

# Caffeine Metabolism in High and Low Caffeine Containing Cultivars of *Camellia sinensis*

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The metabolism of [8-<sup>14</sup>C]adenine and [2-<sup>14</sup>C]caffeine was examined in leaf segments from flush shoots of tea cultivars with high and low caffeine content. The caffeine biosynthesis pathway from AMP *via* theobromine was operative in both high and low caffeine containing cultivars. There was a more rapid rate of caffeine biosynthesis from [8-<sup>14</sup>C]adenine in the high caffeine cultivars while the rate of degradation of both adenine nucleotides and caffeine into CO<sub>2</sub> was greatest in cultivars with a low endogenous caffeine content. Cell-free preparations from tea shoots contained an *N*-methyltransferase, that is a key enzyme in the caffeine biosynthesis pathway; more *in-vitro* activity was detected in preparations from high caffeine containing cultivars. The data obtained suggest that the high caffeine containing cultivars have a more rapid rate of caffeine biosynthesis and a slower rate of caffeine catabolism than cultivars with a low endogenous caffeine content.

## Introduction

Purine alkaloids, such as caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) have been found in more than 60 plant species, including tea (*Camellia sinensis* L.), coffee (*Coffea arabica* L.), cocoa (*Theobroma cacao* L.), maté (*Ilex paraguariensis*) and guarana (*Paullinia sorbilis*) (Suzuki and Waller, 1988; Suzuki *et al.*, 1992; Ashihara, 1993). In recent years, as with coffee, there has been a growing demand for low caffeine containing tea, motivated primarily by the belief that ingestion of large quantities of caffeine, which acts as a stimulus to the central nervous system, can have adverse effects on health (Takeda, 1994).

The caffeine content of the leaves of *ca.* 1500 cultivars of tea has been investigated, the cultivars having been introduced as seeds and preserved as genetic resources at the Japanese National Re-

search Institute of Vegetable, Ornamental Plants and Tea. Nine cultivars with a caffeine content of >5% leaf dry weight were identified and nine cultivars were also found which contained <2% caffeine (Takeda, 1994). This paper reports on a study of the biochemical processes that regulate the accumulation of caffeine in tea, in which the rates of caffeine biosynthesis and catabolism were investigated in cultivars categorized as having either a high or a low endogenous caffeine content.

## Materials and Methods

### Plant material

Young under-developed leaves from flush shoots of tea plants (*Camellia sinensis* L.) were collected in early April at the experimental farm of the National Research Institute of Vegetables, Ornamental Plants and Tea, Makurazaki, Kagoshima Prefecture, Japan.

### Chemicals

[8-<sup>14</sup>C]Adenine (specific activity 2.0 MBq  $\mu\text{mol}^{-1}$ ) and [2-<sup>14</sup>C]caffeine (2.1 MBq  $\mu\text{mol}^{-1}$ )

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were purchased from Moravек Biochemicals Inc., Brea, CA, USA. [Methyl- $^{14}\text{C}$ ]S-adenosyl-L-methionine (SAM) ( $2.2 \text{ MBq } \mu\text{mol}^{-1}$ ) was obtained from Amersham International plc, Amersham, Bucks., UK. Biochemicals used in the study were purchased from Sigma Chemical Co., St. Louis, MO, USA.

#### *Determination of endogenous caffeine levels*

Young leaves from the first flush shoots were steamed and dried. Caffeine was extracted and analyzed quantitatively by reverse phase HPLC (Ikegaya, 1985).

