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A new caffeine biosynthetic pathway in tea leaves: utilisation of adenosine released from the *S*-adenosyl-L-methionine cycle

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Abstract The four-step caffeine biosynthetic pathway includes three methylation steps that utilise *S*-adenosyl-L-methionine (SAM) as the methyl donor. In the process SAM is converted to *S*-adenosyl-L-homocysteine (SAH) which in turn is hydrolysed to L-homocysteine and adenosine. Significant amounts of radioactivity from [methyl-^{14}C]methionine and [methyl-^{14}C]SAM were incorporated into theobromine and caffeine in young tea leaf segments, and very high SAH hydrolase activity was found in cell-free extracts from young tea leaves. Substantial amounts of radioactivity from [adenosyl-^{14}C]SAH were also recovered as theobromine and caffeine in tea leaf segments, indicating that adenosine derived from SAH is utilised for the synthesis of the purine ring of caffeine. From the profiles of activity of related enzymes in tea leaf extracts, it is proposed that the major route from SAM to caffeine is a SAM → SAH → adenosine → adenine → AMP → IMP → XMP → xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway. In addition, direct adenosine kinase-catalysed formation of AMP from adenosine may participate as an alternative minor route. The activity of two of the three *N*-methyltransferase activities involved in caffeine biosynthesis and part of the activities of SAH hydrolase, adenosine nucleosidase, adenine phosphoribosyltransferase and adenosine kinase were located in tea chloroplasts. In contrast, no detectable activity of SAM synthetase was associated with the purified chloroplast fraction. This is a first demonstration that the purine skeleton of caffeine is synthesised from adenosine released from the SAM cycle.

Key words: Caffeine biosynthesis; *S*-Adenosyl-L-methionine; Adenosine; *S*-Adenosyl-L-homocysteine; Subcellular compartmentation

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is a secondary plant product derived from purine nucleotides. The main pathway of caffeine biosynthesis in tea leaves begins with the conversion of xanthosine to 7-methylxanthine with *S*-adenosine-L-methionine (SAM) acting as the methyl donor [1]. Removal of the ribose moiety converts 7-methylxanthine to 7-methylxanthine which undergoes successive methylations, with SAM again acting as the methyl donor, to yield theobromine and then caffeine [1,2]. A number of possible sources for xanthosine have been postulated. These are (i) inosine 5′-monophosphate (IMP) synthesised by de novo purine nucleotide biosynthesis, (ii) intracellular pools of adenine and guanine nucleotides and (iii) purine nucleotides and nucleosides produced by degradation of RNA [3]. Here, we report on a new and important route to caffeine that begins with the release of adenosine from *S*-adenosyl-L-homocysteine (SAH), a product of SAM-dependent methyltransferases. The hydrolysis of SAH also produces L-homocysteine which will be reutilised for SAM synthesis via methionine by the so-called activated methyl cycle [4] or SAM cycle [1].

2. Materials and methods

2.1. Plant material

Tea leaves (*Camellia sinensis* L.) were collected at the experimental farm of the Tokyo Metropolitan Agricultural Experimental Station, Tachikawa, Tokyo, Japan. Young leaves and apices (fresh weight: ca. 100 mg per sample) that had emerged in spring or in autumn were used as experimental material. In some experiments, fully expanded mature leaves in newly emerged shoots and the aged leaves obtained from ca. 1-year-old shoots were also used.

2.2. Chemicals

[8-^{14}C]Adenosine (1.85 MBq/μmol) and [8-^{14}C]adenine (1.96 MBq/μmol) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA). [Methyl-^{14}C]methionine (2.03 MBq/μmol), [methyl-^{14}C]SAM (2.03 MBq/μmol) and [carboxyl-^{14}C]SAM (2.03 MBq/μmol) were from Amersham International plc. (Amersham, Buckinghamshire, UK). [8-^{14}C]SAH was prepared enzymatically according to Poulton and Butt [5] using partially purified tea SAH hydrolase and [8-^{14}C]adenosine. All biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Metabolism of radiolabelled precursors by leaf disks

The metabolism of ^{14}C precursors was investigated as described by Ashihara et al. [6]. Leaf segments (ca. 100–200 mg fresh weight) were incubated in 2 ml of medium, comprising 30 mM potassium phosphate buffer, pH 5.6, 10 mM sucrose and ^{14}C-labelled compounds. Shoot apices were included in the young leaf samples. Incubation conditions of individual experiments are presented in table and figure legends. Analysis of radiolabelled compounds by thin layer chromatography and high performance liquid chromatography (HPLC) was as described by Ashihara et al. [7] except that autoradiography was conducted using an Image-Analyser system (FLA-2000, Fuji Photo Film Co., Ltd., Tokyo, Japan) and HPLC radiocounting used a Ra-

