Caffeine: a well known but little mentioned compound in plant science

Hiroshi Ashihara and Alan Crozier

Caffeine, a purine alkaloid, is a key component of many popular drinks, most notably tea and coffee, yet most plant scientists know little about its biochemistry and molecular biology. A gene from tea leaves encoding caffeine synthase, an *N*-methyltransferase that catalyses the last two steps of caffeine biosynthesis, has been cloned and the recombinant enzyme produced in *E. coli*. Similar genes have been isolated from coffee leaves but the recombinant protein has a different substrate specificity to the tea enzyme. The cloning of caffeine biosynthesis genes opens up the possibility of using genetic engineering to produce naturally decaffeinated tea and coffee.

Hiroshi Ashihara
Metabolic Biology Group, Dept Biology, Faculty of Science, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan.
e-mail: ashihara@cc.ocha.ac.jp

Alan Crozier
Plant Products and Human Nutrition Group, Division of Biochemistry and Molecular Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK G12 8QQ.
e-mail: a.crozier@bio.gla.ac.uk

Caffeine (1,3,7-trimethylxanthine) is one of the few plant products with which the general public is readily familiar, because of its occurrence in beverages such as coffee and tea, as well as various soft drinks. A growing belief that the ingestion of caffeine can have adverse effects on health has resulted in an increased demand for decaffeinated beverages[1]. Unpleasant short-term side effects from caffeine include palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure and insomnia[2,3]. In spite of numerous publications on the long-term consequences of caffeine consumption on human health, no clear picture has emerged, with reports of both protective and deleterious effects[4].

Caffeine was discovered in tea (*Camellia sinensis*) and coffee (*Coffea arabica*) in the 1820s (Ref. 5). Along with other methylxanthines, including theobromine (3,7-dimethylxanthine), paraxanthine (1,7-dimethylxanthine) and methyluric acids (Fig. 1), caffeine is a member of a group of compounds known collectively as purine alkaloids. There are two hypotheses about the role of the high concentrations of caffeine that accumulate in tea, coffee and a few other plant species. The 'chemical defence theory' proposes that caffeine in young leaves, fruits and flower buds acts to protect soft tissues from predators such as insect larvae[6] and beetles[7]. The 'allelopathic theory' proposes that caffeine in seed coats is released into the soil and inhibits the germination of other seeds[8]. The potential ecological role of caffeine is described in Ref. 6.

It is only within the past five years that the biosynthetic and catabolic pathways that regulate the build-up of caffeine in the vacuoles of cells of tea and coffee plants have been elucidated fully. In contrast with the widespread medical interest in caffeine as a dietary component, these developments have received little attention in the plant literature, with the topic being all but neglected in recent biochemistry text books[9–12].

Caffeine is synthesized from xanthosine via a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway; the first, third and fourth steps are catalysed by *N*-methyltransferases that use *S*-adenosyl-L-methionine (SAM) as the methyl donor[13]. A recent important development has been the cloning and expression in *E. coli* of a gene from tea leaves that encodes caffeine synthase, an extremely labile *N*-methyltransferase that catalyses the last two steps in this pathway[14]. In addition, coffee leaf cDNAs of theobromine synthase, which catalyses the penultimate methylation step, have been similarly cloned and expressed in *E. coli*[15,16]. There are also preliminary reports on the cloning of an *N*-methyltransferase from coffee that catalyses the initial methylation step in the pathway[17,18]. These advances in our knowledge of the metabolism of caffeine and related compounds in plants and the potential biotechnological applications of purine alkaloid research are highlighted in this article.

Distribution of purine alkaloids

Purine alkaloids have a limited distribution within the plant kingdom. In some species, the main purine alkaloid is theobromine or methyluric acids rather than caffeine[13]. Among the purine-alkaloid-containing plants, most studies have been carried out with species belonging to the genera *Camellia* and *Coffea*. In *C. sinensis* (Fig. 2), caffeine is found in the highest concentrations in young leaves of first-flush shoots of var. *sinensis* (2.8% of the dry weight). Theobromine is the predominant purine alkaloid in young leaves of cocoa tea (*Camellia ptilophylla*) (5.0–6.8%) and *Camellia irrawadiensis* (<0.8%).

The beans of most cultivars of Arabica coffee (*C. arabica*) (Fig. 3) contain ~1.0% caffeine, whereas *Coffea canephora* cv. Robusta (1.7%) and cv. Guarini (2.4%), *Coffea dewevrei* (1.2%) and *Coffea liberica* (1.4%) contain higher concentrations. By contrast, the caffeine contents of the seeds of other species, such as *Coffea eugenioides* (0.4%), *Coffea salvatrix* (0.7%) and *Coffea racemosa* (0.8%), are lower than that of *C. arabica*. Young expanding leaves of *C. arabica* plants also contain caffeine, with traces of theobromine. In model systems, weak intermolecular complexes form between caffeine and polyphenols[19], and it has been proposed that caffeine is sequestered in the vacuoles of coffee leaves as a chlorogenic acid complex[20]. Mature leaves of *C. liberica*, *C. dewevrei* and *Coffea abeokutae* convert caffeine to the methyluric acids, theacrine (1,3,7,9-tetramethyluric acid), liberine [*O*(2),1,9-trimethyluric acid] and methylliberine [*O*(2),1,7,9-tetramethyluric acid] (Fig. 1).

