAM as a methyl donor. Consequently, it is apparent that the mutants of CmXRS1, that have 3-*N* methylation activity and produce caffeine from paraxanthine as a substrate, need to have replacement of the glutamine residue by histidine at position 161 in the CmXRS1 sequence (Q161H mutation). 3-*N* Methylation activity was not detected in mutants of m1 (P104Q), m3 (L191P), m4 (A23S), and m5 (H219R). Neither the double nor triple mutants that did not have replacement of Q161H, *i.e.* m7 (P104Q + L191P), m9 (A23S + P104Q + H219R) and m11 (A23S + L191P + H219R) mutants, exhibited 3-*N* methylation activity. On the other hand, the double and triple mutants containing Q161H mutation had higher activities than the single Q161H mutant. Especially, m6 (P104Q + Q161H) and m8 (Q161H + L191P) mutants had, respectively, 5- and 3.4-fold activity as compared to the m2 (Q161H) mutant (Fig. 3). The triple mutant m12 (P104Q + Q161H + L191P), which resembled m6 and m8 combined, showed the same level of 3-*N* methylation activity as the m6 mutant, while m10 (A23S + Q161H + H219R) had only the same level of activity as the m2 mutant. The activities of the quadruplex-replaced mutants m13 (A23S + P104Q + Q161H + H219R) and m14 (A23S + Q161H + L191P + H219R) were less than those of either m6 (double mutant) or m12 (triple mutant). However, m15 (A23S + P104Q + Q161H + L191P + H219R) with five residues replaced had the highest activity among all mutants; approx. 6.6-fold the activity of the m2 mutant (Fig. 3). The activities of the mutants using 7-methylxanthine as a substrate were lower than those of the mutants using paraxanthine as a substrate, but the profile of the specific activity against paraxanthine from 8 mutants was consistent with the profile of that against 7-methylxanthine (Fig. 3). No 1-*N* methylation activity could be detected when theobromine was used as a substrate. The obtained results suggest that the additional activity introduced by the mutagenesis is not 1-*N* methylation but 3-*N* methylation activity. To confirm that 7-*N* methyl-

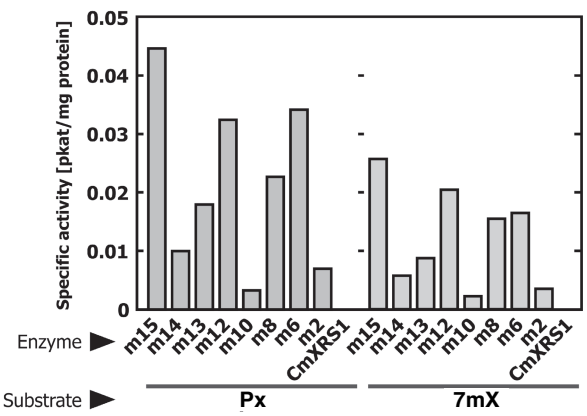


Fig. 3. Activities of CmXRS1 point mutants. As eight CmXRS1 mutants among fifteen mutants have 3-*N* or 1-*N* methylation activity, we analyzed the activities precisely and compared these specific activities. Although mutant m15 which introduced five-points mutations had the highest 3-*N* or 1-*N* methylation activity using paraxanthine or 7-methylxanthine as substrates, the values of the specific activities were approx. 1/700 that of CCS1. Since the activity using theobromine as a substrate was not detected, it seems that mutant m15 has mainly 3-*N* methylation activity.

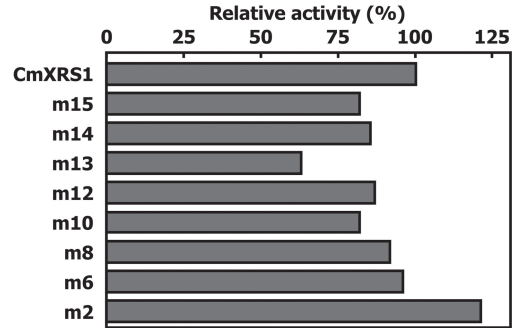


Fig. 4. 7-*N* Methylation activities of CmXRS1 mutants. Reactants treated with CmXRS1 mutants with xanthosine as a substrate were analyzed by HPLC. Relative activities of CmXRS1 mutants are indicated as the percentage of the produced 7-methylxanthine content with wild-type CmXRS1.

ation activity was maintained, the activities of the mutants that had 3-*N* methylation activity, using xanthosine as a substrate, were determined by HPLC (Fig. 4). Mutant enzymes had over 80% of 7-*N* methylation activity compared with the wild-type CmXRS1, except for mutant m13. Although the 3-*N* methylation activity was obtained in individual mutants, the 7-*N* methylation activity of the mutants remained.

