

Review

Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering

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Abstract

Details of the recently elucidated biosynthetic pathways of caffeine and related purine alkaloids are reviewed. The main caffeine biosynthetic pathway is a sequence consisting of xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine. Genes encoding *N*-methyltransferases involved in three of these four reactions have been isolated and the molecular structure of *N*-methyltransferases investigated. Pathways for the catabolism of caffeine have also been studied, although there are currently no reports of enzymatic and genetic studies having been successfully carried out. Metabolism of purine alkaloids in species including *Camellia*, *Coffea*, *Theobroma* and *Ilex* plants is summarised, and evidence for the involvement of caffeine in chemical defense and allelopathy is discussed. Finally, information is presented on metabolic engineering that has produced coffee seedlings with reduced caffeine content, and transgenic caffeine-producing tobacco plants with enhanced disease resistance.

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Keywords: *Camellia sinensis*; Theaceae; *Coffea* sp.; Rubiaceae; *Theobroma cacao*; Sterculiaceae; Review; Metabolism; Caffeine

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1. Introduction

Purine alkaloids are secondary metabolites derived from purine nucleotides (Zulak et al., 2006) that have been found in nearly 100 species in 13 orders of plant kingdom (Ashihara and Crozier, 1999a). Methylxanthines, such as caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), and methyluric acids are classified as purine alkaloids (Fig. 1). They occur in tea, coffee and a number of other non-alcoholic beverages. Caffeine was isolated from tea and coffee in the early 1820s, but the main biosynthetic and catabolic pathways of caffeine were not fully established until 2000. Highly purified caffeine synthase was obtained from tea leaves after which a gene encoding

the enzyme was cloned (Kato et al., 1999, 2000). This facilitated molecular studies on the regulation of caffeine production principally with coffee plants (Ogita et al., 2003). This review describes biosynthesis and catabolism of purine alkaloids, and the genes and molecular structure of *N*-methyltransferases related to caffeine biosynthesis. In addition, details are included on purine metabolism in a number of plants including *Camellia*, *Coffea*, *Theobroma* and *Ilex* species. The emerging role of purine alkaloids *in planta* and metabolic engineering of caffeine are also discussed.

2. Caffeine biosynthesis

Main caffeine biosynthetic pathway is a four step sequence consisting of three methylation and one nucleosidase reactions (Fig. 2). The xanthine skeleton of caffeine is derived from purine nucleotides. The initial step in caffeine biosynthesis is the methylation of xanthosine by a SAM-dependent *N*-methyltransferase. In addition to experiments with radiolabelled precursors, substrate specificities of native (Kato et al., 1999) and recombinant *N*-methyltransferases (Kato et al., 2000; Ogawa et al., 2001; Mizuno et al., 2003a,b; Uefuji et al., 2003) strongly suggest that the major route to caffeine is a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway (Fig. 2). Although the information has been obtained mainly from coffee (*Coffea arabica*) and tea (*Camellia sinensis*), the available evidence indicates that the pathway is essentially the same in other purine alkaloid-forming plants, such as maté (*Ilex paraguariensis* – Ashihara, 1993) and cacao (*Theobroma cacao* – Koyama et al., 2003; Yoneyama et al., 2006).

		Methylxanthines						Uric acid and methyluric acids	
Compounds	Trivial name	R ₁	R ₂	R ₃	R ₄	O-2	Δ ^{2,3}		
Methylxanthines									
	Xanthine	H	H	H	-	-	-		
	1-Methylxanthine	CH ₃	H	H	-	-	-		
	3-Methylxanthine	H	CH ₃	H	-	-	-		
	7-Methylxanthine	H	H	CH ₃	-	-	-		
	1,3-Dimethylxanthine	CH ₃	CH ₃	H	-	-	-		
	1,7-Dimethylxanthine	CH ₃	H	CH ₃	-	-	-		
	3,7-Dimethylxanthine	H	CH ₃	CH ₃	-	-	-		
	1,3,7-Trimethylxanthine	CH ₃	CH ₃	CH ₃	-	-	-		
Uric acid and methyluric acids									
	Uric acid	H	H	H	H	-	-		
	1,3,7-Trimethyluric acid	CH ₃	CH ₃	CH ₃	H	-	-		
	1,3,7,9-Tetramethyluric acid	CH ₃	CH ₃	CH ₃	CH ₃	-	-		
	O(2),1,9-Trimethyluric acid	CH ₃	H	H	CH ₃	CH ₃	Δ ^{2,3}		
	O(2),1,7,9-Tetramethyluric acid	CH ₃	H	CH ₃	CH ₃	CH ₃	Δ ^{2,3}		

Fig. 1. Purine alkaloid structures based on xanthine and uric acid skeletons.

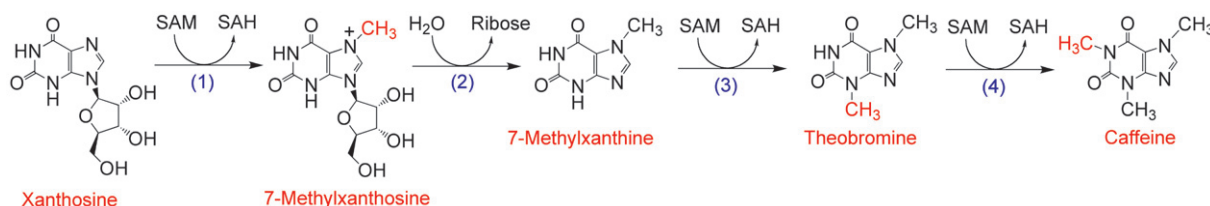


Fig. 2. The major biosynthetic pathway of caffeine from xanthosine: (1) 7-methylxanthosine synthase (xanthosine *N*-methyltransferase); (2) *N*-methylnucleosidase; (3) theobromine synthase (monomethylxanthine *N*-methyltransferase); (4) caffeine synthase (dimethylxanthine *N*-methyltransferase); (3–4) dual-functional caffeine synthase (EC 2.1.1.160). Several *N*-methyltransferases with different substrate specificities contribute to the conversion of xanthosine to caffeine (see Section 3). Recent observations suggest that steps 1 and 2 are catalysed by xanthosine *N*-methyltransferase. SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine.

2.1. Caffeine biosynthesis from xanthosine

The first step in the biosynthetic pathway of caffeine from xanthosine is the conversion of xanthosine to 7-methylxanthosine (Fig. 2). This reaction is catalysed by 7-methylxanthosine synthase (xanthosine 7*N*-methyltransferase, EC 2.1.1.158). The genes encoding 7-methylxanthosine synthase, *CmXRS1* (AB 034699) and *CaXMT1* (AB048793), were isolated from *C. arabica* (Mizuno et al., 2003a; Uefuji et al., 2003). The recombinant proteins obtained from these genes exhibit 7-methylxanthosine synthase activity *in vitro*. Although Schulthess et al. (1996) proposed the inclusion of 7-methyl-XMP in caffeine biosynthesis pathway in coffee, XMP is not converted to 7-methylxanthosine by the recombinant enzymes.

The second step of caffeine biosynthesis involves a nucleosidase which catalyses the hydrolysis of 7-methylxanthosine. Although *N*-methylnucleosidase (EC 3.2.2.25) was partially purified from tea leaves by Negishi et al. (1988), isolation of the native enzyme as well as DNA encoding the enzyme has not yet been achieved. Recent detailed structural studies on coffee 7-methylxanthosine synthase suggested that the methyl transfer and nucleoside cleavage may be coupled and catalysed by a single enzyme (McCarthy and McCarthy, 2007).

The last two steps of caffeine synthesis are also catalysed by SAM-dependent *N*-methyltransferase(s), but this enzyme is different from the *N*-methyltransferase that catalyses the first step in the pathway. Native *N*-methyltransferase activities have been detected in crude and partially purified extracts from tea and coffee plants (see Suzuki et al., 1992; Ashihara and Suzuki, 2004), and a highly purified preparation has been obtained from young tea leaves (Kato et al., 1999). The enzyme, assigned the name caffeine synthase (EC 2.1.1.160), catalyses the last two steps of caffeine biosynthesis, the conversion of 7-methylxanthine to caffeine via theobromine (Fig. 2). The gene encoding caffeine synthase was cloned from young tea leaves by Kato et al. (2000). Since then, plural genes encoding *N*-methyltransferases which have different substrate specificities have been reported (see Section 3.1).