Purine alkaloids are also present in the leaves of maté (*Ilex paraguariensis*), which is used in rural areas of South America, such as the Brazilian Panthanal and

 1360-1385/01/$ – see front matter PII: S1360-1385(01)02055-6

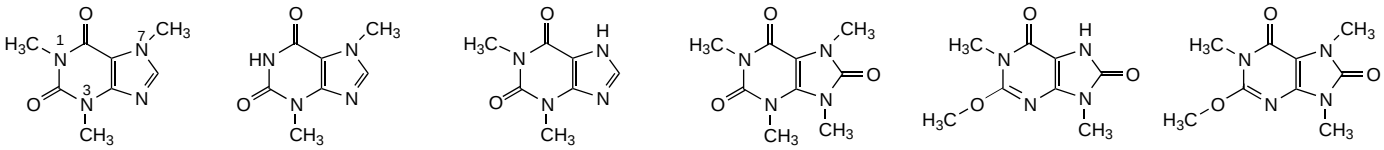

Fig. 1. Structures of the methylxanthines caffeine, theobromine and paraxanthine, and the methyluric acids theacrine, liberine and methylliberine.

the Pampas in Argentina, to produce a herbal tea (http://www.vtek.chalmers.se/~v92tilma/tea/mate.html). Young maté leaves contain 0.8–0.9% caffeine and 0.08–0.16% theobromine. Theobromine is the dominant purine alkaloid in seeds of cocoa (*Theobroma cacao*), with cotyledons of mature beans containing 2.2–2.7% theobromine and 0.6–0.8% caffeine. Caffeine (4.3%) is the major methylxanthine in cotyledons of guaraná (*Paulliania cupana*), extracts of which are used as a refreshing pick-me-up (http://www.rain-tree.com/guarana.htm) and which is, in a dilute form, sold extensively in Brazil as a carbonated drink. Seeds of cola (*Cola nitida*) also contain caffeine (2.2%)[21]. Caffeine has recently been detected in flowers of several citrus species, with the highest concentrations (0.2%) in pollen[22], and is also a fungal metabolite, being the principal alkaloid in sclerotia of *Claviceps sorhicola*, a Japanese ergot pathogen of *Sorghum*[23].

Biosynthesis of purine alkaloids

Origin of the purine ring of caffeine

Caffeine is a trimethylxanthine whose xanthine skeleton is derived from purine nucleotides that are converted to xanthosine, the first committed intermediate in the caffeine biosynthesis pathway. There are at least four routes from purine nucleotides to xanthosine (Fig. 4). The available evidence indicates that the most important routes are the production of xanthosine from inosine 5′-monophosphate, derived from *de novo* purine nucleotide biosynthesis, and the pathway in which adenosine, released from *S*-adenosyl-L-homocysteine (SAH), is converted to xanthosine via adenine, adenosine 5′-monophosphate, inosine 5′-monophosphate and xanthosine 5′-monophosphate[13,24,25].

Recently published data indicate that the conversion of SAH to xanthosine is such that the purine ring of caffeine can be produced exclusively by this route in young tea leaves[25]. The formation of caffeine by this pathway is closely associated with the SAM cycle (also known as the activated-methyl cycle) because the three methylation steps in the caffeine biosynthesis pathway use SAM as the methyl donor (Fig. 4). During this process, SAM is converted to SAH, which in turn is hydrolysed to L-homocysteine and adenosine. The adenosine is used to synthesize the purine ring of caffeine and the L-homocysteine is recycled to replenish SAM levels. Because 3 moles of SAH are produced via the SAM cycle for each mole of caffeine that is synthesized, this pathway has the capacity to be the sole source of both the purine skeleton and the methyl groups required for caffeine biosynthesis in young tea leaves[25].

Purine ring methylation

Xanthosine is the initial purine compound in the caffeine biosynthesis pathway, acting as a substrate for the methyl group donated by SAM. Tracer experiments with labelled precursors and leaf discs from tea and coffee plants have shown that the major route to caffeine is xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine, although alternative minor routes might also operate[26]. However, as well as entering the caffeine biosynthesis pathway, xanthosine is also converted to xanthine, which is degraded to CO_2 and NH_3 via the purine catabolism pathway[27,28] (Fig. 5).

The first methylation step in the caffeine biosynthesis pathway, the conversion of xanthosine

Fig. 2. Commercial tea plantation in Kenya (photograph courtesy of David Werndly, Unilever Research, Colworth, UK).

Fig. 3. Ripening beans of *Coffea arabica* (photograph courtesy of the All Japan Coffee Association, Tokyo, Japan).