Discussion

The histidine residue located at position 160 of CCS1 corresponding to glutamine at position 161 of CmXRS1 is suggested to be essential for 3-*N* methylation activity of caffeine synthase in coffee. Moreover, it appears that the 3-*N* methylation activity of CmXRS1 in the H161Q mutant is enhanced by the additional mutation of P104Q and L191Q in CmXRS1. As shown by the activity of the m10 mutant, the mutations of A23S and H219R of CmXRS1 are not supplementary to the 3-*N* methylation activity of mutant Q161H. As the m15 mutant which has all five residues replaced had the highest 3-*N* methylation activity while the activities of m13 and m14 (quadruplex-replaced mutants) were lower than those of either m6 or m8 (double-replaced mutants), it is assumed that the replacement of A23S + H219R affected the 3-*N* methylation activity of the CmXRS1 mutant under the mutational condition of P104Q + Q161H + L191P. Although the sequence identity between CCS1 which can catalyze 3-*N* methylation and CmXRS1 which does not have any activity is 80.8%, there is 82.1% sequence identity between the amino acid sequence of the m15 mutant with five amino acid residues of CmXRS1 replaced and that of CCS1. The 3-*N* methylation activity is shown by an 1.3% increase of sequence homology. When paraxanthine was used as a substrate, the specific activity of CCS1 was 33.1 pkat/mg protein but that of m15 was just 0.045 pkat/mg protein. The degree of 3-*N* methylation activity in the m15 mutant was no more than approx. 1/700 that of CCS1. As the most different region between CCS1 and CmXRS1 is a deletion sequence of the C-terminal 13-residue in CCS1, the deletion mutant of CCS1 lacking the 13-residue could not catalyze 7-*N* methylation. Furthermore, since the deletion mutant retained 3-*N* methylation activity in spite of lacking the 13-residue sequence, it is possible that 7-*N* methylation activity is correlated with several limited amino acid residues other than the 13-residue.

Amino acid residues related to substrate specificities were identified by the three-dimensional structure of salicylic acid methyltransferase (SAMT) which belongs to motif B' methyltransferases seen in caffeine synthases (Zubieta *et al.*, 2003). The amino acid residues that are bound to *S*-adenosyl-L-methionine (SAM) as a methyl donor are conserved in caffeine synthases, and the corresponding amino acid residues of caffeine synthases are thought to be related to the binding with SAM (Fig. 5). Moreover, using salicylic acid as a methyl acceptor in salicylate-methyl transferase, we found that the amino acid residues act in the binding with salicylic acid (Fig. 5). It is assumed that the corresponding amino acid residues of caffeine synthases, correlated to the binding with salicylic acid in SAMT, act as binding sites for xanthine derivatives as a methyl acceptor. Since the histidine residue located at position 160 (H160) of CCS1, which corresponds to glutamine at position 161 (Q161) of CmXRS1, is essential for 3-*N* methylation activity of caffeine synthase, the salicylic acid binding site in the SAMT sequence, seemingly the region, forms a binding site for xanthine derivatives of caffeine synthases. The obtained 3-*N* methylation activity against paraxanthine was higher than that against 7-methylxanthine, suggesting that H160 of CCS1 was essential for the broad 3-*N* methylation activity as caffeine synthase. However, CTS1 and CTS2 also have a histidine residue which corresponds to Q161 of CmXRS1; their 3-*N* methyla-