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Abbreviations: CS, caffeine synthase; 7NMT, 7-*N*-methyltransferase; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine

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mona 2000 radioactivity monitor (Reytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

2.4. Measurement of enzymatic activity

2.4.1. 7-N-Methyltransferase (7NMT) and caffeine synthase (CS). These activities were measured based on the transfer of a ^{14}C-labelled methyl group from [methyl-^{14}C]SAM to a methyl acceptor as described in an earlier paper [8]. The reaction mixture for these enzymes contained the following components in a total volume of 100 μl: 100 mM Tris–HCl buffer (pH 8.3), 4 μM [methyl-^{14}C]SAM (0.9 kBq) and either 0.2 mM xanthosine (7NMT) or 0.2 mM 7-methylxanthine or 2 mM theobromine (CS).

2.4.2. SAM synthetase and SAH hydrolase. The activities of SAM synthetase and SAH hydrolase were measured based on the formation of SAM from [methyl-^{14}C]methionine and that of SAH from [8-^{14}C]adenosine [9]. The reaction mixture for these enzymes contained the following components in a total volume of 100 μl. SAM synthetase: 50 mM Tris–HCl buffer (pH 8.0) containing 7 mM $MgCl_2$ and 2 mM dithiothreitol (DTT), 40 μM [methyl-^{14}C]methionine, 1.25 mM ATP and 8 mM reduced glutathione (GSH). SAH hydrolase: 50 mM Tris–HCl buffer (pH 8.0) containing 5 mM DTT, 50 μM [8-^{14}C]adenosine (1.8 kBq) and 10 mM homocysteine.

2.4.3. Adenosine nucleosidase, adenine phosphoribosyltransferase, adenosine kinase, adenosine deaminase and adenine deaminase. Activities of these enzymes were determined according to Ashihara et al. [10]. All reaction mixtures were 100 μl in volume and contained 30 mM HEPES–NaOH buffer (pH 7.6), 6.5 mM $MgCl_2$, 1.0 mM DTT. In addition, the following components were included: 45 μM [8-^{14}C]adenosine (1.8 kBq) (assay of adenosine nucleosidase and adenosine deaminase); 0.6 mM 5-phosphoribosyl-1-pyrophosphate (PRPP) and 45 μM [8-^{14}C]adenine (1.8 kBq) (adenine phosphoribosyltransferase); 3.75 mM ATP and 45 μM [8-^{14}C]adenosine (1.8 kBq) (adenosine kinase); 45 μM [8-^{14}C]adenine (1.8 kBq) (adenine deaminase).

2.4.4. AMP deaminase, 5′-nucleotidase and IMP dehydrogenase. The activities of these enzymes were measured spectrophotometrically as described in earlier papers [11,12]. The reaction mixture contained the following components: 10 mM cacodylate buffer (pH 7.1), 5 mM AMP, 5 mM ATP and 0.05% bovine serum albumin (AMP deaminase), 60 mM Tris–HCl buffer (pH 7.5) and 1 mM XMP (5′-nucleotidase), 50 mM tricine–KOH buffer (pH 8.8), 1 mM IMP, 0.2 mM NAD^+, 1 mM allopurinol, 1 mM DTT, 1 mM NaEDTA, 50 mM KCl and 30 mM NaF (IMP dehydrogenase).

2.5. Subcellular fractionation

The procedure of preparation of crude and purified chloroplasts and assay of marker enzymes and chlorophyll were the same as used in an earlier study [13]. Total enzymatic activities were measured after the homogenate of young tea leaves was filtered through a layer of Miracloth. The activities of enzymes in chloroplasts were estimated using chloroplasts obtained after centrifugation at $1000 \times g$ for 10 min and two gentle washes with extraction buffer. Localisation of enzyme activities in chloroplasts was confirmed using a Percoll gradient centrifugation. Organelles in homogenate and crude and purified chloroplasts were ruptured by freezing with liquid nitrogen and thawing before determination of enzyme activities.

