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Caffeine biosynthesis and adenine metabolism in transgenic *Coffea canephora* plants with reduced expression of *N*-methyltransferase genes

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Abstract

In anti-sense and RNA interference transgenic plants of *Coffea canephora* in which the expression of *CaMXMT1* was suppressed, caffeine biosynthesis from [8-^{14}C]adenine was investigated, together with the overall metabolism of [8-^{14}C]adenine. Compared with wild type control plants, total purine alkaloid biosynthesis from adenine and conversion of theobromine to caffeine were both reduced in the transgenic plants. As found previously, [8-^{14}C]adenine was metabolised to salvage products (nucleotides and RNA), to degradation products (ureides and CO_2) and to purine alkaloids (theobromine and caffeine). In the transgenic plants, metabolism of [8-^{14}C]adenine shifted from purine alkaloid synthesis to purine catabolism or salvage for nucleotides. HPLC analysis revealed a significantly reduced caffeine content in the transgenic plants. A small quantity (less than 20 nmol g^{-1} fresh weight) of xanthosine had accumulated in at least one of the transgenic plants.

Keywords: *Coffea canephora*; Rubiaceae; Coffee; Transgenic plants; RNAi; Caffeine biosynthesis; *N*-methyltransferase; Purine metabolism

1. Introduction

Biosynthesis of caffeine (**9**) begins with the methylation of xanthosine (**5**) derived from purine nucleotides; three *N*-methyltransferases and a nucleosidase are involved in the pathway (see Fig. 1, Suzuki et al., 1992; Ashihara and Crozier, 1999, 2001; Ashihara and Suzuki, 2004; Ashihara, 2006). Several *N*-methyltransferase genes for caffeine (**9**) biosynthesis have been isolated from coffee plants (Mizuno et al., 2001, 2003a,b; Ogawa et al., 2001; Uefuji et al., 2003). Ogita et al. (2002) recently reported the construction of coffee plants in which the expression of *N*-methyltransferase genes of caffeine (**9**) biosynthesis is repressed by anti-sense or RNAi methods, using the sequences of 7-methylxanthine (**7**) *N*-methyltransferase (*CaMXMT1*) RNAi. They found a marked decrease in theobromine (**8**) and caffeine (**9**) contents in the transgenic plantlets of *Coffea canephora* (Ogita et al., 2003, 2004). Since caffeine (**9**) biosynthesis is closely related to the purine nucleotides, we examine whether purine nucleotide metabolism in transgenic plants differs from controls, and whether abnormal compound(s) are produced in the transgenic plants. The results show that biosynthesis of caffeine (**9**) from [8-^{14}C]adenine (**1**) is reduced in the transgenic plants. However, no accumulation of unusual compounds specific to this transgenic plant was found. In the transgenic plants, adenine (**1**) metabolism shifts to the synthesis of nucleotides and to degradation to CO_2 (**19**) via the conventional catabolic pathway proceeding via ureides.

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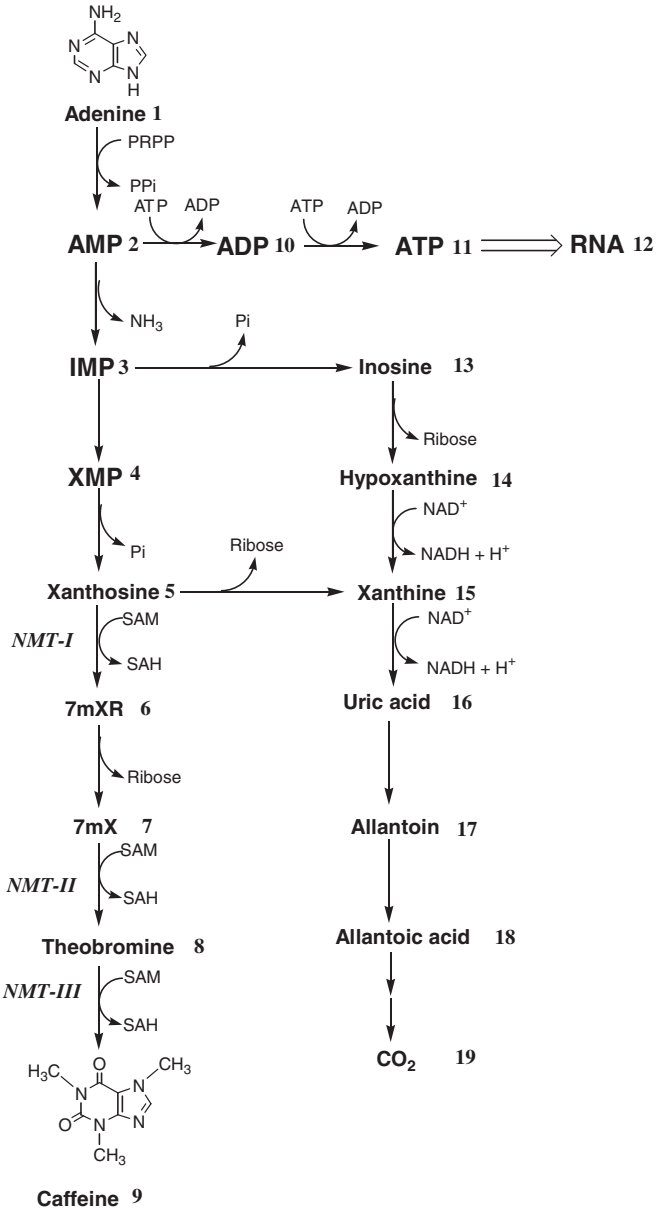