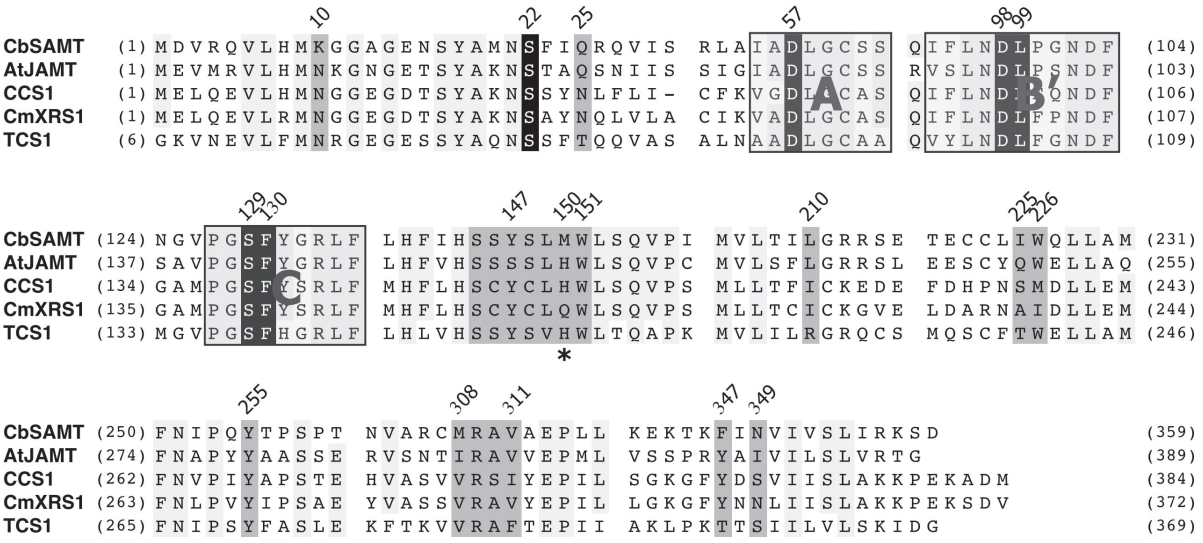


Fig. 5. Comparison of amino acid sequences among motif B' *N*-methyltransferases. Residues indicated by white letters on closed boxes are SAM/SAH-binding residues in SAMT. The large boxes A, B' and C indicate SAM-binding regions. Residues highlighted by shaded boxes are salicylate-binding residues in SAMT, nominated substrates binding sites in other members of the motif B' methyltransferase family, and additional active site residues of SAMT. The shaded boxes represent conserved amino acid residues, and dashes represent gaps that have been optimal aligned. The asterisk indicates the 160-histidine residue of the CCS1 sequence. The methionine residue is located at position 150 in CbSAMT. CbSAMT is *Clarkia* SAMT (Ross *et al.,* 1999), AtJAMT is *Arabidopsis* JAMT (Seo *et al.,* 2001). CCS1 and CmXRS1 are *N*-methyltransferases in the coffee caffeine synthase family (Mizuno *et al.,* 2003a, b). TCS1 is tea TCS1 (Kato *et al.,* 2000). This figure is redrawn from Zubieta *et al.* (2003).

tion activity against paraxanthine was low. Therefore, one possibility is that the specific amino acid residue(s) which interfere with the 3-*N* methylation activity against paraxanthine are present in CTS1 and CTS2. Another possibility is that the specific amino acid residue(s) contribute to the enhancement of 3-*N* methylation activity against only 7-methylxanthine in CTS1 and CTS2. It was proposed that H160 of CCS1 is located in the vicinity of the substrate-recognizing site that was estimated by the three-dimensional structure of caffeine synthase (McCarthy and McCarthy, 2007). Although the crystal structure suggests that H160 of CCS1, corresponding to Q161 of CmXRS1, is an important amino acid residue for the binding of methyl donor, the hypothesis was confirmed by this study.

In the tea plant, an enzyme possessing 7-*N* methylation activity corresponding to coffee CmXRS1 has not been identified. The amino acid sequence of theobromine synthase (named PCS1) from cocoa tea (*Camellia ptilophylla*) that accumulates theobromine but not caffeine is more than 90% identical to that of caffeine synthase (named TCS1) from tea the plant (*Camellia sinensis*) (Yoneyama *et al.*, 2005). However, theobromine synthase from *C. ptilophylla* showed low homology with that from *Theobroma cacao*. R221 of TCS1, corresponding to L210 which is related to the binding of salicylic acid in SAMT, did not show caffeine synthase activity (1-*N* methylation activity) by the replacement of one histidine residue in PCS1 (Yoneyama *et al.*, 2005). Caffeine synthase (1-*N* and 3-*N* methylation) and theobromine synthase (only 3-*N* methylation) from coffee have arginine residues while CmXRS1 (only 7-*N* methylation) has a histidine residue. The CmXRS1 mutant in which the histidine residue H219 was replaced with an arginine residue (m5 mutant) did not exhibit 3-*N* methylation activity. Consequently, the difference between caffeine synthase activity and theobromine synthase activity in coffee may be determined by another amino acid residue found in tea.