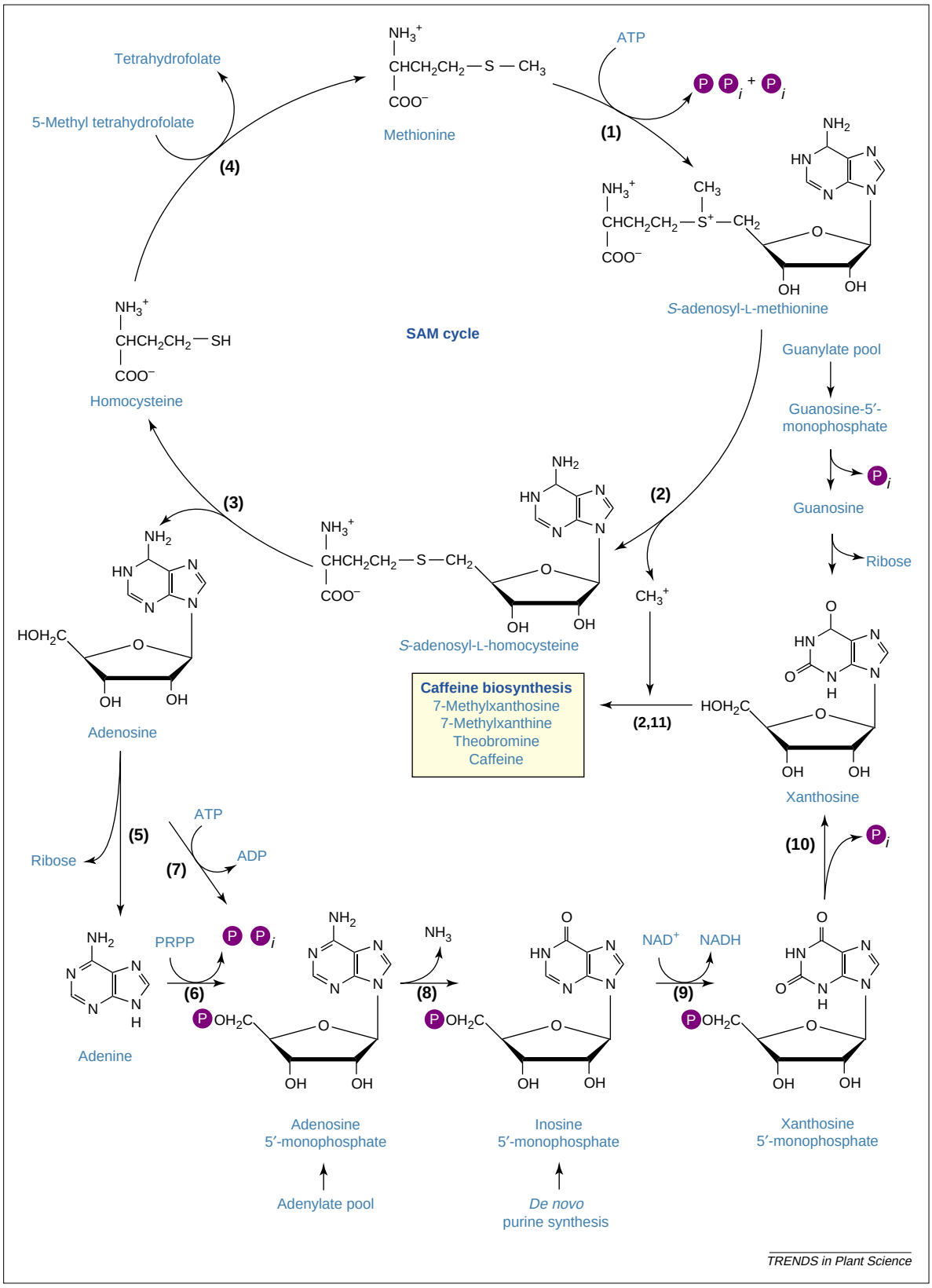

Fig. 4. Proposed new major pathway for the biosynthesis of purine alkaloids in which adenosine derived from the *S*-adenosyl-L-methione (SAM) cycle is metabolized to xanthosine, which is converted to caffeine by a route that involves three SAM-dependent methylation steps. In addition, xanthosine is synthesized from inosine 5′-monophosphate produced by *de novo* purine synthesis. Small amounts of xanthosine might also be derived from the guanylate and adenylate pools. Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD; PRPP, 5-phosphoribosyl-1-diphosphate. Enzymes: (1) SAM synthetase; (2) SAM-dependent *N*-methyltransferases; (3) *S*-adenosyl-L-homocysteine hydrolase; (4) methionine synthase; (5) adenosine nucleosidase; (6) adenine phosphoribosyltransferase; (7) adenosine kinase; (8) adenine 5′-monophosphate deaminase; (9) inosine 5′-monophosphate dehydrogenase; (10) 5′-nucleotidase; (11) 7-methylxanthosine nucleosidase.