#### *Metabolism of [8- $^{14}\text{C}$ ]adenine and [2- $^{14}\text{C}$ ]caffeine*

Pulse-chase experiments using [8- $^{14}\text{C}$ ]adenine were carried out as described by Ashihara and Kubota (1986). Leaf segments, *ca.* 200 mg fresh weight, from high and low caffeine-containing tea cultivars were incubated in 2 ml medium, comprising 30 mM potassium phosphate buffer, pH 5.6, 10 mM sucrose and 9.2 mM [8- $^{14}\text{C}$ ]adenine, in 30 ml Erlenmeyer flask, in a shaking water bath at 27 °C. After a 4 h incubation, the buffer was replaced with fresh medium containing non-radioactive adenine, and the leaf samples incubated for a further 20 h. Measurement of released  $\text{CO}_2$  and extraction and analysis of radiolabelled metabolites were carried out as described previously (Ashihara and Kubota, 1986; Ashihara and Nobusawa, 1981).

In studies on the metabolism of caffeine, labelled adenine in the incubation medium was replaced with 4.5  $\mu\text{M}$  [2- $^{14}\text{C}$ ]caffeine, and leaf segments were incubated 24 h at 27 °C after which metabolites were extracted with 6% perchloric acid and analyzed by TLC after neutralization with KOH.

#### *Determination of N-methyltransferase activity*

Tea leaves were frozen with liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$ . Cell-free preparations for the assay of N-methyltransferase activity were prepared according to the method of Suzuki and Takahashi (1975). Frozen leaves (1 g f.w.) were ground in a pre-chilled mortar with a small amount of acid washed sea sand and 10 ml potassium phosphate buffer pH 7.3 containing 5 mM 2-mercaptoethanol, 5 mM EDTA, 1 g insoluble polyvinylpyrrolidone

done and 0.5% (w/v) sodium ascorbate. The homogenate was squeezed through a layer of nylon cloth and centrifuged at  $20,000\times g$  for 20 min at 4 °C. Aliquots of the supernatant (2.5 ml) were applied to a prepacked Sephadex G-25 gel filtration column, with a 9 ml bed volume, equilibrated with 10 mM potassium phosphate buffer pH 7.3 containing 2 mM 2-mercaptoethanol and 2 mM EDTA. Protein eluted with 3.5 ml equilibration buffer was used for enzyme assays.

The N-methyltransferase assay was based on the transfer of the  $^{14}\text{C}$ -labelled methyl group from [ $^{14}\text{C}$ ]SAM to paraxanthine (1,7-dimethylxanthine) to form [3-methyl- $^{14}\text{C}$ ]caffeine. The assay was carried out in an Eppendorf tube in 100  $\mu\text{l}$  100 mM Tris-HCl, pH 8.3 containing desalted enzyme preparation, 2 mM  $\text{MgCl}_2$ , 0.2 mM paraxanthine and 4  $\mu\text{M}$  [ $^{14}\text{C}$ ]SAM. The reaction was initiated by the addition of the enzyme preparation and the mixture was incubated for 1 min at 27 °C. The blank rate was determined by a parallel incubation from which paraxanthine was omitted. The reaction was terminated by vortexing tubes after the addition of 40  $\mu\text{g}$  bovine serum albumin and 1 ml chloroform. A bi-phasic mixture was obtained by brief centrifugation. Caffeine partitions into the lower organic extract while SAM remains in the upper aqueous phase. An 0.5 ml aliquot of chloroform was transferred to a scintillation vial, evaporated to dryness at 60 °C, dissolved in 1 ml distilled water, scintillation cocktail added and [ $^{14}\text{C}$ ]caffeine measured by liquid scintillation counting.

## **Results and Discussion**

#### *Analysis of endogenous caffeine*

Tea cultivars, previously identified as having a high caffeine content ( $>5\%$  d.w.) or a low caffeine content ( $<2\%$  d.w.) (Takeda, 1994), were re-investigated in the present study using leaves harvested in 1991 and 1992. The data obtained, presented in Table I, demonstrate that the caffeine content of the Zai-17 and Zai-133 cultivars of *C. sinensis* var. *sinensis* was  $<2\%$  while, with the exception of PKS-274 in 1992, the PKS cultivars of *C. sinensis* var. *assamica* contained  $>5\%$  caffeine.