Judging from these results and by comparing the amino acid sequences among caffeine synthetic enzymes, we propose that the candidate amino acid residues correlated with the expres-

sion of 1-*N* or 3-*N* methylation activity are D230 and F232 in the CCS1 sequence (corresponding to V231 and L233 of CmXRS1). Furthermore, there is a possibility that V328, S331 and R362 in the CCS1 sequence (corresponding to S316, A318 and P349 of CmXRS1) located near the additional active site proposed by Zubieta *et al*. (2003) are related to substrate specificities (Fig. 5). These nominated residues may be useful targets for mutational analysis in future experiments.

Acknowledgement

This work was supported by Grants-in-Aid from All Japan Coffee Association.

Ashihara H., Gillies F. M., and Crozier A. (1997), Metabolism of caffeine and related purine alkaloids in leaves of tea (*Camellia sinensis* L.). Plant Cell Physiol. **38**, 413–419.

Bradford M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **7**, 248–254.

Kato M. and Mizuno K. (2004), Caffeine synthase and related methyltransferases in plants. Front. Biosci. **9**, 1833–1842.

Kato M., Kanehara T., Shimizu H., Suzuki T., Gillies F. M., Crozier A., and Ashihara H. (1996), Caffeine biosynthesis in young leaves of *Camellia sinensis*: *in vitro* studies on *N*-methyltransferase activity involved in the conversion of xanthosine to caffeine. Physiol. Plant **98**, 629–636.

Kato M., Mizuno K., Fujimura T., Iwama M., Irie M., Crozier A., and Ashihara H. (1999), Purification and characterization of caffeine synthase from tea leaves. Plant Physiol. **120**, 579–586.

Kato M., Mizuno K., Crozier A., Fujimura T., and Ashihara H. (2000), Caffeine synthase gene from tea leaves. Nature **406**, 956–957.

McCarthy A. A. and McCarthy J. G. (2007), The structure of two *N*-methyltransferases from the caffeine biosynthetic pathway. Plant Physiol. **144**, 879–889.

Mizuno K., Tanaka H., Kato M., Ashihara H., and Fujimura T. (2001), cDNA cloning of caffeine (theobromine) synthase from coffee (*Coffea arabica* L.). In: ASIC Proceedings of the 19th International Scientific Colloquium on Coffee, pp. 815–818.

Mizuno K., Akira O., Kato M., Yoneyama N., Tanaka H., Ashihara H., and Fujimura T. (2003a), Isolation of a new dual-functional caffeine synthase gene encoding an enzyme for the conversion of 7-methylxanthine to caffeine from coffee (*Coffea arabica* L.). FEBS Lett. **534**, 75–81.

Mizuno K., Kato M., Irino F., Yoneyama N., Fujimura T., and Ashihara H. (2003b), The first committed step reaction of caffeine biosynthesis: 7-methylxanthosine synthase is closely homologous to caffeine synthases in coffee (*Coffea arabica* L.). FEBS Lett. **547**, 56–60.

Ross J. R., Nam K. H., D'Auria J. C., and Pichersky E. (1999), *S*-Adenosyl-L-methionine: salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. Arch. Biochem. Biophys. **367**, 9–16.

Seo H. S., Song J. T., Cheong J. J., Lee Y. H., Lee Y. W., Hwang I., Lee J. S., and Choi Y. D. (2001), Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. USA **98**, 4788–4793.

Suzuki T., Ashihara H., and Waller G. R. (1992), Purine and purine alkaloid metabolism in *Camellia* and *Coffea* plants. Phytochemistry **31**, 2575–2584.

Uefuji H., Herai Y., Koizumi N., Kusano T., and Sano H. (2003), Molecular cloning and functional characterization of three distinct *N*-methyltransferase involved in the caffeine biosynthetic pathway in coffee plants. Plant Physiol. **132**, 372–380.

Yoneyama N., Morimoto H., Ye C. X., Ashihara H., Mizuno K., and Kato M. (2005), Substrate specificity of *N*-methyltransferase involved in purine alkaloids synthesis is dependent upon one amino acid residue of the enzyme. Mol. Gen. Genomics **275**, 125–135.

Zheng X. Q. and Ashihara H. (2004), Distribution, biosynthesis and function of purine and pyridine alkaloids in *Coffea arabica* seedlings. Plant Sci. **166**, 807–813.

Zubieta C., Ross J. R., Koscheski P., Yang Y., Pichersky E., and Noel J. P. (2003), Structural basis for substrate recognition in the salicylic acid carboxyl methyltransferase family. Plant Cell **15**, 1704–1716.