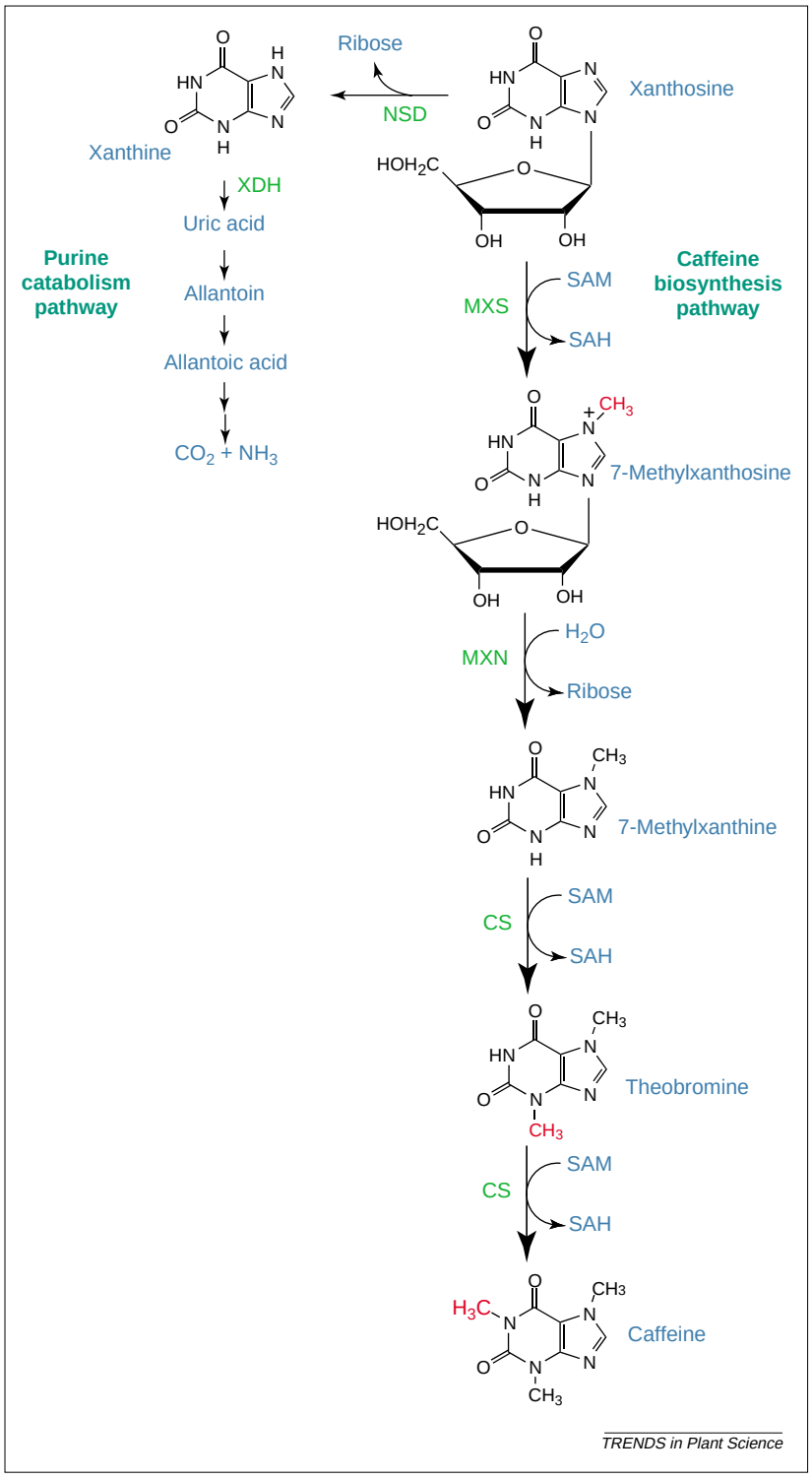

Fig. 5. Biosynthesis of caffeine from xanthosine and the conversion of xanthosine to xanthine and its breakdown to CO_2 and NH_3 via the purine catabolism pathway. Abbreviations: CS, caffeine synthase; MXS, methylxanthosine synthase; MXN, methylxanthosine nucleotidase; NSD, inosine–guanosine nucleosidase; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methione; XDH, xanthine dehydrogenase.

to 7-methylxanthosine, is catalysed by an *N*-methyltransferase, 7-methylxanthosine synthase (MXS). MXS has been extracted from tea and coffee leaves, and exhibits high substrate specificity for xanthosine as the methyl acceptor and for SAM as the methyl donor. It has low activity and is extremely labile, therefore achieving even partial purification has proved a difficult task[29]. However, the purification of MXS from coffee leaves has been achieved[17,18]. The pH optimum of the purified enzyme was 7.0 and the K_m values for xanthosine and SAM were 22 μM and 15 μM, respectively. The next enzyme, methylxanthine nucleosidase, which has been partially purified from tea leaves, catalyses the hydrolysis of 7-methylxanthosine to 7-methylxanthine[30].

The activities of the *N*-methyltransferases that catalyse the conversions of 7-methylxanthine to theobromine and theobromine to caffeine were first shown in crude extracts of tea leaves by Takeo Suzuki and Ei-ichi Takahashi, 26 years ago[31]. However, like MXS, the activity is extremely labile and it was not until 1999 that an enzyme from young tea leaves was purified to apparent homogeneity[32]. This *N*-methyltransferase, caffeine synthase (CS), is monomeric, has an apparent molecular mass of 41 kDa and displays a sharp pH optimum of 8.5. It exhibits *N*-3- and *N*-1-methyltransferase activities, and a broad substrate specificity, showing high activity with paraxanthine, 7-methylxanthine and theobromine, and low activity with 3-methylxanthine and 1-methylxanthine (Table 1). Furthermore, the enzyme has no MXS activity towards either xanthosine or xanthosine-5′-monophosphate[32]. The $V_{ma} \div K_m$ value of tea CS is highest for paraxanthine and so paraxanthine is the best substrate for CS (Ref. 32). However, there is limited synthesis of endogenous paraxanthine from 7-methylxanthine and therefore, *in vivo*, paraxanthine is not an important methyl acceptor[28].

The effects of the concentration of SAM and several methyl acceptors on the activity of CS show typical Michaelis–Menten kinetics, and there is no feedback inhibition by caffeine. It is therefore unlikely that allosteric control of the CS activity is operating in tea leaves. One of the major factors affecting the activity of CS *in vitro* appears to be a product inhibition by SAH. CS is inhibited completely by low concentrations of SAH. Therefore, control of the intracellular SAM:SAH ratio is one possible mechanism for regulating the activity of CS *in vivo*. CS is a chloroplast enzyme but CS activity is not affected by light *in situ* and caffeine is synthesized in the darkness[33].