3. Results and discussion

Methylation of putative caffeine precursors by tea leaf segments was demonstrated in situ using methyl-^{14}C-labelled methionine and SAM. More than 50% of radioactivity taken up by the segments of young tea leaves in a 2 h incubation with [methyl-^{14}C]methionine was incorporated into the purine alkaloids theobromine and caffeine, with most radioactivity being associated with caffeine. The incorporation was greatly reduced in mature and aged leaves (Fig. 1). Similar trends were observed when [methyl-^{14}C]SAM was used instead of [methyl-^{14}C]methionine (data not presented).

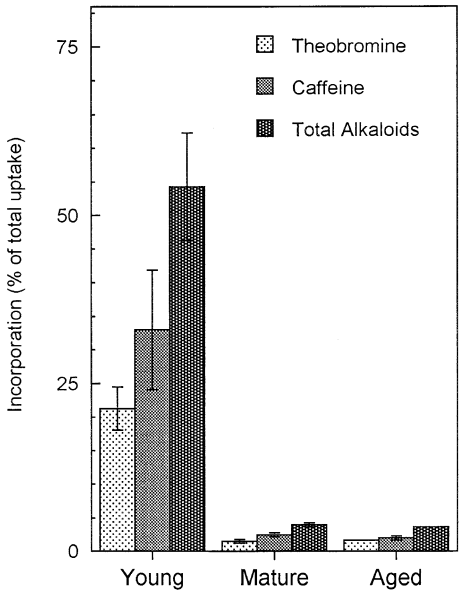

Fig. 1. Incorporation of 9 μM [methyl-^{14}C]methionine (specific activity 2.00 MBq/μmol) into theobromine, caffeine and total purine alkaloids in young, mature and aged tea leaves. Segments of tea leaves were incubated for 2 h with [methyl-^{14}C]methionine and 10 mM sucrose in 2.0 ml of 30 mM potassium phosphate buffer (pH 5.6) at 27°C. The rates of incorporation are expressed as a percentage of total radioactivity taken up by the leaf segments ± S.D. ($n = 3$). Young leaves (newly emerged, small expanding leaves in small size, ca. 100 mg/leaf), mature leaves (young fully expanded leaves, ca. 450 mg/leaf), and aged leaves (1 year old leaves, ca. 480 mg/leaf) were used. Total uptakes by the segments of the young, mature, and aged leaves were 14.1 ± 4.4, 25.9 ± 3.1 and 38.0 ± 0.9 kBq/g fresh weight, respectively.

Data obtained in a time course study of the incorporation of [methyl-^{14}C]SAM into the theobromine and caffeine by young tea leaves indicated that the caffeine biosynthesis increased in a linear manner up to 18 h and, as in the previous experiment, there was higher incorporation of label into caffeine than into theobromine (Table 1). In addition to its involvement in caffeine biosynthesis, SAM is utilised in the biosynthesis of polyamines and ethylene in plants with the

Table 1
Incorporation of radioactivity from 9 μM [methyl-^{14}C]SAM and 9 μM [carboxyl-^{14}C]SAM into purine alkaloids and CO_2 in tea leaves

Precursor	Incubation (h)	Theobromine	Caffeine	CO_2
[Methyl-^{14}C]SAM	2	880 ± 20 (6.9)	1540 ± 140 (12.0)	60 ± 10 (0.4)
	6	2240 ± 380 (11.4)	4630 ± 520 (23.5)	110 ± 10 (0.5)
	18	3060 ± 150 (6.8)	16330 ± 870 (36.4)	600 ± 270 (1.3)
[Carboxyl-^{14}C]SAM	2	–	–	720 ± 20 (7.6)
	6	–	–	2240 ± 380 (9.8)
	18	–	–	10670 ± 730 (24.0)

The rates of incorporation are expressed as Bq/g fresh weight ± S.D. ($n = 3$) and as a percentage of total radioactivity taken up by the leaf segments (parentheses). Leaf segments were incubated for 2, 6 or 18 h with radioactive SAM and 10 mM sucrose in 2.0 ml of potassium phosphate buffer (pH 5.6).

carboxyl group of SAM being removed as CO_2 in reactions catalysed by SAM decarboxylase and 1-aminocyclopropane-1-carboxylic acid oxidase, respectively [14]. The data obtained on $^{14}CO_2$ output in experiments using [carboxyl-^{14}C]SAM suggest that these pathways also operate in tea leaves, but that the proportion of SAM utilised is lower than that involved in caffeine biosynthesis (Table 1). Upon donation of the methyl group to acceptor molecules, SAM is converted to SAH. As SAH is a potent inhibitor of methyltransferases [15] including caffeine synthase [2], removal of SAH by SAH hydrolase is essential for the continued operation of the caffeine biosynthesis *N*-methyltransferases. The SAH hydrolase reaction is reversible and the equilibrium lies heavily in favour of SAH synthesis from adenosine and homocysteine [16]. Removal of adenosine and homocysteine is, therefore, essential for the effective hydrolysis of SAH [5] and this is achieved by metabolism of homocysteine to methionine which is further converted to SAM thereby completing the SAM regeneration cycle.