Fig. 1. Overall metabolism of adenine (**1**) in coffee plants. Adenine (**1**) is converted to AMP (**2**) and is utilised for (a) caffeine (**9**) biosynthesis (**2** → **9**), (b) ATP (**11**) and RNA (**12**) synthesis (**2** → **12**), and (c) catabolism via allantoin (**2** → **19**). NMT-I, II and III are three methylation steps catalysed by *N*-methyltransferases. Transgenic plants are used, prepared by the antisense and RNAi with *CaMXMT1*, a gene of the enzyme which catalyses step NMT-II. In these transgenic plants, transcripts for all enzymes which catalysed steps NMT I, II and III are reduced. 7mXR, 7-methylxanthosine (**6**); 7mX, 7-methylxanthine (**7**).

2. Results and discussion

2.1. Biosynthesis of purine alkaloids from [8-^{14}C] adenine (*1*)

Fig. 2 shows the in situ biosynthetic activity of purine alkaloids, estimated from the incorporation of radioactivity from [8-^{14}C]adenine (**1**). Adenine (**1**) is the most efficient exogenously supplied precursor for caffeine (**9**) synthesis (Suzuki et al., 1992), and recent research suggests that adenine (**1**) derived from the SAM cycle is a precursor of caffeine (**9**) biosynthesis in vivo (Koshiishi et al., 2001a). The experiments were performed in October 2004 (Experiment 1) and February 2005 (Experiment 2). Caffeine (**9**) biosynthetic activity was generally higher in Experiment 1 than in the Experiment 2, but caffeine (**9**) biosynthetic activity in the transgenic plants was significantly lower than in wild type controls in most examples. In Experiment 1, incorporation of radioactivity from [8-^{14}C]adenine (**1**) into caffeine (**9**) in RNAi-S #1 and antisense #1 was lower than in wild type #1, although radioactivity in total purine alkaloids (theobromine (**8**) plus caffeine (**9**)) in antisense #1 was similar to that in wild type #1. The velocity of caffeine (**9**) biosynthesis may be slower in antisense #1 than in wild type #1, because most of the radioactivity was found in theobromine (**8**), a direct precursor of caffeine (**9**), in antisense #1 plants. In Experiment 2, caffeine (**9**) biosynthetic activity of wild types #2 and #3 was lower than in wild type #1 in Experiment 1. This activity probably depends on the season in which the experiments were performed, i.e., October and February. Although we used similar sizes of leaves for the experiments, caffeine (**9**) biosynthetic activity is reduced in winter. Nevertheless, the biosynthetic activity in the transgenic plants (antisense #2, antisense #3 and RNAi-L #1) was markedly less than in wild type plants (#2 and #3). No accumulation of radioactivity was found in other purine alkaloids, including 7-methylxanthosine (**6**) or 7-methylxanthine (**7**). The transgenic plants prepared with anti-sense or RNAi of the theobromine synthase gene (*CaMXMT1*) therefore displayed not only reduced theobromine synthase activity but also reduced activity of other *N*-methyltransferases in caffeine (**9**) biosynthesis involving similar nucleotide sequences. This observation is consistent with the facts that RNAi or antisense sequence of *CaMXMT-1* affects the expression of other *N*-methyltransferase genes involved in caffeine (**9**) biosynthesis (Ogita et al., 2004).