Activity of the recombinant theobromine synthase (CTS1 and CaMXMT, EC 2.1.1.159) is specific for the conversion of 7-methylxanthine to theobromine. In contrast, the recombinant caffeine synthases (CCS1 and CaDMXT1) can utilise paraxanthine, theobromine and 7-methylxanthine as shown with tea caffeine synthase (TCS1) (see Section 3.1). Although paraxanthine is the most active substrate of this recombinant enzyme, only limited amounts of paraxanthine are synthesized in coffee cells so *in vivo* caffeine synthase is involved principally in the conversion of 7-methylxanthine to caffeine via theobromine.

Radiolabelled tracer experiments with theobromine-accumulating cacao and a Chinese tea (*Camellia ptilophylla*) showed a limited conversion of theobromine to caffeine. *N*-Methyltransferase activity which catalysed the conversion of 7-methylxanthine to theobromine, but not

theobromine to caffeine, was detected in crude extracts from *C. ptilophylla* (Ashihara et al., 1998). Recently, genes homologous to caffeine synthase have been isolated from several theobromine-accumulating plants including cacao (Yoneyama et al., 2006). The recombinant enzymes derived from these genes have only 3-*N*-methyltransferase activity. Therefore, in these theobromine-accumulating plants, specific theobromine synthases are present. Caffeine does not occur in *C. ptilophylla* (Ashihara et al., 1998) although it is present in leaves and fruits of cacao along with theobromine (Koyama et al., 2003; Zheng et al., 2004). Genes encoding caffeine synthase appear, therefore, to be present in cacao but not the Chinese tea plants.

2.2. Supply of xanthosine for caffeine biosynthesis

Xanthosine, the initial substrate of purine alkaloid synthesis, is supplied by at least four different pathways: *de novo* purine biosynthesis (*de novo* route), the degradation pathways of adenine nucleotides (AMP route) and guanine nucleotides (GMP route), and the *S*-adenosyl-L-methionine (SAM) cycle (SAM route) (Fig. 3).

Purine nucleotides are synthesized by *de novo* and salvage pathways (Ashihara and Crozier, 1999a; Stasolla et al., 2003; Zrenner et al., 2006). The production of purine nucleotides is the result of *de novo* synthesis from non-purine precursors, namely, CO₂, 10-formyltetrahydrofolate, 5-phosphoribosyl-1-pyrophosphate and the amino acids, glycine, glutamine, and aspartate.

Utilisation of IMP, which is formed by the *de novo* purine biosynthetic pathway, for caffeine biosynthesis was demonstrated in young tea leaves using ¹⁵N-glycine and ¹⁴C-labelled precursors and inhibitors of *de novo* purine biosynthesis (Ito and Ashihara, 1999). Xanthosine is formed by an IMP → XMP → xanthosine pathway. IMP dehydrogenase (1.1.1.205) and 5'-nucleotidase (EC 3.1.3.5) may catalyse these reactions, IMP dehydrogenase being implicated as an inhibitor of the enzyme reduced the rate of caffeine biosynthesis in tea and coffee leaves (Keya et al., 2003).

A portion of the xanthosine used for caffeine biosynthesis is derived from the adenine and guanine nucleotide pools which are produced by the *de novo* and salvage pathways. There are several potential pathways for xanthosine synthesis from AMP, but the AMP → IMP → XMP → xanthosine route is likely to predominate. All three enzymes involved in the conversion, AMP deaminase (EC 3.5.4.6), IMP dehydrogenase and 5'-nucleotidase, have been detected in tea leaves (Koshiishi et al., 2001).

Xanthosine for caffeine biosynthesis is also produced from guanine nucleotides by a GMP → guanosine → xanthosine pathway. Guanosine deaminase (EC 3.5.4.15) activity found in cell-free extracts from young tea leaves (Negishi et al., 1994), but GMP deaminase activity has not been detected (Stasolla et al., 2003; Zrenner et al., 2006). The absence of GMP deaminase suggests that a GMP → IMP → XMP → xanthosine route is not functional in plants.

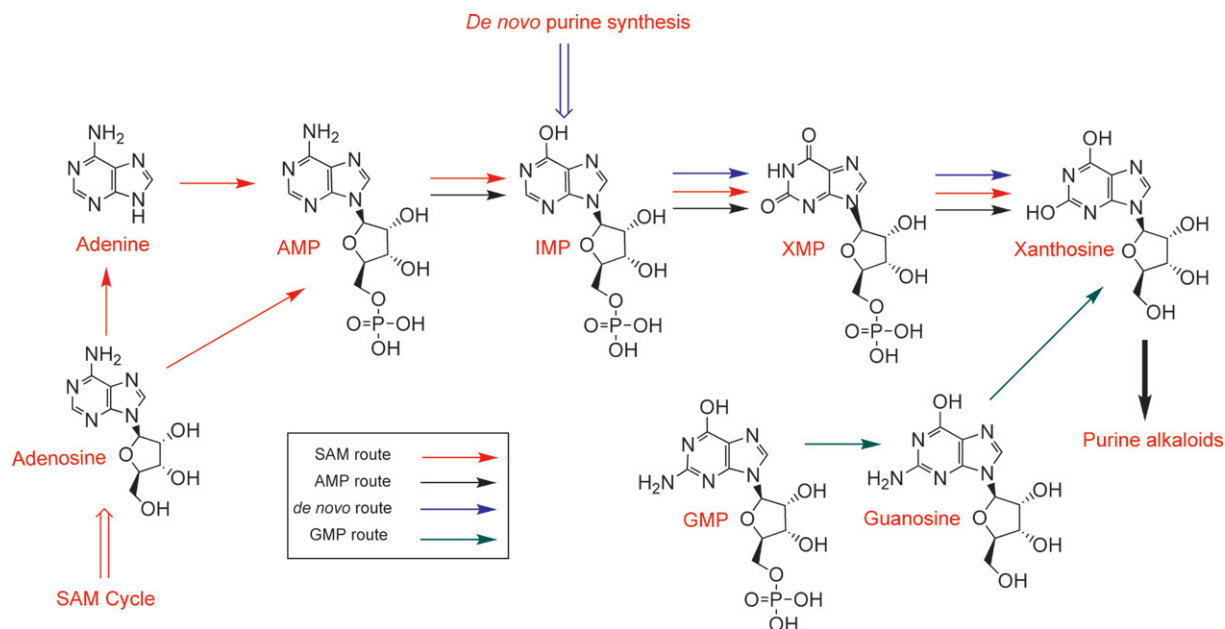


Fig. 3. Purine alkaloids are synthesized from xanthosine which is produced via at least four routes: from adenosine released from the SAM cycle (SAM route); from IMP originating from *de novo* purine synthesis (*de novo* route), from the cellular adenine nucleotide pool (AMP route) and from the guanine nucleotide pool (GMP route).

Many studies on caffeine biosynthesis have utilised radiolabelled purine bases and nucleosides. This is because it is believed that most of exogenous purine nucleotides are rapidly hydrolysed by plant tissues forming purine nucleoside and/or bases which are then utilised in pathways that in most instances lead indirectly to xanthosine. Most of these purine compounds, including adenine, adenosine, inosine, hypoxanthine and guanine, are converted to their respective nucleotides by salvage enzymes, including adenine phosphoribosyltransferase (EC 2.4.2.7), hypoxanthine/guanine phosphoribosyltransferase (EC 2.4.2.8), adenosine kinase (EC 2.7.1.20) and inosine/guanosine kinase (EC 2.4.2.1), which usually have high activities in plant cells. The resultant nucleotides then enter the purine alkaloid biosynthetic pathway. In the case of guanosine, it may be directly deaminated to xanthosine and utilised for caffeine biosynthesis (Fig. 3) (Ashihara et al., 1997b).

SAM is the methyl donor for the three methylation steps in the caffeine biosynthetic pathway. In the process SAM is converted to *S*-adenosyl-L-homocysteine (SAH), which is in turn hydrolysed to homocysteine and adenosine. Homocysteine is recycled via the SAM cycle to replenish SAM levels and adenosine is released from the cycle. Adenosine released from the cycle is converted to AMP directly and/or indirectly via adenine. AMP is converted to xanthosine and used for caffeine biosynthesis. Since three moles of SAH are produced via the SAM cycle for each mole of caffeine that is synthesized, in theory 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 (Koshiishi et al., 2001).