Table 2 summarises the enzyme activities of enzymes involved in the SAM cycle and enzymes catalysing the metabolism of adenosine to xanthosine in young, mature and aged tea leaves. The presence of SAM synthetase, SAM:xanthosine 7-*N*-methyltransferase (7NMT), caffeine synthase and SAH hydrolase activities was demonstrated in the tea leaf extracts. Although methionine synthase activity was not determined, it must be present in tea leaves as it also participates in the de novo methionine biosynthesis [14]. The SAM cycle can, therefore, be operative in tea leaves. The activities of most enzymes were higher in the young leaves, where active caffeine biosynthesis takes place, than in mature and aged leaves (Table 2). This trend was particularly evident with SAH hydrolase, 7NMT and caffeine synthase.

In order to determine the metabolic fate of adenosine released from SAH, [8-^{14}C]SAH was supplied and its metabolism investigated. After an 18 h incubation, one third of recovered radioactivity had been incorporated into caffeine and theobromine, with the remainder associated with SAM, adenine nucleotides, nucleic acids and CO_2 (Table 3). This demonstrates that substantial quantities of adenosine, released in the conversion of SAH to homocysteine, are utilised for caf-

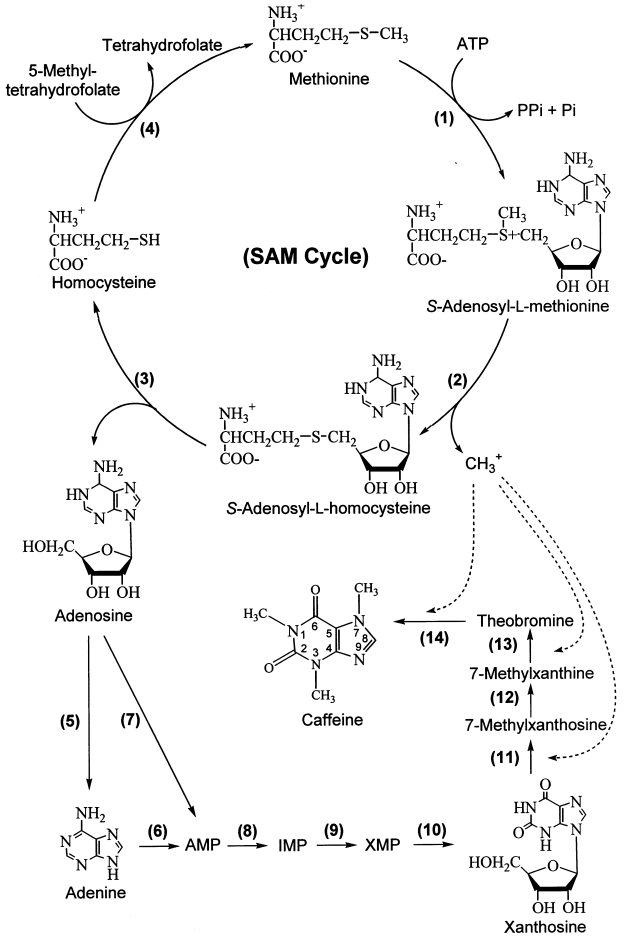

Fig. 2. Proposed new pathway for the biosynthesis of purine alkaloids in which adenosine derived from the SAM cycle is metabolised to xanthosine that is converted to caffeine via a route that involves three SAM-dependent methylation steps. Enzymes: (1) SAM synthetase; (2) SAM-dependent *N*-methyltransferases; (3) SAH hydrolase; (4) methionine synthase; (5) adenosine nucleosidase; (6) adenine phosphoribosyltransferase; (7) adenosine kinase; (8) AMP deaminase; (9) IMP dehydrogenase; (10) 5′-nucleotidase; (11) xanthosine 7-*N*-methyltransferase; (12) 7-methylxanthosine nucleosidase; (13) 7-methylxanthine 3-*N*-methyltransferase (caffeine synthase); (14) theobromine 1-*N*-methyltransferase (caffeine synthase).