Cloning of caffeine synthase and related genes

Using 3′ rapid amplification of cDNA ends with degenerate gene-specific primers based on the *N*-terminal residues of purified tea CS, a 1.31 kb sequence of cDNA has been obtained[14]. The 5′ untranslated sequence of the cDNA fragment was isolated by 5′ rapid amplification of cDNA ends. The total length of the isolated cDNA, termed *TCS1* (GenBank Accession No. AB031280), is 1438 bp and it encodes a protein of 369 amino acids. The deduced amino acid sequence of TCS1 shows low homology with other *N*-, *S*- and *O*-methyltransferases from plants and microorganisms, with the exception of

Table 1. Substrate specificity of native and recombinant *N*-methyltransferases from tea and coffee[a]

Source	Substrate (methylation position)								
	7-mX (*N*-3)	3-mX (*N*-1)	1-mX (*N*-3)	Tb (*N*-1)	Tp (*N*-7)	Px (*N*-3)	X (*N*-3)	XR	Refs
Tea leaves (native)	100	17.6	4.2	26.8	TR	210.0	TR	ND	32
Tea leaf TCS1 (recombinant)	100	1.0	12.3	26.8	TR	230.0	*	ND	14
Coffee leaf CTS1 (recombinant)	100	ND	ND	ND	ND	1.4	ND	ND	15
Coffee leaf CTS2 (recombinant)	100	ND	ND	ND	ND	1.1	ND	ND	15
Coffee leaf CaMXMT (recombinant)	100	ND	ND	ND	ND	15.0	ND	ND	16

[a]Enzyme activities of each source are presented as a percentage of the activity when 7-mX is used as the substrate.
Abbreviations: 1-mX, 1-methylxanthine; 3-mX, 3-methylxanthine; 7-mX, 7-methylxanthine; *, not determined; ND, not detected; Px, paraxanthine; Tb, theobromine; Tp, theophylline; TR, trace; X, xanthine; XR, xanthosine.

salicylic acid *O*-methyltransferase[34], with which it shares 41.2% sequence homology. To determine whether the isolated cDNA encoded an active CS protein, *TCS1* was expressed in *E. coli* and lysates of the bacterial cells were incubated with a variety of xanthine substrates in the presence of SAM, which served as a methyl donor. The substrate specificity of the recombinant enzyme was similar to that of purified CS from young tea leaves (Table 1). The recombinant enzyme mainly catalysed *N*-1- and *N*-3-methylation of mono- and dimethylxanthines. No 7-*N*-methylation activity was observed when xanthosine was used as the methyl acceptor. These results provide convincing evidence that *TCS1* encodes CS.

Recently, four *CS* genes from young coffee leaves have been cloned[15]. The predicted amino acid sequences of these genes showed ~40% homology with that of TCS1. Two of the coffee genes, *CTS1* and *CTS2*, were expressed in *E. coli*. The substrate specificity of the recombinant coffee enzymes was much more restricted than that of recombinant tea CS because they used only 7-methylxanthine as a methyl acceptor, converting it to theobromine (Table 1). Therefore, coffee *N*-3-methyltransferases are referred to as theobromine synthases. Independently, another laboratory has cloned similar genes from coffee leaves[16]. Upon expression in *E. coli*, one of the genes, *CaMXMT*, was found to encode a protein possessing *N*-3-methylation activity. The *N*-terminal sequence of CaMXMT shows similarities (35.8%) to that of tea CS and also shares 34.1% homology with salicylic acid *O*-methyltransferase.

Cloning of the *MXS* gene

The coffee *MXS* gene, which participates in the first methylation step of the caffeine biosynthesis pathway, has been cloned[17,18]. The cDNA encoded a protein of 371 amino acids that does not exhibit significant homology to other known proteins, including CS. *C. arabica* callus independently transformed with antisense *MXS* secreted caffeine into the incubation medium in amounts ranging from that produced by untransformed callus to ~2% of the normal levels[18]. This indicates that the antisense cDNA can inhibit caffeine production in coffee callus. However, in the absence of information, either about the substrate specificity of the recombinant enzyme or about whether the conversion of xanthosine to 7-methylxanthosine is blocked in transgenic antisense coffee plants, it cannot yet be concluded that the cloned gene encodes MXS.

Catabolism of caffeine

In tea and coffee plants, caffeine is mainly produced in young leaves and immature fruits, and it continues to accumulate gradually during the maturation of these organs. However, it is slowly catabolized by the removal of the three methyl groups, resulting in the formation of xanthine (Fig. 6)[35]. Several demethylases seem to participate in these sequential reactions but no such enzyme activity has been isolated to date from higher plants. Xanthine is further degraded by the conventional purine catabolism pathway to CO_2 and NH_3 via uric acid, allantoin and allantoate (Fig. 6). Exogenously supplied [8-^{14}C]theophylline is degraded to CO_2 far more rapidly than [8-^{14}C]caffeine, indicating that the initial step in the caffeine catabolism pathway, the conversion of caffeine to theophylline, is the major rate-limiting step. This is not the case in the low-caffeine-containing leaves of *C. eugenioides*, which, unlike *C. arabica*, metabolize [8-^{14}C]caffeine rapidly, with much of the label being incorporated into CO_2 within 24 h (Ref. 36). *C. eugenioides* therefore appears to have far higher levels of *N*-7-demethylase activity than *C. arabica*, and thus can efficiently convert endogenous caffeine to theophylline, which is rapidly metabolized further.

Several species of caffeine-degrading bacteria have been isolated, including *Pseudomonas cepacia*, *Pseudomonas putida* and *Serratia marcescens*. Bacterial degradation is different from that operating in higher plants because it appears to involve a caffeine → theobromine → 7-methylxanthine → xanthine →→ NH_3 pathway (Fig. 6). Bacterial *N*-1-demethylase activity catalysing the metabolism of caffeine to theobromine has been isolated from *Pseudomonas putida*[37].