3. *N*-Methyltransferases involved in caffeine biosynthesis

3.1. Genes

Genes encoding enzymes involved in caffeine biosynthesis were successfully isolated from plants of the coffee family by PCR and library screening methods (Ogawa et al., 2001; Uefuji et al., 2003; Mizuno et al., 2003a,b). This involved designing degenerated primers based on conserved regions in tea caffeine synthase (AB031280) and *Arabidopsis* unknown proteins. Small PCR products were then used as the probe to isolate full length cDNA from a library, resulting in the identification of all three *N*-methyltransferase genes (Fig. 2). To date, seven cDNAs have been characterized from coffee plants; one for xanthosine methyltransferase (XMT/XRS), three for 7-methylxanthine methyltransferase or theobromine synthase (MXMT/CTS), and three for 3,7-dimethylxanthine methyltransferase or caffeine synthase (DXMT/CCS) (Table 1). Their properties are well described in a recent review by Kato and Mizuno (2004), so this article will only briefly summarise some aspects of the cloned genes and their products.

First, it should be mentioned that two research groups independently designated identified genes/proteins and different nomenclatures were adopted. For instance, an enzyme catalyzing the second methylation step, converting 7-methylxanthine to 3,7-dimethylxanthine (theobromine), was named both 7-methylxanthine methyltransferase (MXMT) and coffee theobromine synthase (CTS) (see Table 1). In the future this will be resolved according to the international rules of gene nomenclature, but in this article the enzymes will be referred to by their original name.

Table 1
Nomenclature of *N*-methyltransferases and encoding genes involved in caffeine biosynthesis^a

Enzyme	Gene
Xanthosine methyltransferase (7-methylxanthosine synthase) (EC 2.1.1.158)	<i>CaXMT</i> (AB048793) <i>CmXRS1</i> (AB034699)
7-Methylxanthine methyltransferase (theobromine synthase) (EC 2.1.1.159)	<i>CaMXMT1</i> (AB048794) <i>CTS1</i> (AB034700) <i>CaMXMT2</i> (AB084126) <i>CTS2</i> (AB054841)
3,7-Methylxanthine methyltransferase (caffeine synthase) (EC 2.1.1.160)	<i>CaDXMT1</i> (AB984125) <i>CCS1</i> (AB086414) <i>CtCS7</i> (AB086415)

^a *CaXMT*, *Coffea arabica* xanthosine methyltransferase; *CmXRS*, coffee 7-methylxanthosine synthase; *CaMXMT*, *C. arabica* 7-methylxanthine methyltransferase; *CTS*, coffee theobromine synthase; *CaDXMT*, *C. arabica* 3,7-dimethylxanthine methyltransferase; *CCS*, coffee caffeine synthase; *CtCS*, coffee tentative caffeine synthase.

A single gene encoding xanthosine methyltransferase (XMT/*CmXRS*) has been identified, and it encodes a polypeptide consisting of 372 amino acids with an apparent molecular mass of 41.8 kDa. It is expressed almost uniformly in aerial tissues of *C. arabica*, including leaves, floral buds and immature but not mature beans. In contrast, at least three genes encoding 7-methylxanthine methyltransferase (theobromine synthase) have been isolated. The number of amino acids in the putative polypeptides are 378 for MXMT1 (42.7 kDa) and 384 for MXMT2 and CTS2 (43.4 kDa). They differ by insertion or deletion of blocks of several residues in the C-terminal region. Their catalytic properties as judged from kinetic parameters, such as K_m values, are apparently distinct each other. They are expressed in young leaves, floral buds and immature but not mature beans. Three genes were also identified for 3,7-dimethylxanthine methyltransferase (caffeine synthase); *DXMT*, *CCS1* and *CtCS7*, each encoding a 43-kDa polypeptide consisted of 384 amino acids. However, their kinetic properties differ with, for example, *DXMT* and *CCS1* showing K_m values for theobromine of 1200 and 157 μ M, respectively. Expression profiles are also distinct, *DXMT* being expressed exclusively in immature beans, while *CCS1* expression is ubiquitous, occurring in all tissues. The presence of isoforms of these enzymes with different properties suggests that caffeine is synthesized through multiple pathways depending on availability and concentration of the substrates (Mizuno et al., 2001, 2003a,b; Ogawa et al., 2001; Uefuji et al., 2003).

The deduced polypeptides of these enzymes have more than 82% similarity and phylogenetic analysis indicates that they are more closely related to *C*-methyltransferases, including those for jasmonic acid, salicylic acid and benzoic acid, than to other *N*-methyltransferases. This suggests that coffee *N*-methyltransferases constitute a distinct sub-group within the plant methyltransferase family. Their cellular localization was determined by the green-fluorescence protein fusion method and this showed that all three enzymes are localized in the cytoplasm (Ogawa et al., 2001; Kodama

et al., 2007). Caffeine, thus, appears to be synthesized in the cytoplasm, and translocated to vacuoles via not yet identified mechanisms.

3.2. Molecular structure of caffeine biosynthesis enzymes

Despite of the marked similarity in amino acid sequences, each enzyme catalyzes only the methylation of its specific substrate. This raises a question as to how the substrate specificity of each enzyme is determined. The importance of histidine-160 was pointed out after comparing amino acid sequences of proteins related to MXMT/*CTS*, and it was suggested that substrate specificity may be determined by a small number of key amino acids (Ogawa et al., 2001). Recent crystallography analyses have established that histidine-160 is of importance for substrate binding (McCarthy and McCarthy, 2007). This study revealed that a single amino acid in each enzyme is critical for substrate selectivity as exemplified by serine-316 in XMT being the central to the recognition of xanthosine.

Crystallographic data also elucidated the structure of XMT and DXMT, each of which was shown to consist of two domains, the methyltransferase domain and the α -helical cap domain. The overall structure was found to resemble that of salicylic acid methyltransferase, which exists as a dimer in solution (Zubieta et al., 2003). It has been proposed that XMT and DXMT also form dimers (McCarthy and McCarthy, 2007). Further analyses by bimolecular fluorescence complementation showed that all three enzymes, XMT, MXMT and DXMT, are capable of forming homo-dimers *in planta* (Kodama et al., 2007). Furthermore, each enzyme was also found to form a hetero-dimer with each of the other two enzymes. To test whether or not enzymatic activity is influenced by hetero-dimer formation, competition analysis was performed using the MXMT and XMT proteins. Preliminary results showed no difference in MXMT activity in the presence or absence of XMT, suggesting that hetero-dimer formation might not interfere with the individual enzymatic activities (Kodama et al., 2007). These observations imply that a hetero-dimer may act as a dual-functional enzyme, thereby rapidly and efficiently catalyzing caffeine biosynthesis *in planta*.

Another notable feature of XMT is that it has a dual function, exhibiting both methyltransferase and nucleoside cleavage activities (McCarthy and McCarthy, 2007). Although plants often possess nucleosidase activity with low-substrate specificity (Kim et al., 2006), this finding accounts for the biosynthesis of caffeine in transgenic tobacco plants, transformed with genes for three *N*-methyltransferases but not with a nucleotidase-encoding gene (Uefuji et al., 2005).