#### *Biosynthesis of caffeine*

The capacity of the high and low caffeine cultivars to synthesize caffeine was estimated with

Table I. Endogenous caffeine levels in tea selected from genetic resources exhibiting high- and low-caffeine content. Data expressed as percentage caffeine  $\text{g}^{-1}$  dry weight in leaves of first flush shoots harvested in the spring of 1991 and 1992.

Plant material	1991	1992
<i>Camellia sinensis</i> var. <i>sinensis</i>		
Zai-17	1.96	1.99
Zai-133	1.89	1.96
<i>Camellia sinensis</i> var. <i>assamica</i>		
PKS-224	5.07	5.28
PKS-274	5.04	4.62
PKS-283	5.25	5.13
PKS-423	5.00	5.38

[8- $^{14}\text{C}$ ]adenine as adenine has been shown to be a very effective precursor of caffeine in tea leaves (Suzuki and Takahashi, 1976). The results of a pulse-chase experiment using leaves of the high caffeine cultivar PKS-283 are illustrated in Fig. 1. Similar data were obtained in pulse-chase feeds

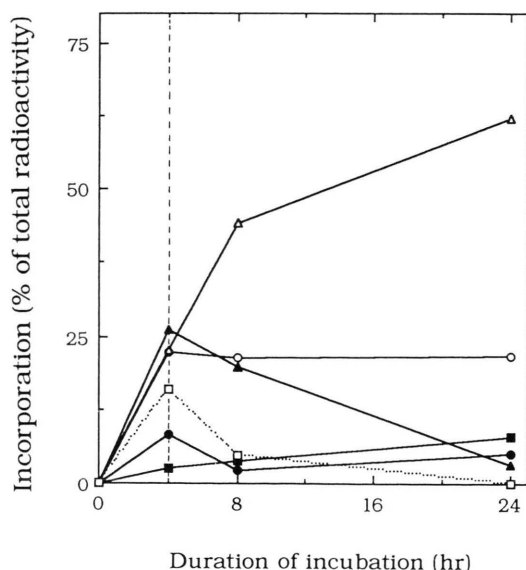


Fig. 1. Distribution of radioactivity associated with [8- $^{14}\text{C}$ ]adenine metabolites after a 'pulse-chase' experiment with leaves of high caffeine containing cultivar of tea, PKS-283. Leaves were incubated with [8- $^{14}\text{C}$ ]adenine for 4 h after which the radioactivity was 'chased' in a non-radiolabelled medium for a further 20 h. Incorporation of radioactivity expressed as a percentage of the total radioactivity taken up by the leaf samples ( $28.1 \pm 0.5 \text{ kBq g}^{-1} \text{ f.w.}$ ). ○ – Nucleic acids, ● – nucleotides, △ – caffeine, ▲ – theobromine, ■ –  $\text{CO}_2$ , □ – adenine.

with other low- and high caffeine cultivars. In all instances, the level of radioactivity associated with nucleotides, theobromine and adenine declined after the 6 h pulse when the leaves were transferred to a non-radioactive medium. In contrast, incorporation of radioactivity into caffeine and  $\text{CO}_2$  increased during the 6–24 h chase period. These observations are consistent with an "AMP pathway" for caffeine biosynthesis involving an  $\text{AMP} \rightarrow \text{IMP} \rightarrow \text{XMP} \rightarrow \text{xanthosine} \rightarrow 7\text{-methylxanthosine} \rightarrow 7\text{-methylxanthine} \rightarrow \text{theobromine} \rightarrow \text{caffeine}$  route (see Fig. 2) [Fujimori *et al.*, 1991; Negishi *et al.*, 1992].