Future perspectives: biotechnology of caffeine

Genes encoding CS and other *N*-1 and *N*-3 methyltransferases have been cloned. This development opens up the possibility of using genetic

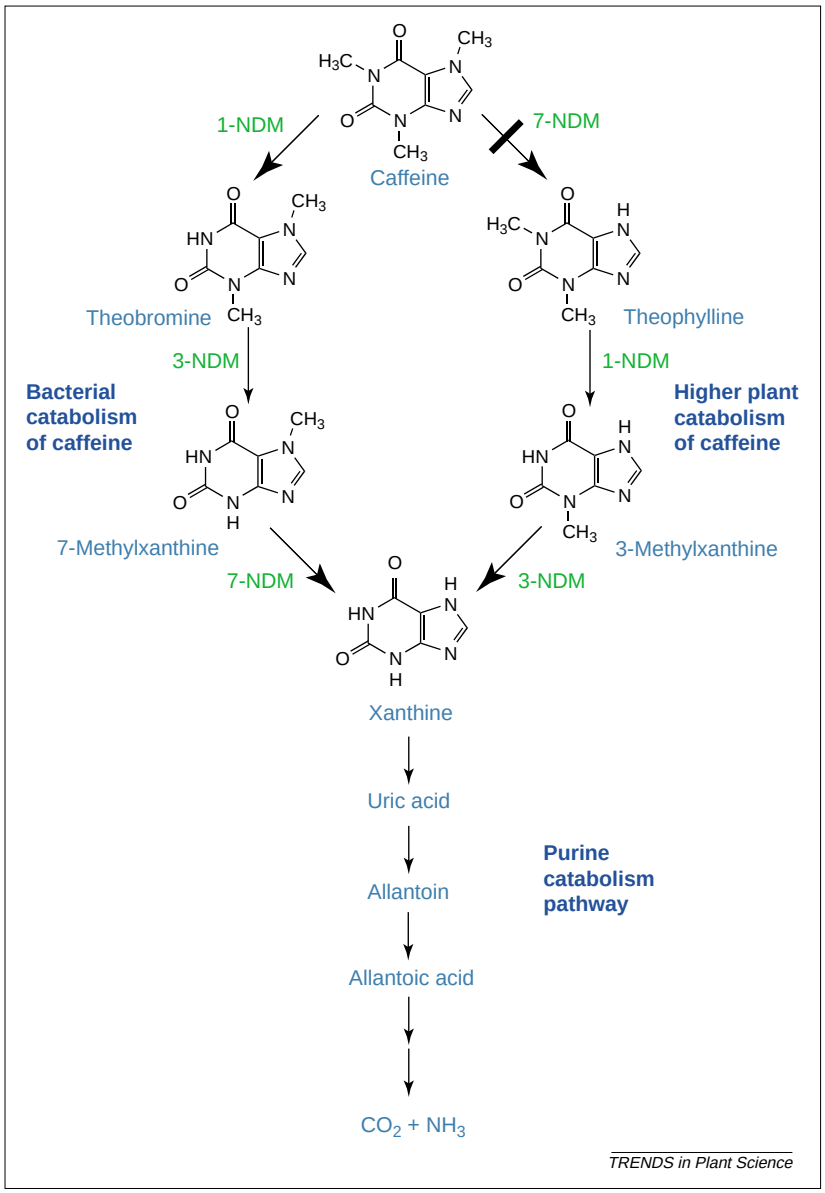

Fig. 6. Bacterial and higher plant caffeine catabolism pathways. The bar between caffeine and theophylline indicates a rate-limiting step in *Coffea arabica* and *Camellia sinensis*. As a consequence, caffeine accumulates in these species because it is converted to theophylline in only limited quantities. In bacteria, such as *Pseudomonas putida*, the initial degradation step is *N*-1-demethylation, which results in the conversion of caffeine to theobromine rather than theophylline. In higher plants, theobromine is a precursor rather than a catabolite of caffeine. Abbreviations: 1-NDM, *N*-1-demethylase; 3-NDM, *N*-3-demethylase; 7-NDM, *N*-7-demethylase.

engineering to produce transgenic tea and coffee plants that are naturally deficient in caffeine. The use of such genetic engineering to make fully flavoured caffeine-free beverages will be of interest to the increasing numbers of consumers who are concerned about the potentially adverse effects of caffeine consumption on their health.

Since the early 1970s, demand for decaffeinated coffee and tea has increased rapidly and, in the case of coffee, 'decaf' sales in the USA in 1999 had a 23% share of the market, estimated to be worth more than US$4 billion. The latest decaffeination method involves the use of supercritical fluid extraction with carbon dioxide to eliminate the health problems posed by the toxicity of residues from extraction solvents. However, for a commercial-scale operation, this process is expensive and, to discerning customers, flavours and aromas will still be lost. In the long term, the increasing demand for decaffeinated coffee and tea could probably be better met by the use of *Coffea* and *Camellia* species that produce low levels of caffeine. In the case of *Coffea*, such material is available from species such as *C. eugenioides* but none are suitable for commercial exploitation because of the poor quality and bitter taste of the resultant beverage and/or the form and low productivity of the trees. There is also a similar situation with tea. Although a breeding programme to obtain low-caffeine-producing plants is feasible, there are genetic barriers and it would probably take 20 years or more to establish and stabilize the desired traits. In the circumstances, the use of genetic engineering to produce transgenic caffeine-deficient tea and coffee might ultimately be a more practical proposition.