4. Catabolism of caffeine

Caffeine is produced in young leaves and immature fruits, and continues to accumulate gradually during the

maturation of these organs. However, it is very slowly degraded with the removal of the three methyl groups, resulting in the formation of xanthine. Catabolism of caffeine in coffee leaves was first reported by Kalberer (1965). Since then a number of tracer experiments using ^{14}C -labelled purine alkaloids have been reported (Suzuki and Waller, 1984; Ashihara et al., 1996, 1997a; Mazzafera, 1998; Vitória and Mazzafera, 1998) which demonstrate that the major catabolic pathway is caffeine \rightarrow theophylline \rightarrow 3-methylxanthine \rightarrow xanthine. Xanthine is further degraded by the conventional purine catabolism pathway

to CO_2 and NH_3 via uric acid, allantoin and allantoate (Fig. 4) (Ashihara and Crozier, 1999a; Stasolla et al., 2003; Zrenner et al., 2006). Caffeine catabolism usually begins with its conversion to theophylline catalysed by *N*7-demethylase. The involvement of the P450-dependent mono-oxygenase activity for this reaction was suggested by Huber and Baumann (1998) and Mazzafera (2004). However, activity of this enzyme has not yet determined even in cell-free extracts. $[8\text{-}^{14}\text{C}]$ Theophylline is degraded to CO_2 far more rapidly than $[8\text{-}^{14}\text{C}]$ caffeine, indicating that the conversion of caffeine to theophylline is the major

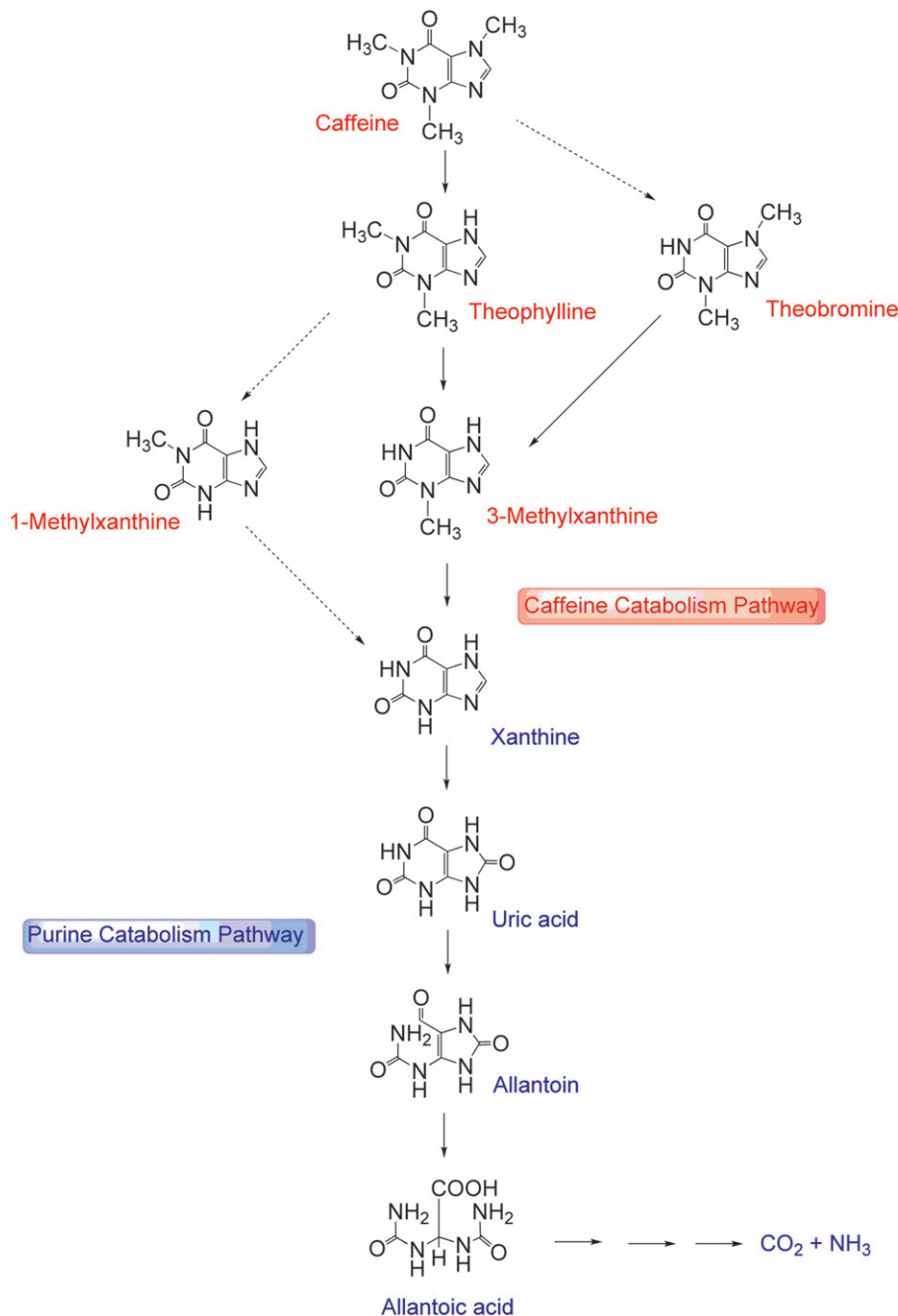


Fig. 4. Caffeine catabolic pathways. Caffeine is mainly catabolised to xanthine via theophylline and 3-methylxanthine. Xanthine is further degraded to CO_2 and NH_3 by the conventional oxidative purine catabolic pathway. Dotted arrow shows minor routes. Typically, the conversion of caffeine to theophylline is the major rate limiting step and as a consequence caffeine accumulates in species such as *Coffea arabica* and *Camellia sinensis*.

rate-limiting step of caffeine catabolism and the reason why caffeine accumulates in high concentrations in tissues of *C. sinensis* and *C. arabica* (Ashihara et al., 1996; Ito et al., 1997).

5. Metabolism of purine alkaloids in individual species

In addition to major pathways of purine alkaloid biosynthesis and catabolism, species dependent minor pathways also exist. In this section, species-specific metabolism and physiological studies will be reviewed.

5.1. *Camellia* species

Caffeine is the major purine alkaloid in tea, but some *Camellia* species accumulate theobromine instead of caffeine (see Table 2). *C. ptilophylla* was discovered in 1988 and named cocoa tea (Chang et al., 1988). Theobromine accumulates in young shoots of this species in concentrations as high as 7% of the dry weight, which is well above the levels observed in *T. cacao* (Ye et al., 1997). An unusual Chinese tea known as kucha (*Camellia assamica* var. kucha) contains theacrine (1,3,7,9-tetramethyluric acid) at a concentration of 2.8% of dry weight in expanding buds and young leaves (Zheng et al., 2002). The stamens and petals of flowers of several species of *Camellia* plants also contain caffeine and/or theobromine; and the species-to-species pattern of purine alkaloid distribution is very similar to that found in young leaves (Fujimori and Ashihara, 1990; Ashihara and Kubota, 1987). In the dry fruit, the pericarp contains the most alkaloids, but there are also considerable amounts in the seed coat and, to a lesser extent, the fruit stalk and the seed. Germinating seeds also contain significant amounts of the alkaloids (Suzuki and Waller, 1985).

Experiments on the incorporation of [¹⁴C]adenine into purine alkaloids by *C. sinensis* leaves showed that caffeine synthesis occurs only in young tissues (Ashihara and Kubota, 1986). Seasonal variations in caffeine biosynthetic activity have been investigated using leaf discs, prepared at monthly intervals over the course of a one year period, from plants grown under natural field conditions in Japan (Fujimori et al., 1991). Incorporation of radioactivity into theobromine and caffeine occurred only in the young leaves

harvested between April and June. Furthermore, the activities of the three *N*-methyltransferases associated with caffeine biosynthesis were detected in *C. sinensis* leaves only during this limited three month period. This indicates that in field-grown plants, caffeine biosynthesis is restricted to young leaves of new shoots in the spring. The relative amounts of transcripts of *TCSI* which encode caffeine synthase are more than three-fold higher in young leaves compared to mature leaves suggesting that high caffeine biosynthesis in young shoots is mainly dependent upon expression of the *TCSI* gene (Li et al., accepted for publication).

In young tea leaves, [8-¹⁴C]theophylline and [2-¹⁴C]xanthine are also utilised for caffeine biosynthesis via 3-methylxanthine (Ashihara et al., 1997a). As theophylline, 3-methylxanthine and xanthine are catabolites of caffeine, these purine alkaloids may, therefore, be re-utilised for the caffeine synthesis in young leaves. Although conversion of xanthosine to theobromine, the initial three steps of the caffeine biosynthetic pathway, occurs only in young leaves, metabolism of [¹⁴C]theobromine to caffeine takes place even in mature tea leaves (Ashihara et al., 1997a). Thus, the last step of caffeine synthesis, unlike the first three, is not restricted to young leaves.

Caffeine is synthesized in stamens and petals of tea flowers and biosynthetic activity is high prior to the opening of the flowers (Fujimori and Ashihara, 1990). In fruits of *C. sinensis*, the levels of caffeine and theobromine increase markedly during the growing season until the fruit is fully mature and dry (Suzuki and Waller, 1985). Caffeine biosynthesis has been detected in pericarp and seeds of tea fruits with the highest biosynthetic capacity occurring in young tissues (Terrasaki et al., 1994).