Incorporation of label from [8- $^{14}\text{C}$ ]adenine into caffeine was slightly higher in caffeine-rich cultivars, PKS-224 and PKS-283 than in the low caffeine cultivars Zai-17 and Zai-133, while the converse applied to the release of  $^{14}\text{CO}_2$  (Fig. 3). Higher plants contain little or no adenine deaminase activity (Le Floch *et al.*, 1982), [8- $^{14}\text{C}$ ]adenine is therefore probably converted to adenine

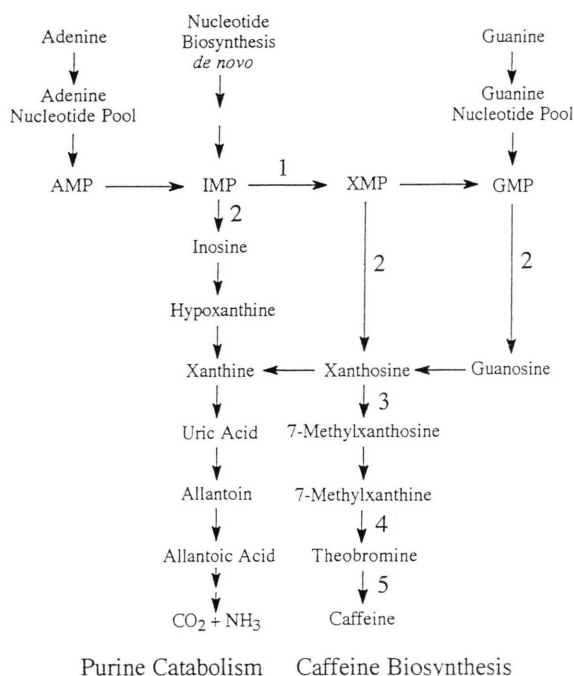


Fig. 2. Metabolic pathways illustrating both the biosynthesis of caffeine from purine nucleotides and the catabolism of purine nucleotides to  $\text{CO}_2$  and  $\text{NH}_3$ . 1 – IMP dehydrogenase, 2 – 5'-nucleotidase, 3 – SAM:xanthosine 7-N-methyltransferase, 4 – SAM:7-methylxanthine 1-N-methyltransferase, 5 – SAM:theobromine 1-N-methyltransferase.

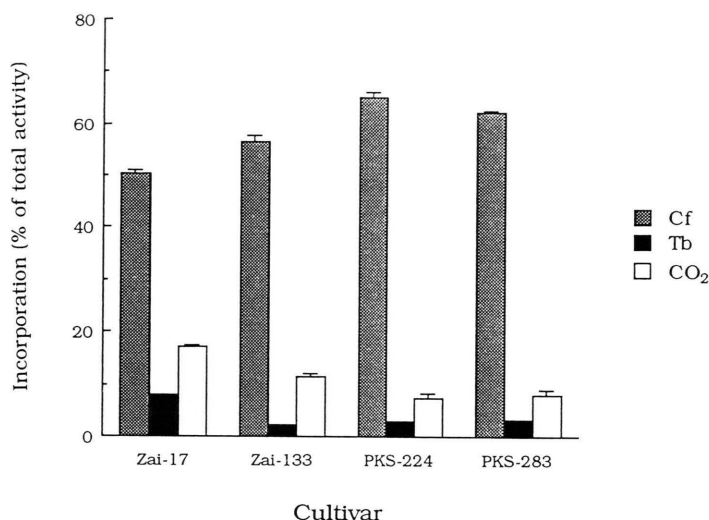


Fig. 3. Incorporation of radioactivity from [8-<sup>14</sup>C]adenine into caffeine, theobromine, and CO<sub>2</sub> after a 'pulse-chase' experiment with leaf segments from high- (PKS-224 and PKS-283) and low caffeine containing cultivars (Zai-17 and Zai-133) of tea. Leaves were incubated with [8-<sup>14</sup>C]adenine for 4 h after which the radioactivity was 'chased' in a non-radiolabelled medium for a further 20 h. Incorporation of radioactivity expressed as a percentage of the total radioactivity taken up by the leaf sample. PKS-224, 24.4 kBq g<sup>-1</sup> (f.w.); PKS-283 28.1 kBq g<sup>-1</sup>; Zai-17 28.2 kBq g<sup>-1</sup>; Zai-133 37.9 kBq g<sup>-1</sup>.