2.2. Overall metabolism of [8-^{14}C]adenine (*1*)

To compare the metabolism of adenine (**1**) in wild type and transgenic coffee leaves, we used RNAi-S #1 and Antisense #3 as caffeine (**9**) biosynthesis reduced transgenic plants, and wild type #1 and #2 as controls. The results of our previous studies of adenine (**1**) metabolism in coffee leaves (Fujimori and Ashihara, 1994; Ashihara et al., 1996) are summarised in Fig. 1. These studies indicate that [8-^{14}C]adenine (**1**) is converted to AMP (**2**) by adenine phosphoribosyltransferase, and enters the adenylate pool consisting of AMP (**2**), ADP (**10**) and ATP (**11**). Some adenylate is utilised for RNA (**12**) synthesis, some is deaminated to IMP (**3**) by AMP deaminase, and is then converted to XMP (**4**) by IMP dehydrogenase. XMP (**4**) is dephosphorylated by 5-nucleotidase, and xanthosine (**5**) enters the caffeine (**9**) biosynthetic pathway. Some IMP (**3**) is dephosphorylated by 5-nucleotidase, and inosine

(**13**) is formed. Inosine (**13**) is converted to hypoxanthine (**14**) and then enters the typical purine catabolic pathway. Radioactive ^{14}C from [8-^{14}C]adenine is finally released as $^{14}CO_2$ (**19**) via allantoin (**17**) and allantoic acid (**18**), which are in the pathway.

In Experiment 1, incorporation of radioactivity from [8-^{14}C]adenine into caffeine (**9**) was markedly reduced, but incorporation into ureides (allantoin (**17**) plus allantoic acid (**18**)) is significantly increased in the RNAi-S#1 leaves (Table 1). This suggests that xanthosine (**5**) accumulated in the transgenic plant leaves enters the purine catabolic pathway. In Experiment 2, caffeine (**9**) biosynthesis from adenine in both samples was lower than in Experiment 1. Incorporation of radioactivity into two purine alkaloids (theobromine (**8**) and caffeine (**9**)) was, nevertheless, much reduced in the Antisense #3 leaves. Nearly half of the total radioactivity from [8-^{14}C]adenine (**1**) was recovered in catabolites (ureides plus CO_2 (**19**)) in both samples, but degradation of ureides was faster in the Antisense #3 leaves. In contrast to the RNAi-S #1, substrates that were not utilised for caffeine (**9**) synthesis may be salvaged to nucleotides, and some may be utilised for RNA **(12)** synthesis in the Antisense #3 leaves.

In both experiments, detectable radioactivity was recovered in common metabolites, and no accumulation of radioactivity was found in unusual metabolites. Purine and caffeine (**9**) metabolism in leaves of coffee and tea plants varies during leaf development and at different seasons (Ashihara et al., 1996; Fujimori et al., 1991; Fujimori and Ashihara, 1994). In this study, we used young leaves of similar size from the same coffee trees as much as possible, but it is impractical to use leaves of exactly the same morphological and physiological characteristics. The present results suggest that, if caffeine (**9**) biosynthesis is restricted, the substrate of caffeine (**9**) biosynthesis may be utilised for the nucleotide synthesis or be degraded via the purine catabolic pathway.

2.3. Endogenous levels of purine compounds

Table 2 shows endogenous levels of the major purine compounds in wild type and transgenic coffee leaves found in Experiment 1. The level of caffeine (**9**) was much higher than for other purine nucleotides, and was clearly reduced in transgenic coffee leaves. Total adenine nucleotide levels in Antisense #1 and RNAi-S #1 leaves are higher than in wild type #1. Adenine nucleotide (**2**, **10**, **11**) levels in the transgenic plants were not statistically different (by t test, $P < 0.05$) from levels in wild type, except that the ADP (**10**) level in RNAi S #1was lower than in the control. Xanthosine (**5**) could not be detected in the wild type leaves, but a small amount (14–20 nmol g^{-1} fresh weight) was found in both transgenic leaves; the standard deviation in Antisense #1 was large.

2.4. Concluding remarks

Our data indicate that caffeine (**9**) biosynthesis from purine nucleotides is reduced in the low caffeine (**9**) transgenic plants. In coffee plants and probably in other caffeine (**9**) producing plants, the caffeine (**9**) biosynthetic pathway may be a supplementary pathway to the usual pathways for

Table 1
Overall metabolism of [8-^{14}C]adenine (**1**), by leaf segments from wild type and transgenic *Coffea canephora* plants