It is well established that artificial shading of tea plants prior to harvest produces young shoots that yield a higher quality beverage (Anan and Nakagawa, 1974). The biosynthesis of the flavan-3-ols, (–)-epicatechin and (–)-epicatechin-3-gallate, (–)-epigallocatechin and (–)-epigallocatechin-3-gallate in young tea leaves is enhanced by light (Saijo, 1980). In contrast, shading generally increases caffeine levels in tea leaves when expressed as percentage of dry weight (Anan and Nakagawa, 1974). However, since light does not have any significant influence on caffeine biosynthesis *per se* the light-mediated changes in

Table 2
Purine alkaloids in young leaves of *Camellia* species^a

Latin name	Common name	Major purine alkaloid	Reference
<i>C. sinensis</i> var. <i>sinensis</i>	Tea	Caffeine (2.8%)	Nagata and Sakai (1984)
<i>C. assamica</i>	Assam tea	Caffeine (2.4%)	Nagata and Sakai (1984)
<i>C. taliensis</i>		Caffeine (2.5%)	Nagata and Sakai (1984)
<i>C. kissi</i>		Caffeine (<0.02%)	Nagata and Sakai (1984)
<i>C. irrawadiensis</i>		Theobromine (<0.8%)	Nagata and Sakai (1984)
<i>C. ptilophylla</i>	Cocoa tea	Theobromine (5.9%)	Ye et al. (1997)
<i>C. assamica</i> var. <i>kucha</i>	Kucha	Theacrine (2.8%)	Zheng et al. (2002)
<i>C. japonica</i>	Camellia	None (0%)	Nagata and Sakai (1984)
<i>C. sasanqua</i>	Sasangua	None (0%)	Nagata and Sakai (1984)

^a Italicised figures in parentheses represent the concentration of purine alkaloids expressed as a % of dry weight.

caffeine content may be an indirect consequence of changes in the levels of other compounds (Koshiishi et al., 2000).

In *C. pitilophylla*, theobromine synthesis in young and mature leaves of flush shoots from 1-year-old plants is 10 times more rapid than in aged leaves. Theobromine is synthesised from [8-¹⁴C]adenine. Neither cell-free extracts nor segments from *C. pitilophylla* leaves can convert [8-¹⁴C]theobromine to [¹⁴C]caffeine (Ashihara et al., 1998). Recently, genes encoding theobromine synthase which catalyse only 3*N*-methylation were isolated from theobromine accumulating *Camellia* plants including *C. pitilophylla* and *C. irrawadiensis* (Yoneyama et al., 2006).

Theacrine (1,3,7,9-tetramethyluric acid) and caffeine are the major purine alkaloids in kucha (*C. assamica* var. kucha) leaves (Zheng et al., 2002). Endogenous levels of theacrine (2.8% dry weight) and caffeine (0.6–2.7%) in expanding buds and young leaves are higher than in mature leaves. Leaf discs incorporate [methyl-¹⁴C]SAM into theacrine as well as theobromine and caffeine, indicating that SAM acts as the methyl donor not only for caffeine biosynthesis but also for theacrine production. [8-¹⁴C]Adenosine and [8-¹⁴C]caffeine are both converted to theacrine with the highest incorporation occurring in expanding buds. These results indicate that theacrine is synthesized from purine nucleotide via caffeine. Conversion of caffeine to theacrine probably occurs by successive oxidation and methylation with 1,3,7-trimethyluric acid as the intermediate (Fig. 5) (Zheng et al., 2002). Metabolism of theacrine has not yet been investigated in kucha.

Relatively little information is available for the catabolism of purine alkaloids in *C. sinensis* although the pathways have been investigated using various ¹⁴C-labelled purine alkaloids (Ashihara et al., 1997a). The results obtained suggest that the caffeine degradation pathway via theophylline is functional in tea leaves. In young leaves, sizable amounts of [8-¹⁴C]theophylline are salvaged for the synthesis of caffeine via 3-methylxanthine and theobromine. In the theobromine accumulated-species, *C. pitilophylla*, catabolism of [2-¹⁴C]theobromine was very slow and only small amounts were converted to xanthine which enters the conventional purine catabolic pathway to be degraded to CO₂ and NH₃ (Ashihara et al., 1998).

5.2. *Coffea* species

The caffeine content of seeds of different *Coffea* species varies from 0.4% to 2.4%, some examples are presented in Table 3. Beans of *C. arabica* and *Coffea canephora* contain ca. 1% and ca. 2% caffeine, respectively. Young expanding leaves of *C. arabica* plants also contain theobromine, though in lower levels than caffeine (Frischknecht et al., 1986; Fujimori and Ashihara, 1994). In *C. arabica* seedlings, caffeine occurs mainly in leaves and cotyledons at concentrations varying from 0.8% to 1.9% dry wt. Caffeine is essentially absent in roots and the older brown parts of shoots (Zheng and Ashihara, 2004). Methyluric

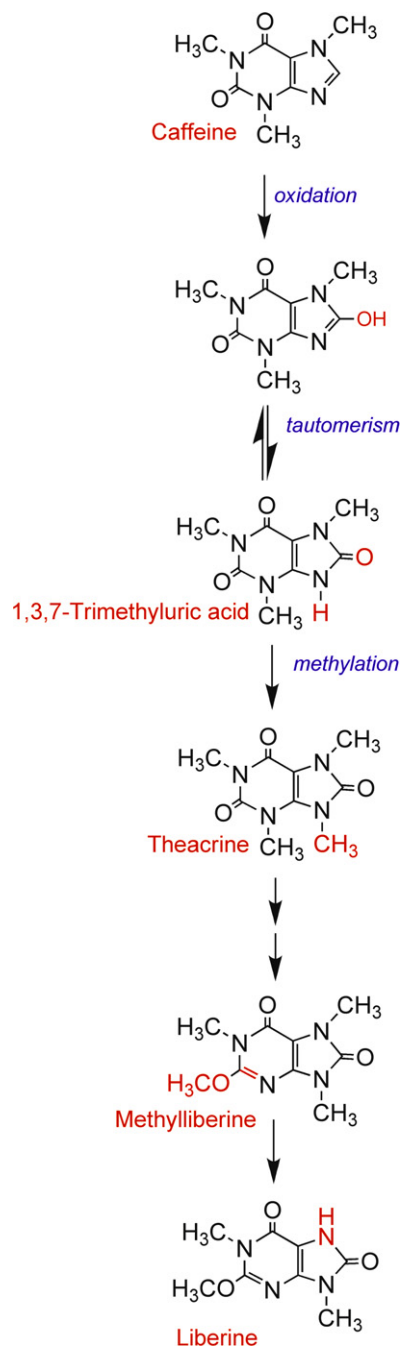


Fig. 5. The possible pathway for the conversion of caffeine to methyluric acids in kucha and some *Coffea* species.

acids, such as liberine ($\Delta^{2,3},O(2),1,9$ -trimethyluric acid) and methyliberine ($\Delta^{2,3},O(2),1,7,9$ -tetramethyluric acid) occur in several species of coffee including *Coffea liberica*, *Coffea dewevrei* and *Coffea abeokuta* (Baumann et al., 1976; Petermann and Baumann, 1983).

Caffeine is synthesized in young leaves of *C. arabica* seedlings and accumulates in mature leaves (Frischknecht et al., 1986; Fujimori and Ashihara, 1994). Active caffeine biosynthesis occurs in the upper leaves and the upper part of the stem but is absent in the second and third leaves, cotyledons, lower stem and roots (Zheng and Ashihara, 2004).

Table 3
Purine alkaloids in mature, ripened beans of *Coffea* species^a

Latin name	Common name	Major purine alkaloid	Reference
<i>C. arabica</i>	Arabica coffee	Caffeine (1.0%)	Koshiro et al. (2006)
<i>C. canephora</i>	Robusta coffee	Caffeine (1.9%)	Koshiro et al. (2006)
<i>C. kianjavatensis</i>	Mascarocoffea	Caffeine (0.7%)	Clifford et al. (1991)
<i>C. dewevrei</i>		Caffeine (1.2%)	Mazzafera and Carvalho (1992)
<i>C. liberica</i>		Caffeine (1.4%)	Mazzafera and Carvalho (1992)
<i>C. eugenoides</i>		Caffeine (0.4%)	Mazzafera and Carvalho (1992)
<i>C. salvatrix</i>		Caffeine (0.7%)	Mazzafera and Carvalho (1992)
<i>C. racemosa</i>		Caffeine (0.8%)	Mazzafera and Carvalho (1992)

^a Italicised figures in parentheses represent the concentration of purine alkaloids expressed as a % of dry weight. Mature leaves of *C. dewevrei* and *C. liberica* accumulate theacrine, liberine and methylxanthine.