Table 2
Profiles of enzyme activities involved in the SAM cycle and adenosine metabolism in tea leaves of different growth stages

Enzyme	Young	Mature	Aged
(1) *SAM cycle enzymes*			
SAM synthetase (EC 2.5.1.6)	114 ± 4.0	20 ± 1.0	7 ± 1
7-*N*-Methyltransferase (–)[a]	9 ± 0.5	1 ± 0.1	nd
Caffeine synthase (7mX) (–)	75 ± 1.0	15 ± 1.0	nd
Caffeine synthase (Tb) (–)	15 ± 0.5	1 ± 0.1	nd
SAH hydrolase (3.3.1.1)	17500 ± 700	380 ± 13	10 ± 0
(2) *Adenosine metabolism enzymes*			
Adenosine nucleosidase (3.2.2.7)	31800 ± 910	2810 ± 58	1430 ± 54
Adenine phosphoribosyltransferase (2.4.2.7)	545 ± 12	294 ± 5.0	50 ± 0.5
Adenosine kinase (2.7.1.20)	1020 ± 50	226 ± 19	8 ± 0.5
AMP deaminase (3.5.4.6)	61 ± 0.5	31 ± 1.0	14 ± 3.5
Adenylate kinase (2.7.4.3)	1580 ± 54	1450 ± 100	1500 ± 25
5′-Nucleotidase (XMP) (3.1.3.5)[b]	3330 ± 240	2830 ± 60	2110 ± 110
IMP dehydrogenase (1.1.1.205)	307 ± 4	10 ± 1	14.1
Adenine deaminase (3.5.4.2)	nd	nd	nd
Adenosine deaminase (3.5.4.4)	nd	nd	nd

Enzymatic activity is expressed as pkat/g fresh weight ± S.D. ($n = 3$). nd: not detected.
[a](–): EC number has not yet been given.
[b]The activity of 5′-nucleotidase shown was measured with xanthosine 5′-monophosphate (XMP) as a substrate. Growth stages of tea leaves are compatible as those shown in Fig. 1.

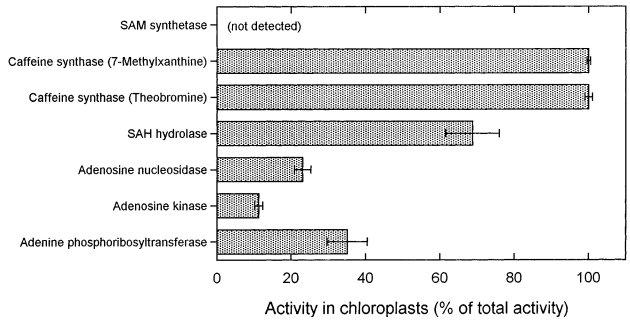

Fig. 3. Localisation of enzymes involved in caffeine biosynthesis and the SAM cycle in chloroplasts of young tea leaves. Total enzymatic activity in the washed chloroplast fraction is shown as a percentage of total activity observed in leaf homogenate.

feine biosynthesis. As 3 mol of adenosine is produced via the SAM cycle for each mol of caffeine synthesis, it is feasible that this pathway has the capacity to be the sole source of both the purine skeleton and the methyl moieties required for caffeine biosynthesis in young tea leaves. The results also suggest that almost all the SAH formed from SAM, as a product of *N*-methyltransferase activity, is hydrolysed to homocysteine and adenosine. In addition to caffeine skeleton formation, adenosine is utilised for the synthesis of adenine nucleotides and nucleic acids. Many adenine nucleotides are rapidly catabolised and, as a consequence, as shown in Table 3, substantial amounts of CO_2 are released.