Metabolites	Experiment 1		Experiment 2	
	Wild type #1	RNAi-S #1	Wild type #2	Antisense #3
ATP/ADP (**11**, **10**)	6.5 ± 0.7	10.0 ± 1.0	8.0 ± 0.2	15.4 ± 3.4
AMP/1MP/XMP (**2**, **3**, **4**)	17.0 ± 0.8	17.3 ± 0.8	9.8 ± 0.5	12.1 ± 0.2
Theobromine (**8**)	15.3 ± 0.4	17.9 ± 0.4	14.5 ± 1.5	0.3 ± 0.0
Caffeine (**9**)	14.5 ± 0.1	3.0 ± 0.1	2.6 ± 0.1	0.2 ± 0.0
Xanthine (**15**)	0.3 ± 0.0	0.5 ± 0.2	0.0 ± 0.0	2.2 ± 0.5
Ureides (**17**, **18**)	10.8 ± 0.9	27.6 ± 3.1	48.9 ± 2.2	23.6 ± 5.0
CO_2 (**19**)	4.6 ± 0.8	2.3 ± 0.1	1.0 ± 0.1	23.0 ± 1.4
RNA (**12**)	29.2 ± 0.2	19.8 ± 0.9	12.8 ± 0.0	20.2 ± 1.6
Total uptake	30.9 ± 3.6	19.3 ± 2.0	17.6 ± 0.2	29.4 ± 6.2

Values are percentage of radioactivity taken up by the leaf segments. Total uptake is expressed as kBq per 100 mg fresh weight. Average and SD are shown.
Less than 3% of total radioactivity was distributed in several spots that are not shown.

Table 2
Endogenous levels of purine nucleotides, nucleosides and caffeine in young leaves of wild type and transgenic *Coffea canephora* plants

	ATP (**11**)	ADP (**10**)	AMP/IMP (**2**, **3**)	Xanthosine (**5**)	Guanosine	Caffeine (**9**)
Wild type #1	190 ± 23	76 ± 7	87 ± 16	nd	18 ± 1	40,300 ± 2400
Antisense #1	221 ± 2	57 ± 16	98 ± 19	14 ± 12	12 ± 1*	27,100 ± 1600*
RNAi S #l	242 ± 7	30 ± 10*	120 ± 26	20 ± 2	14 ± 6	22,400 ± 1100*

Values are expressed as nmol per g fresh weight. Average and SD ($n = 3$) are shown.
* Indicates values that differ significantly from values for Wild type #1 ($P < 0.05$).

nucleotide salvage and catabolism. Therefore, if caffeine (**9**) synthesis is reduced, the substrate is readily metabolised by other common pathways, although a small amount of xanthosine (**5**), which is a substrate of the first committed step reaction of caffeine (**9**) biosynthesis, was detected in the transgenic plants. These observations suggest that no harmful compounds will be produced in transgenic coffee plants having reduced caffeine (**9**) synthesis. These transgenic plant lines can therefore be used as new decaffeinated coffee varieties in coffee beverages.

3. Experimental

3.1. Plant materials

Construction of transgenic *Coffea canephora* plants (antisense, RNAi-S, and RNAi-L) with reduced caffeine (**9**) content has been demonstrated in previous papers (Ogita et al., 2002, 2003, 2004). We used 4-year-old coffee trees, which had been grown, in pods under a natural photoperiod in a greenhouse of the Nara Institute of Science and Technology as materials. As shown in Fig. 2, we chose three wild type, three antisense transgenic, and two RNAi transgenic trees at random. Young leaves of similar size were collected at the same time. Young leaves (ca. 300 mg fresh weight per leaf) were collected in October 2004 (Experiment 1) and in February 2005 (Experiment 2) and were used as experimental materials. Basic genetic experiments, including determination of the transcript level, have been performed in previous work (Ogita et al., 2002, 2003, 2004).

3.2. Tracer experiments

Metabolism of [8-^{14}C]adenine (**1**) in leaf segments was studied with triplicate samples using the methods published in our previous papers (Fujimori and Ashihara, 1994; Ashihara et al., 1996). Leaf segments (ca. 100 mg fresh wt) and 2.0 ml of 20 mM Na–Pi buffer (pH 5.6) containing 10 mM sucrose and 0.5% sodium ascorbate were placed in the main compartment of a 30-ml Erlenmeyer flask. The flask was fitted with a small glass tube that contained a piece of filter paper impregnated with 0.1 ml of 20% KOH in the centre well, to collect $^{14}CO_2$. Each reaction was started by adding a solution of [8-^{14}C]adenine (**1**) to the main compartment. The flasks were incubated in an oscillating water bath at 27 °C for 18 h. After incubation, the plant materials were harvested using a stainless steel tea strainer, then washed with distilled H_2O and frozen with liquid N_2. They were then stored at −80 °C prior to extraction. $KHCO_3$ that had been absorbed by the filter paper was allowed to diffuse into H_2O overnight, and aliquots of the resulting solution were used for determination of the radioactivity. The radioactivity was measured with a liquid scintillation analyser.