The pattern of caffeine synthesis during fruit development in *C. arabica* and *C. canephora* is similar. Active caffeine biosynthesis occurs from pericarp expansion to endosperm formation and in young seeds through to maturation (Koshiro et al., 2006). The transcripts of two *N*-methyltransferase genes *CmXRS1* and *CCS1* are present in fruits of every stage of growth except fully ripened tissues. The pattern of expression of these genes during growth is roughly related to the *in situ* synthesis of caffeine from adenine nucleotides, although exceptions were found in the very early and the later stages of fruit growth. Since the level of *CTS2* transcripts encoding theobromine synthase is very low in fruits, the alternative *CCS1* gene encoding dual-functional caffeine synthase may be operative for the last two steps of caffeine biosynthesis.

In developing *C. arabica* fruits, the level of transcripts of *CmXRS1* and *CCS1* are higher in seeds than in pericarp. Native caffeine synthase (3*N*-methyltransferase) activity is distributed in both organs in a similar manner. Therefore, caffeine accumulating in ripened coffee seeds appears to be synthesized within the developing seeds (Koshiro et al., 2006).

Petermann and Baumann (1983), using ¹⁴C-labelled purine alkaloids, demonstrated that caffeine is converted to liberine via theacrine and methylxanthine in leaves of *C. dewevrei*, *C. liberica* and *C. abeokutae*. In young leaves caffeine is synthesized from theobromine. Caffeine is gradually replaced by theacrine at the next growth stage, and then theacrine was converted to liberine which is the predominant purine alkaloid in young leaves of seedlings with developed branches. Low amount of methylxanthine, a putative precursor of liberine, is always present in mature leaves. The results suggest a caffeine → theacrine → methylxanthine → liberine pathway occurs in these species of *Coffea* (Fig. 5).

In *C. arabica* leaves, the main caffeine catabolism pathway via theophylline is operative (Ashihara et al., 1996). The conversion of caffeine to theophylline is the rate-limiting step in purine alkaloid catabolism and provides a ready explanation for the high concentration of endogenous caffeine found in *C. arabica* leaves. Although theobromine is converted primarily to caffeine, a small portion of the theobromine pool appears to be degraded to xanthine by a caffeine-independent pathway. In addition to being broken down to CO₂ via the purine catabolism pathway, xanthine

is recycled by being metabolised to 7-methylxanthine. In leaves and fruits of *C. arabica* and *C. dewevrei*, incorporation of the radioactivity from [2-¹⁴C]caffeine into 7-methylxanthine was also detected by Vitória and Mazzafera (1998). In *C. dewevrei*, 7-methylxanthine appears to be not involved in caffeine catabolism as it does not accumulate when purine catabolism is inhibited by allopurinol (Mazzafera, 1993). It may, however, be re-utilised for caffeine biosynthesis.

In young and mature leaves of *Coffea eugenoides* which contain low levels of caffeine, [8-¹⁴C]caffeine is catabolised rapidly primarily by the main caffeine catabolic pathway via theophylline. This suggests that the low caffeine accumulation in *C. eugenoides* is a consequence of rapid degradation of caffeine perhaps accompanied by a slow rate of caffeine biosynthesis (Ashihara and Crozier, 1999b).

5.3. *Theobroma* and related species

Theobromine is the dominant purine alkaloid in seeds of cacao (*T. cacao*). The cotyledons of mature beans contain 2.2–2.7% on a dry weight basis and 0.60–0.8% caffeine while shells contain 0.6–0.7% theobromine and 0.5–0.6% caffeine (Senanayake and Wijesekera, 1971). Examination of several *T. cacao* genotypes representing the three horticultural races, Criollo, Forastero and Trinitario revealed considerable variations in the purine alkaloid content of the seed with slightly higher levels found within the Criollo types (Hammerstone et al., 1994).

Cupu (*Theobroma grandiflorum*), contains 0.25% liberine in cotyledons and 0.08% in the nut shells (Baumann and Wanner, 1980). Hammerstone et al. (1994) reported that theacrine is the principal purine alkaloid in seeds of eleven species of *Theobroma* and nine species of *Herrania*. Little or no accumulation of purine alkaloids was found in the leaves of these species. Zheng et al. (2004) investigated the purine alkaloid content of *T. cacao* fruits at different growth stages. The major purine alkaloid in small-sized young fruits, consisting mainly of pericarp was theobromine, followed by caffeine. The theobromine content of the pericarp decreased sharply with the age of tissue while the caffeine content declined gradually. Large amounts of theobromine (0.4% fresh weight) accumulate

in the cotyledon-rich seeds of mature fruits. Theobromine was also found in the seed coats and placenta.

Koyama et al. (2003) examined the purine alkaloid content of cacao leaves at various ages. The major purine alkaloid in young leaves is theobromine (0.08% fresh weight), followed by caffeine (0.015% fresh weight). More than 75% of purine alkaloids disappeared with subsequent leaf development. In young cacao leaves, radioactivity from ^{14}C -labelled adenine, adenosine, guanine, guanosine, hypoxanthine and inosine was incorporated into 3- and 7-methylxanthine, 7-methylxanthosine and theobromine, in addition to salvage (nucleotides and nucleic acids) and degradation products (ureides and CO_2). In contrast, ^{14}C -labelled xanthine and xanthosine were completely degraded, but nearly 20% of $[8\text{-}^{14}\text{C}]$ xanthosine was converted to purine alkaloids. These observations are consistent with the following biosynthetic pathways for theobromine:

- (a) $\text{AMP} \rightarrow \text{IMP} \rightarrow \text{XMP} \rightarrow \text{xanthosine} \rightarrow 7\text{-methylxanthosine} \rightarrow 7\text{-methylxanthine} \rightarrow \text{theobromine}$.
- (b) $\text{GMP} \rightarrow \text{guanosine} \rightarrow \text{xanthosine} \rightarrow 7\text{-methylxanthosine} \rightarrow 7\text{-methylxanthine} \rightarrow \text{theobromine}$.
- (c) $\text{xanthine} \rightarrow 3\text{-methylxanthine} \rightarrow \text{theobromine}$.

Although no caffeine biosynthesis from ^{14}C -labelled purine bases and nucleosides was observed during 18 h incubations, exogenous $[8\text{-}^{14}\text{C}]$ theobromine was converted to caffeine by young leaves. Metabolism of theobromine to caffeine may, therefore, be slow in cacao leaves. No purine alkaloid synthesis was observed in older leaves. Significant catabolism of purine alkaloids was found in both developing intermediate-sized and fully developed leaves, in which $[8\text{-}^{14}\text{C}]$ theobromine was degraded to CO_2 via 3-methylxanthine, xanthine and allantoinic acid (Koyama et al., 2003).

The theobromine synthase encoding genes *TcCaf-1* (CK144298) and *BCS1* (AB096699) are expressed only in young *T. cacao* leaves (Bailey et al., 2005; Iwasaki, 2006). Caffeine production is stimulated by treatment of young actively growing cacao leaves with salicylic acid, which is the endogenous signal that induces pathogen defense responses in plants. This suggests that caffeine biosynthesis in cocoa is inducible by pathogen attack, wounding and salicylic acid (Aneja and Gianfagna, 2001) and it may be part of the defense response of cacao to herbivory and infection (see Section 5.1).

Catabolism of purine alkaloids in *T. cacao* has been investigated using fully developed leaves and pericarp of mature fruits. $[8\text{-}^{14}\text{C}]$ Theobromine was incorporated in 3-methylxanthine, ureides and CO_2 (Koyama et al., 2003; Zheng et al., 2004). This indicates that a theobromine \rightarrow 3-methylxanthine \rightarrow xanthine pathway is operative.

5.4. Maté

Infusions of maté leaves are widely consumed as a stimulant in rural regions of Brazil, Uruguay, Argentina and Paraguay. Young maté leaves contain caffeine (0.8–0.9%),

theobromine (0.08–0.16%) and theophylline (<0.02%). Much lower levels are found in mature leaves, and trace amounts of caffeine and theobromine occur in fruit, bark, and wood (Mazzafera, 1994). However, variations in the concentration of caffeine and theobromine between individual genotypes have been reported (Marx et al., 2003). Caffeine and theobromine were found in leaf epicuticular waxes of maté (Athaydea et al., 2000).