nucleotides before being converted to allantoin and CO<sub>2</sub> via the conventional purine catabolism pathway. The higher accumulation of [<sup>14</sup>C]caffeine and the lower rate of release of <sup>14</sup>CO<sub>2</sub> by the high-compared to low caffeine cultivars, implies that the degree to which purine nucleotide catabolites are directed to the caffeine biosynthesis pathway, rather than to further catabolism to CO<sub>2</sub>, may be an important factor in the regulation of caffeine production. IMP dehydrogenase and 5'-nucleotidase would be key enzymes in this regulatory process (see Fig. 2).

#### Catabolism of caffeine

Caffeine is degraded by a series of demethylation steps leading to xanthine and the purine catabolism pathway which leads ultimately to the release of CO<sub>2</sub> (see Fig. 2) (Crozier *et al.*, 1995). It has been reported that the rate of catabolism of caffeine, as well as its rate of biosynthesis, is an important factor in the degree of accumulation of endogenous caffeine in several species of coffee (Mazzafera *et al.*, 1991). *Coffea* species with a high endogenous caffeine content, such as *C. arabica* and *C. canephora*, catabolise [8-<sup>14</sup>C]caffeine much more slowly than low caffeine-containing species such as *C. dewevrei* and *C. bengalensis*.

Data obtained on the rate of catabolism of caffeine in the high and low caffeine containing cultivars of tea, as indicated by the release of <sup>14</sup>CO<sub>2</sub> from leaf segments incubated with [2-<sup>14</sup>C]caffeine,

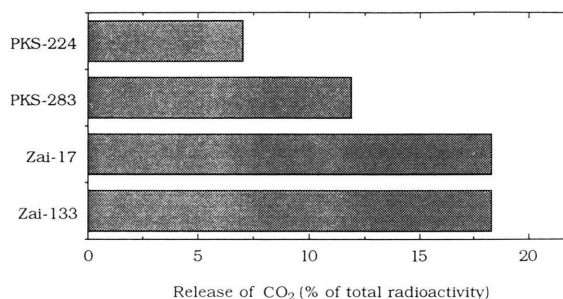


Fig. 4. Release of <sup>14</sup>CO<sub>2</sub> from [2-<sup>14</sup>C]caffeine incubated with leaf segments from high- (PKS-224 and PKS-283) and low caffeine containing cultivars (Zai-17 and Zai-133) of tea. Samples were incubated with [2-<sup>14</sup>C]caffeine for 24 h. Incorporation expressed as percentage of radioactivity taken by the leaf segments. PKS-22 46.6 ± 0.8 kBq g<sup>-1</sup> (f.w.); PKS-283 43.0 ± 2.5 kBq g<sup>-1</sup>; Zai-17 43.0 ± 2.5 kBq g<sup>-1</sup>; Zai-133 45.6 ± 1.1 kBq g<sup>-1</sup>.

are illustrated in Fig. 4. Higher rates of caffeine catabolism were observed in the two low-caffeine cultivars, Zai-17 and Zai-133. No radiolabelled metabolites were detected in the perchloric acid extracts of leaf segments. This suggests that the initial demethylation of caffeine is a rate-limiting step in the degradation pathway leading to CO<sub>2</sub> and NH<sub>3</sub> via xanthine and uric acid.