The cloning of the *CS* gene is an important advance towards the production of transgenic caffeine-deficient tea and coffee through gene silencing with antisense mRNA or RNA interference. One potential complication is that antisense CS plants might accumulate 7-methylxanthine instead of caffeine. There are few studies of the clinical effects of 7-methylxanthine, although one recent study suggests that it can counter deterioration of eyesight in the elderly by improving the quality of sclera collagen[38]. The situation is potentially more straightforward with the *MXS* gene because antisense expression will produce transgenic plants in which the conversion of xanthosine to 7-methylxanthosine is blocked. Xanthosine might not accumulate because it can be converted to xanthine, which will be degraded by the purine catabolism pathway (Fig. 5).

An alternative way to produce transgenic caffeine-deficient coffee and tea plants would be to overexpress a gene encoding an *N*-demethylase associated with the degradation of caffeine. Expression of the *Pseudomonas putida N*-1-demethylase activity in either *C. arabica* or *C. sinensis* is unlikely to result in caffeine deficiency because caffeine will be degraded to theobromine, which is the immediate precursor of caffeine in both species. However, expression of the *N*-7-demethylase encoding gene from *C. eugenioides* in transgenic tea and coffee plants is much more likely to lead to a reduced caffeine content because the *C. eugenioides* gene product will catalyse the metabolism of caffeine to theophylline, which the native enzymes will catabolize to CO_2 and NH_3 (Fig. 6).

The future use of such material to produce fully flavoured caffeine-free tea and coffee will appeal to many consumers who wish to avoid the risks of adverse side effects associated with caffeine.

Acknowledgements
We thank Kouichi Mizuno (University of Tsukuba) and Misako Kato (Ochanomizu University) for their valuable comments during the preparation of this article, and also Takao Yokota (Teikyo University) for his help in the design and preparation of Fig. 4. Some of the work referred to in this article was supported by Grants in Aid to H.A. from the Ministry of Education, Science, Sports and Culture of Japan (08454255 and 10640627). A.C. was supported by UK–Japan travel grants from the British Council and the Royal Society.

Consumption of decaffeinated tea should also be considered from a more long-term health prospective because it is possible that the protective effects of tea, especially green tea, against heart disease (which are attributed to catechins and related polyphenols[39–42]) might be enhanced by a lack of the potentially hypertensive caffeine[43,44]. It is also feasible that the reported anticancer effects of drinking tea[45] would be amplified by an absence of caffeine.

The cloning of the caffeine biosynthesis genes also opens up the possibility of studying the cellular and subcellular localization of the *N*-methyltransferases and the molecular mechanisms that regulate the production of caffeine and, as such, will add an extra dimension to data already obtained in biochemical studies. Initial studies have shown that CS transcripts are commonest in young tea shoots and decline sharply as the leaves mature, in parallel with decreasing caffeine biosynthesis *in vivo*[46]. More transcripts of *CTS1* and *CTS2* accumulate in young coffee leaves and flower buds than in mature and aged leaves[15]. Similarly, *CaMXMT* transcripts accumulate in young leaves and stems but not roots and old leaves of coffee plants[16]. The availability of transgenic caffeine-deficient *C. arabica* and *C. sinensis* plants should also enable the proposed roles of caffeine as a chemical protectant against insects and as an allelopathic agent to be thoroughly evaluated.