There is only one published report on the purine alkaloid biosynthesis of maté in which the metabolism of ^{14}C -labelled adenine, guanosine and hypoxanthine by different aged leaves was investigated. Young leaves, but not mature dark-green leaves, incorporated each precursor into theobromine and caffeine, and no significant degradation of caffeine was detected (Ashihara, 1993).

Maté is a shade-tolerant species, frequently cultivated in agro-forestry systems and monocultures. The effect of light intensity on the purine alkaloid content has been investigated and it was shown that at very low light intensities the caffeine and theobromine content increased. Although a negative correlation was found between biomass accumulation and purine alkaloid content, the total purine alkaloid yield per plant was not altered (Coelho et al., 2007).

5.5. Other species

There are reports on the distribution of purine alkaloids in *Paullinia cupana* (guaraná), *Paullinia yoco*, *Paullinia pachycarpa*, *Cola* species and various *Citrus* plants (Baumann et al., 1995; Kretschmar and Baumann, 1999; Weckerle et al., 2003). In seeds of guaraná, caffeine occurs mainly in the cotyledons (4.3%) and testa (1.6%). *Citrus* flowers accumulate up to 0.17% purine alkaloids on a fresh weight basis, almost exclusively in the androecium. The main purine alkaloid is caffeine but theophylline is also present. Trace quantities of caffeine have also found in the nectar (Weckerle et al., 2003). Selected examples of purine alkaloids in species other than *Camellia* and *Coffea* are presented in Table 4.

6. Role of purine alkaloids in planta

The physiological role of purine alkaloids in *planta* has until recently remained largely undetermined and it may be that they may be waste end products produced in a limited number of plant species during the course of evolution. Degradation of caffeine is relatively slow even in aged leaves of most species, and it appears not to act as a nitrogen reserve since considerable amounts remain in leaves after abscission. There are two hypotheses concerning the role of caffeine in plants, the “chemical defence” and “allelopathic function” theories.

6.1. Chemical defence

The “chemical defence theory” proposes that the high concentrations of caffeine in young leaves, fruits and

Table 4
Purine alkaloids in cacao, cupu, *Herreania* sp., maté, guaraná and pomelo

Latin name	Common name	Organ	Major alkaloid	Reference
<i>Theobroma cacao</i>	Cacao	Cotyledons	Caffeine (2.5%)	Senanayake and Wijesekera (1971)
<i>Theobroma grandiflorum</i>	Cupu	Cotyledons	Theacrine (0.25%)	Baumann and Wanner (1980)
<i>Herreania</i> sp.		Seeds	Theacrine (<2.0%)	Hammerstone et al. (1994)
<i>Ilex paraguariensis</i>	Maté	Leaves	Caffeine (0.9%)	Mazzafera (1994)
<i>Paullinia cupana</i>	Guaraná	Cotyledons	Caffeine (4.3%)	Baumann et al. (1995)
<i>Citrus maxima</i>	Pomelo, Chinese grapefruit	Anthers	Caffeine (0.9%) ^a	Kretschmar and Baumann (1999)

^a Italicised figures in parentheses represent the concentration of caffeine expressed as a % of dry weight.

flower buds of species such as *C. arabica* and *C. sinensis* act as a defence to protect young soft tissues from pathogens and herbivores. It has been shown that spraying tomato leaves with a 1% solution of caffeine deters feeding by tobacco hornworms, while treatment of cabbage leaves and orchids with 0.01–0.1% solutions of caffeine acts as a neurotoxin and kills or repels slugs and snails (Hollingsworth et al., 2002). This work has now been extended and convincing evidence for the chemical defence theory has recently been obtained with transgenic caffeine-producing tobacco plants (see Section 7.2) (Uefuji et al., 2005; Kim and Sano, 2007).

Another possible example of caffeine and chemical defense occurs when tea plants are attacked by adult female shot-hole borer beetles (*Xyleborus fornicatus*), which bore into the stem. This is often followed by infection of the stem with the fungus, *Monacrosporium ambrosium*, a symbiote of the beetle. The growth of the fungus is inhibited by caffeine and it has been proposed that accumulation of caffeine in tea stems after beetle attack could be a plant defense strategy (Kumar et al., 1995).

The role of caffeine as a chemical defense for coffee against the berry borer *Hypothenemus hampei* has also been investigated (Guerreiro Filho and Mazzafera, 2003). No positive correlation was observed between resistance and caffeine content in experiments in which seeds from several coffee species, containing varying levels of caffeine, were exposed to adult insects. It was suggested that the berry borer has evolved an adaptation to handle the toxic effects of caffeine.

Crinipellis pernicios, which is the causal agent of witches' broom disease, attacks actively growing young shoots, flowers and developing fruits of cacao. Growth of *C. pernicios* is significantly inhibited by caffeine and infected stem tissue contains 7–8 times more caffeine than healthy stems. Aneja and Gianfagna (2001) propose that this may be part of the defense response of this species to herbivory and/or infection. The report, however, does not mention what affect infection had on the level of theobromine, the major purine alkaloid in cacao.

6.2. Allelopathy

The “allelopathic or autotoxic function theory” proposes that caffeine in seed coats and falling leaves is released into the soil to inhibit germination of seeds around

the parent plants (for review, see Anaya et al., 2006). Although there is experimental evidence from laboratory studies to support this suggestion, it is unclear to what extent caffeine is involved in allelopathy in natural ecosystems, especially as soil bacteria such as *Pseudomonas putida* can degrade the purine alkaloid (Hohnloser et al., 1980; Gluck and Lingens, 1988).

7. Genetic engineering of caffeine

7.1. Decaffeinated coffee plants

Identification of genes encoding enzymes for caffeine biosynthesis facilitated engineering the caffeine biosynthetic pathway to either suppress or enhance production. The first approach was to construct transgenic coffee plants with reduced caffeine content by the RNA interference method, in which mRNA of the target gene is selectively degraded by small double-stranded RNA species (Ogita et al., 2003, 2004, 2005). The 3'-untranslated region and the coding region of *CaMXMT* cDNA were selected to design the RNAi constructs. Two different RNAi constructs, RNAi-S having a short insert with 150 bp, and RNAi-L with a long insert of 360 bp, were inserted into the pBIH1-IG vector (Ohta et al., 1990), which was introduced into the EHA101 strain of *Agrobacterium tumefaciens* to transform *C. arabica* and *C. canephora* plants. The resulting transformed lines were assayed for expression of *N*-methyltransferase genes by RT-PCR, and it was found that the *CaMXMT*-RNAi construct suppressed transcripts for not only *CaMXMT* but also *CaXMT* and *CaDXMT*. The homogeneity between *CaMXMT*, *CaXMT* and *CaDXMT* is over 90% in the coding region (Uefuji et al., 2003), suggesting that the primary small double-stranded *CaMXMT*-RNA progressively produces many secondary small double-stranded RNAs spanning its coding region to the adjacent sequence of the initiator region, which in turn destroys mRNAs for *CaXMT* and *CaDXMT*. The reduced level of transcripts suggested decreased activities of the corresponding enzymes, and this was confirmed by directly measuring their products, theobromine and caffeine, using HPLC. The caffeine content in the controls was approximately 8.4 mg/g fresh leaf tissue, while that in both RNAi-S and RNAi-L was 4.0 mg, showing an average 50% reduction. However, the amount was

variable depending on the line, with one notable example of *C. canephora* showing up to 70% reduction (Ogita et al., 2003). The caffeine biosynthetic activity in leaves of these low caffeine transgenic plants is reduced. Metabolic studies indicate that substrates of caffeine biosynthesis are catabolised via the conventional purine catabolic pathway and, as a consequence, other than reduced caffeine content, these plants have the same profile of cellular metabolites as wild-type tissues (Ashihara et al. 2006). When, in due course these plants reach maturity, it is expected that they will produce normal coffee beans except for low caffeine content.