#### N-methyltransferase activities in cell-free preparations

It has been proposed that the activity SAM:xanthosine 7-N-methyltransferase, SAM:7-methyl-

xanthine 3-*N*-methyl-transferase and SAM:theobromine 1-*N*-methyltransferase are key factors in the regulation of caffeine biosynthesis (see Fig. 2) (Fujimori *et al.*, 1991). The activity of all three enzymes has been detected in cell-free preparations from tea leaves (Negishi *et al.*, 1985; Suzuki and Takahashi, 1975). The three *N*-methyltransferases elute as a single unresolved peak when subjected to anion-exchange and gel-filtration FPLC, suggesting that a single enzyme may be responsible for all the methylation steps (Ashihara, 1995).

The assay of *N*-methyltransferase activities in cell-free preparations has previously involved separation and quantification of the methylated products by paper chromatography (Suzuki and Takahashi, 1975; Negishi *et al.*, 1985; Fujimori *et al.*, 1991) and as a consequence is laborious. To overcome this problem and facilitate rapid and effective screening of *N*-methyltransferase activity a modified assay procedure was used in the current study. The reaction product, [ $^{14}$ C]caffeine, was separated from [ $^{14}$ C]SAM by partitioning the aqueous incubation medium against chloroform. Although not an intermediate in the biosynthesis of caffeine *in vivo* (Suzuki and Takahashi, 1975), paraxanthine was used as the *in vitro* methyl acceptor (see Materials and Methods). The *N*-methyltransferase from both tea and coffee preparations has a lower apparent  $K_m$  for paraxanthine than 7-methylxanthine and theobromine making it a more suitable assay substrate [Suzuki and Takahashi, 1975; Roberts and Waller, 1979].

The *N*-methyltransferase activity in cell-free extracts from leaves of high and low caffeine containing cultivars of tea was investigated using the modified assay procedure. The data obtained, presented as  $\text{kat g}^{-1}$  fresh weight in Fig. 5 demonstrate that preparations from the leaves of high caffeine cultivars contain 2–5 times more *N*-methyltransferase activity than those from low caffeine cultivars.

### Conclusions

The results obtained indicate the endogenous caffeine content of tea leaves is regulated by the

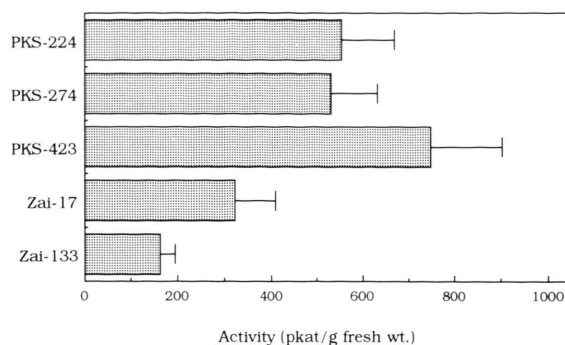


Fig. 5. Caffeine biosynthesis *N*-methyltransferase activity in cell-free preparations from young leaves of high- (PKS-224 and PKS-283) and low caffeine (Zai-17 and Zai-133) containing cultivars of tea. Activity expressed as  $\text{pkat g}^{-1}$  (f.w.).

rates of caffeine biosynthesis and degradation. Cultivars with a high caffeine content synthesize caffeine from [ $8\text{-}^{14}\text{C}$ ]adenine more rapidly and also exhibit higher *N*-methyltransferase activity than low caffeine-containing cultivars. In addition, the rate of [ $2\text{-}^{14}\text{C}$ ]caffeine catabolism is more rapid in cultivars with low – than a high caffeine content. It is evident that the application of molecular biology to produce transgenic tea plants naturally deficient in caffeine could bring about reduced expression of genes encoding the *N*-methyltransferases associated with caffeine biosynthesis. A reduced caffeine content could also be accomplished by limiting the availability of substrates for the caffeine production. One approach could be to down-regulate IMP dehydrogenase and enhance 5'-nucleotidase activity so that IMP is directed to inosine and the purine catabolism pathway at the expense of caffeine biosynthesis (see Fig. 2). Endogenous caffeine pools in tea could also be reduced by activation of the demethylases responsible for the catabolism of caffeine to xanthine.

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