In the analysis of the radioactive metabolites from [8-^{14}C]adenine (**1**), segments were homogenised with 6%

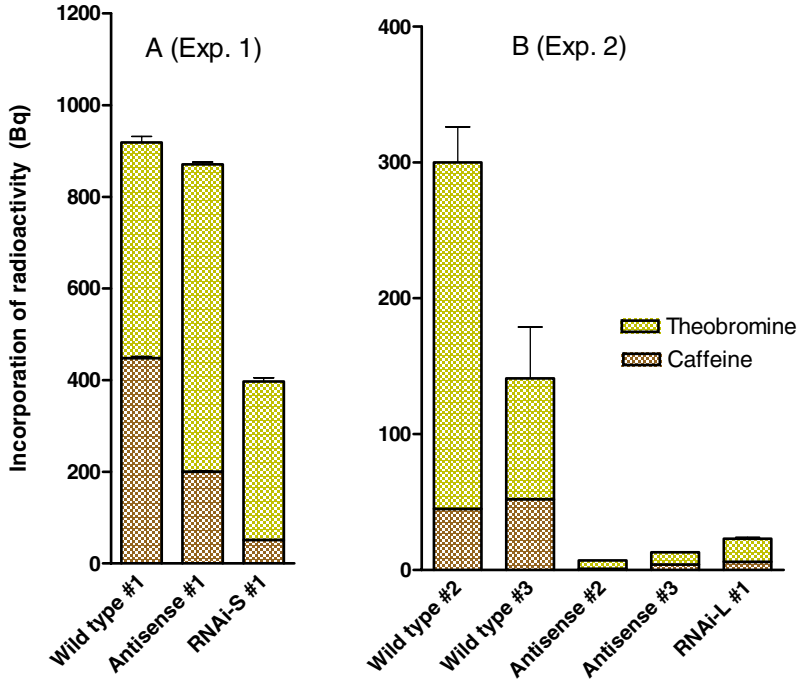

Fig. 2. Caffeine (**9**) biosynthetic activity in young leaves of wild type and transgenic antisense and RNAi *Coffea canephora* plants. Activity is estimated from the incorporation of ^{14}C from [8-^{14}C]adenine (**1**) into theobromine (**8**) and caffeine (**9**) during 18 h incubation. A, Experiment 1 (October); B, Experiment 2 (February). Incorporation of radioactivity is expressed as Bq per 100 mg fresh weight leaf segments. SD for total purine alkaloids (theobromine (**8**) and caffeine (**9**)) is also shown.

$HClO_4$ using a mortar and pestle on ice. The cold $HClO_4$-insoluble materials were washed successively with a mixture of EtOH–Et_2O (1:1, v/v) at 50 °C for 15 min. The washed insoluble fraction containing nucleic acids was hydrolysed with 6% $HClO_4$ at 100 °C for 20 min according to Schneider (1945). The cold $HClO_4$-soluble small molecular weight purine metabolites and the hot $HClO_4$-soluble purine base residues of nucleic acids were neutralised with KOH, and fractionated by TLC.

In screening of the purine alkaloid synthesis from [8-^{14}C]adenine (**1**), leaf segments were homogenised in MeOH–H_2O [4:1] and the soluble extracts were analysed by TLC.

TLC was performed with microcrystalline cellulose plates (200 × 200 mm) (Merck, Darmstadt, Germany). The solvent systems used were *n*-BuOH–HOAc–H_2O (4:1:2, v/v) and *n*-BuOH–MeOH–H_2O–NH_3 (60:20:20:1). Distribution of radioactivity in metabolites on the TLC sheet was determined using a Bio-Imaging Analyser (Type FLA-2000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

4. HPLC analysis

Nucleotides, nucleosides and nucleobases were extracted with 6% $HClO_4$ from fresh leaves (500 mg fresh weight). They were analysed using HPLC systems that were originally established by Ashihara et al. (1987, 1990) for plant nucleotides and nucleoside analysis. To analyse nucleotides, an anion-exchange column (WAX-1, Shimadzu Corp., Kyoto, Japan) was used, and for nucleosides, a poly vinyl alcohol gel column (Asahipack GS-320, Showa Denko Corp., Tokyo, Japan) was used. These methods were originally applied to tea leaves, in our previous paper (Koshiishi et al., 2001b). Purine alkaloids were extracted with MeOH–H_2O (4:1) and analysed with the HPLC system using ODS Hypersil column (Shandon, Runcorn, UK) as shown in our previous paper (Zheng and Ashihara, 2004).

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