7.2. Natural pesticides

The second approach was to construct caffeine-producing transgenic plants, which originally did not synthesize the purine alkaloid (Uefuji et al., 2005; Kim et al., 2006). This idea was based on a report that exogenously applied caffeine markedly increased the resistance of plants against several pests, and thereby enhanced their growth and survival (Nathanson, 1984). To this end, the first step was to construct a multi-gene transfer vector, pBIN-NMT777, with three genes, *CaXMT*, *CaMXMT1* and *CaDXMT1*. Tobacco (*Nicotiana tabacum* cv. Xanthi) leaf discs were transformed with pBIN-NMT777 using *Agrobacterium*. After appropriate culture and selection, fifteen kanamycin-resistant transgenic plantlets were shown by RT-PCR to express all three *N*-methyltransferase genes. The selected lines were grown to maturity and the anticipated accumulation of purine alkaloids in leaves demonstrated. Subsequently, the caffeine content of individual leaves at different developmental stages was determined. In mature leaves of the transgenic plants, the average caffeine content was 0.2 µg/g fresh weight and when plants aged and entered the reproductive stage forming flower buds, this increased to over 5 µg/g. No caffeine was detected in control plants.

Next, the feeding behaviour of caterpillars of tobacco cutworms (*Spodoptera litura*) (Fig. 6A) was tested using transgenic lines which produced different levels of caffeine. Third-instar cutworm larvae were starved for 3 h and then allowed to select and feed on leaf discs prepared from transgenic or control plants. While the larvae fed on the control leaf discs they positively avoided the transgenic leaves (Fig. 6B). A quantitative estimation indicated that the consumed leaf area was up to 1.1 cm² for the control leaf tissue, and <0.02 cm² for the transgenic discs containing 5 µg/g caffeine. Transgenic discs accumulating as little as 0.4 µg/g caffeine were also effectively in repelled caterpillars (Fig. 6C).

In order to have a lethal effect on the caterpillars, exogenous caffeine had to be mixed with an artificial food paste at a concentration of 10 mg/g (Uefuji et al., 2005). This is at least three orders of magnitude higher than the concentration of endogenous caffeine that was required to repel the insects. To address this apparent paradox, the biochem-

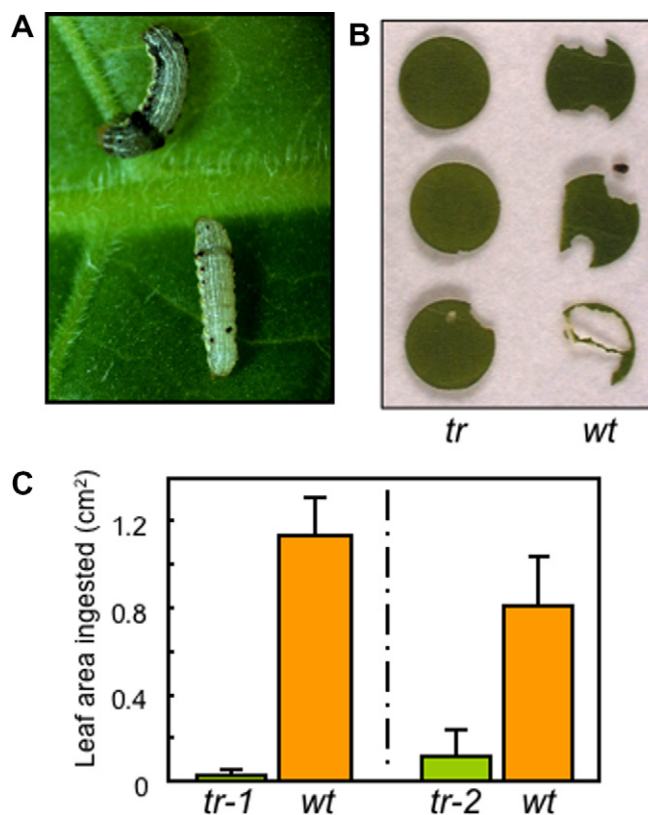


Fig. 6. Repellent effects of transgenic caffeine-producing tobacco plants against tobacco cutworm (*Spodoptera litura*). (A) Tobacco cutworm larvae at the third instar stage of development. (B) Larvae were starved for 3 h then allowed to feed for 3 h in darkness on three wild-type (*wt*) and three transgenic leaf discs producing 5 µg caffeine/g fresh weight (*tr*) after which the leaf discs were photographed. (C) Quantification of feeding behaviour. Twenty replicate tests were performed, and the leaf areas consumed by the larvae (*y* axis) calculated with the aid of an image analyzer and expressed in cm² ± standard error. The *x* axis indicates discs from transgenic plants (green) containing 5 µg caffeine/g fresh weight (*tr* – 1) and 0.4 µg caffeine/g fresh weight (*tr* – 2), and from wild-type plants (orange) (*wt*).

ical properties of transgenic lines were investigated, and it was found that transcripts for pathogenesis-related (PR) proteins and proteinase inhibitor (PI) accumulated constitutively (Kim and Sano, 2007). Since PI-II is known to cause digestion dysfunction in larvae guts (Green and Ryan, 1972), this at least partially explains why the caterpillars avoided the transgenic leaves despite the seemingly low levels of endogenous caffeine. As expression of PR proteins is usually associated with disease resistance, the effects of pathogen infection of the transgenic tobacco lines was investigated and this revealed that the plants were resistant against infection by tobacco mosaic virus and the bacterial pathogen, *Pseudomonas syringae*. This suggests that caffeine stimulates endogenous defence mechanisms against not only herbivores but also pathogens by activating the expression of defence-related genes. The effects appear to resemble those induced by signalling molecules such as jasmonic acid and salicylic acid. However, caffeine is not generally thought to affect gene expression, and further investigation is necessary to determine the molecular

mechanisms involved. Further study is also required to determine to what extent the effects observed with transgenic caffeine-producing tobacco occur in other species, especially those of agricultural importance.

8. Summary and perspectives

It is just over seven years since the first report on the cloning of caffeine synthase from tea, the gene encoding the enzyme regulating the final two methylation steps in the caffeine biosynthesis pathway (Kato et al., 2000). Since this pioneering study there has been a veritable explosion of research, most notably by Japanese scientists, that has led to the successful cloning of a number of methyltransferase-encoding genes from coffee, tea and cacao. Much of the wide spread interest in this research has been fuelled by the possibilities of using genetic engineering to obtain transgenic, low caffeine coffee and tea that could be used to produce “natural” decaffeinated beverages.

To-date, transgenic *C. canephora* plants with leaves with a 50–70% reduced caffeine content have been obtained (Ogita et al., 2003) but as yet there are no reports on the caffeine content of beans produced by such plants. When this does occur, a more substantial suppression of caffeine production will be required as a >90% reduction is necessary for commercial coffees to qualify to be sold under the “decaffeinated” label. It will also be more appropriate to produce transgenic decaffeinated beans using *C. arabica* that yield a more superior beverage than Robusta coffee from *C. canephora*. An alternative route to decaffeinated coffee may be the naturally caffeine-deficient *C. arabica* detected in a screening programme in Brazil by Silvarolla et al. (2004).

The recent report of Kim and Sano (2007) that tobacco plants genetically engineered to produce caffeine repel insect pests and exhibit resistance to viral and bacterial infection is potentially of major importance. Almost 40% of world agricultural production is lost because of herbivores and diseases (Baker et al., 1997; Jouanin et al., 1998) and a practical and economic means of pest control is one of the most urgent measures required to obtain a reliable and increasing supply of foodstuffs for the World's expanding population. The production of a low level of caffeine by agriculturally important crops may have the potential to offer protection against both insect pests and pathogens and from an environmental prospective it would certainly safer and cheaper than treatment of crops with pesticides and fungicides. Such developments would have interesting political ramifications because in Europe, at least, there are lobby groups, arguably naïve, who speak intransigently against the use of genetically modified crops because of what they perceive as their dramatic adverse effects on the environment.

The finer details of the caffeine biosynthesis pathway have now been elucidated and, although it has not progressed to the same extent, an increasing amount of data

are available on the catabolism of caffeine in a variety of purine alkaloid-containing plants. Information on cellular metabolic organization of caffeine biosynthesis and catabolism links to purine nucleotide metabolism, intercellular translocation and accumulation mechanisms at specific cellular sites, such as chloroplasts and vacuoles, have yet to be fully revealed. Cell-, tissue-, and organ-specific synthesis and catabolism of purine alkaloids may be regulated by unique and unknown developmental- and environmental-specific control mechanisms. A great deal of fascinating purine alkaloid biology in plants remained to be discovered.

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