References

1 Mazzafera, P. *et al.* (1991) Caffeine metabolism in *Coffea arabica* and other species of coffee. *Phytochemistry* 30, 3913–3916
2 Chou, T.M. and Benowitz, N.L. (1994) Caffeine and coffee: effects on health and cardiovascular disease. *Comp. Biochem. Physiol.* 109C, 173–189
3 Nurminen, M-L. *et al.* (1999) Coffee, caffeine and blood pressure. *Eur. J. Clin. Nutr.* 53, 831–839
4 Eskenzai, B. (1999) Caffeine – filtering the facts. *New Engl. J. Med.* 341, 1688–1689
5 Kihlman, B.A. (1977) *Caffeine and Chromosome*, Elsevier
6 Harborne, J.B. (1993) *Introduction to Ecological Biochemistry* (4th edn), Academic Press
7 Hewavitharanage, H. *et al.* (2000) Effect of caffeine on shot-hole borer beetle (*Xyleborus fornicatus*) of tea (*Camellia sinensis*). *Phytochemistry* 51, 35–41
8 Waller, G.R. (1989) Biochemical frontiers of allelopathy. *Biol. Plant.* 31, 418–447
9 Dennis, D.T. *et al.* (1997) *Plant Metabolism* (2nd edn), Longman
10 Dey, P.M. and Harborne, J.B. (1997) *Plant Biochemistry*, Academic Press
11 Heldt, H-W. (1997) *Plant Biochemistry and Molecular Biology*, Oxford University Press
12 Buchanan, R.B. *et al.*, eds (2000) *Biochemistry and Molecular Biology of Plants*, American Society of Plant Physiologists, Rockville, MD, USA
13 Ashihara, H. and Crozier, A. (2000) Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. In *Advances in Botanical Research* (Vol. 30) (Callow, J.R., ed.), pp. 117–205, Academic Press
14 Kato, M. *et al.* (2000) Caffeine synthase gene from tea leaves. *Nature* 406, 956–957
15 Mizuno, K. *et al.* cDNA cloning of caffeine (theobromine) synthase from coffee (*Coffea arabica* L.). In *Proceedings of the 19th International Scientific Colloquium on Coffee*, Association Scientifique Internationale du Café, Paris, France (in press)
16 Ogawa, M. *et al.* (2001) 7-Methylxanthine methyltransferase of coffee plants. Gene isolation and enzymatic properties. *J. Biol. Chem.* 276, 8213–8218
17 Moisyadi, S. *et al.* (1998) Cloning and characterization of a cDNA encoding xanthosine-*N*7-methyltransferase from coffee (*Coffea arabica*). *Acta Hortic.* 461, 15HB 367–377
18 Moisyadi, S. *et al.* (1999) Cloning and characterization of xanthosine-*N*7-methyltransferase, the first enzyme of the caffeine biosynthetic pathway. In *Proceedings of the 18th International Scientific Colloquium on Coffee*, pp. 327–331, Association Scientifique Internationale du Café, Paris, France
19 Haslam, E. (1998) *Practical Polyphenols. From Structure to Molecular Recognition and Physiological Action*, Cambridge University Press
20 Mosli Waldhauser, S.S. and Baumann, T.W. (1996) Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. *Phytochemistry* 42, 985–996
21 Suzuki, T. and Waller, G.R. (1988) Metabolism and analysis of caffeine and other methylxanthines in coffee, tea, cola, guarana and cacao. In *Modern Methods of Plant Analysis* (Vol. 8) (Linskens, H.F. and Jackson, J.F., eds), pp. 184–220, Springer-Verlag
22 Kretschmar, J.A. and Baumann, T.W. (1999) Caffeine in *Citrus* flowers. *Phytochemistry* 52, 19–23
23 Bogo, A. and Mantle, P.G. (2000) Caffeine: also a fungal metabolite. *Phytochemistry* 54, 937–939
24 Ito, E. and Ashihara, H. (1999) Contribution of purine nucleotide biosynthesis *de novo* to the formation of caffeine in young tea (*Camellia sinensis*) leaves. *J. Plant Physiol.* 154, 145–151
25 Koshiishi, C. *et al.* (2001) A new caffeine biosynthetic pathway in tea leaves: utilization of adenosine release from the *S*-adenosyl-L-methionine cycle. *FEBS Lett.* 499, 50–54
26 Kato, M. *et al.* (1996) Caffeine biosynthesis in young leaves of *Camellia sinensis* L.: *in vitro* studies on *N*-methyltransferase activity involved in the conversion of xanthosine to caffeine. *Physiol. Plant.* 98, 629–636
27 Ashihara, H. *et al.* (1996) Biosynthesis of caffeine in leaves of coffee. *Plant Physiol.* 111, 747–753
28 Ashihara, H. *et al.* (1997) Metabolism of caffeine and related purine alkaloids in leaves of tea (*Camellia sinensis* L.). *Plant Cell Physiol.* 38, 413–419
29 Mosli Waldhauser, S.S. *et al.* (1997) Separation of the *N-7* methyltransferase, the key enzyme in caffeine biosynthesis. *Phytochemistry* 45, 1407–1414
30 Negishi, O. *et al.* (1985) Methylation of xanthosine by tea-leaf extracts and caffeine biosynthesis. *Agric. Biol. Chem.* 49, 887–890
31 Suzuki, T. and Takahashi, E. (1975) Biosynthesis of caffeine by tea-leaf extracts: enzymic formation of theobromine from 7–methylxanthine and of caffeine from theobromine. *Biochem. J.* 146, 87–96
32 Kato, M. *et al.* (1999) Purification and characterization of caffeine synthase from tea leaves. *Plant Physiol.* 120, 579–586
33 Koshiishi, C. *et al.* (2000) Effects of light on purine alkaloid biosynthesis in young leaves of *Camellia sinensis*. *J. Plant Res.* 113, 217–221
34 Ross, J.R. *et al.* (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
35 Ashihara, H. *et al.* (1996) Catabolism of caffeine and related purine alkaloids in leaves of *Coffea arabica* L. *Planta* 198, 334–339
36 Ashihara, H. and Crozier, A. (1999) Biosynthesis and catabolism of caffeine in low-caffeine containing species of *Coffea*. *J. Agric. Food Chem.* 47, 3425–3431
37 Asano, Y. *et al.* (1994) Enzymes involved in theobromine production from caffeine by *Pseudomonas putida*. *Biosci. Biotechnol. Biochem.* 58, 2303–2304
38 Trier, K. *et al.* (1999) Biochemical and ultrastructural changes in rabbit sclera after treatment with 7-methylxanthine, theobromine, acetazolamide or L-ornithine. *Br. J. Ophthalmol.* 83, 1370–1375
39 Stensvold, I. *et al.* (1992) Tea consumption. Relationship to cholesterol, blood pressure, and coronary and total mortality. *Prev. Med.* 21, 546–553
40 Hertog, M.G.L. *et al.* (1993) Dietary antioxidant flavonoids and the risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 342, 1007–1010
41 Vinson, J.A. and Dabbagh, Y.F. (1998) Effect of green and black tea supplementation on lipids, lipid oxidation and fibrinogen in the hamster: mechanisms for the epidemiological benefits of tea drinking. *FEBS Lett.* 433, 44–46
42 Leenen, R. *et al.* (2000) A single dose of tea with or without milk increases plasma antioxidant activity in humans. *Eur. J. Clin. Nutr.* 54, 87–92
43 Lane, J.D. *et al.* (1998) Caffeine raises blood pressure at work. *Psychosom. Med.* 60, 327–330
44 James, J.E. (1997) Is habitual caffeine use a preventable cardiovascular risk factor? *Lancet* 349, 279–281
45 Fujiki, H. (1999) Two stages of cancer prevention with green tea. *J. Cancer Res. Clin. Oncol.* 125, 589–597
46 Kato, M. *et al.* (2000) cDNA cloning and expression analysis of caffeine synthase gene from tea leaves. *Plant Cell Physiol.* 41 (Suppl.), S39