Theoretically, there are several possible pathways for caffeine biosynthesis from adenosine. However, as adenosine deaminase and adenine deaminase activity could not be detected in tea leaf extracts (Table 2), conversion of adenosine to xanthosine at the nucleoside or nucleobase level would appear to be excluded as has been demonstrated in other plant materials [1]. The enzyme activity profiles shown in Table 2 suggest that adenosine is converted to AMP and then deaminated to IMP by AMP deaminase, and that xanthosine is synthesised via XMP by the sequential reactions catalysed by IMP dehydrogenase and 5′-nucleotidase. Adenosine may be salvaged to AMP by two routes, one is direct formation catalysed by adenosine kinase, the other is a two-step conversion via adenine catalysed by adenosine nucleosidase and adenine phosphoribosyltransferase. The presence of all three of these enzymes was demonstrated in tea extracts with the highest activities observed in preparations from young tea leaves. It has been reported that tea leaves contain an adenosine-specific nucleosidase that can use deoxyadenosine but do not react with any other nucleosides [17]. As adenosine nucleosidase activity is ca. 30-fold higher than that of adenosine kinase (Table 2), it would appear to indicate that most adenosine is hydrolysed to adenine which is salvaged by adenine phosphoribosyltransferase. We, therefore, propose the following new pathway of caffeine biosynthesis from SAM: SAM → SAH → adenosine → adenine → AMP → IMP → XMP → xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine, as illustrated in Fig. 2. An alternative minor route involving direct phosphorylation of adenosine to produce AMP cannot be excluded because adenosine kinase activity in extracts from young tea leaves is higher than that of adenine phosphoribosyltransferase (Table 2).

Table 3
Metabolism of 6.4 μM [8-^{14}C]SAH by segments of young tea leaves

Metabolite	Incorporation	
	(kBq/g f.w.)	(%)
Caffeine	17.6 ± 1.5	(31.0)
Theobromine	1.3 ± 0.0	(2.4)
Adenine nucleotides	0.7 ± 0.2	(1.2)
Adenine	0.5 ± 0.1	(0.8)
Adenosine	0.1 ± 0.0	(0.2)
SAH+SAM	3.3 ± 1.4	(5.8)
Nucleic acids	11.9 ± 0.5	(21.0)
CO_2	21.4 ± 1.9	(37.7)
Total uptake	56.7 ± 1.8	(100)

Leaf segments were incubated with [8-^{14}C]SAH and 10 mM sucrose in 2.0 ml of 30 mM potassium phosphate buffer (pH 5.6) at 27°C for 18 h.

Finally, compartmentation of the SAM cycle and caffeine biosynthesis was examined. We have already demonstrated that caffeine synthase, a key enzyme catalysing the final two steps of caffeine biosynthesis, is located in chloroplasts [13]. The data in Fig. 3 show that in addition to the caffeine synthase activity, more than 70% of total activity of SAH hydrolase and parts of adenosine kinase, adenosine nucleosidase and adenine phosphoribosyltransferase activities were associated with Percoll-purified chloroplasts. In contrast, SAM synthetase was detected exclusively in the cytosol.

Localisation of adenosine kinase and adenine phosphoribosyltransferase activities in chloroplasts from spinach leaves has been reported [18]. There are several biochemical studies on SAH hydrolase [3,19] and adenosine nucleosidase [17,20], but none on the subcellular localisation of these enzymes in leaves. Recently, genes encoding SAH hydrolase were cloned from tobacco [21] and parsley [22]. The *N*-terminal sequences lack the typical features of transit peptide signals, suggesting that the genes may encode the cytosol form of SAH hydrolase in these plants. However, the data presented in Fig. 3 indicate that tea SAH is more likely to be a chloroplastic enzyme.

As SAM synthetase is a cytosolic enzyme (Fig. 3), SAM must be produced in the cytosol of tea leaves. Nothing is known about the subcellular localisation of methionine synthetase. We propose a model in which homocysteine produced in chloroplasts is transported to the cytosol, however, transport of methionine cannot be excluded. Conversion of methionine to SAM takes place in the cytosol of young tea leaves and SAM returns to chloroplasts where it serves as a methyl donor in caffeine biosynthesis and in the process is converted to SAH. The available evidence also indicates that adenosine derived from SAH may also be converted to adenine and AMP in tea chloroplasts. Three models for the compartmentation of methionine and SAM synthesising and recycling enzymes has been proposed by Ravanel et al. [22]. These models are similar to ours in that they suggest that homocysteine and/or methionine are transported across the chloroplast membrane. The main difference between our model and those of Ravanel et al. [22] is the position of SAH hydrolase. They suggest it is cytosolic, but our data indicate a significant amount of this enzyme is located in chloroplasts.

In conclusion, the data presented in this report establish that in young tea leaves not only the methyl groups but also the purine ring of caffeine is derived from SAM. The capacity of the pathway is such that it is possible that the purine ring of caffeine is produced exclusively by this route.

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