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SUBCELLULAR LOCALIZATION OF THE *N*-3 METHYL-TRANSFERASE INVOLVED IN CAFFEINE BIOSYNTHES IN TEA

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Abstract—Caffeine biosynthesis consists of sequential methylations at *N*-7, *N*-3 and *N*-1 of the xanthine ring catalysed by *S*-adenosyl-L-methionine-dependent methyltransferase activities. In preparations from young leaves of *Camellia sinensis*, *N*-3-methyltransferase was associated with a purified chloroplast preparation obtained by using differential centrifugation and a discontinuous Percoll density gradient. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The biosynthesis of the purine alkaloid, caffeine, has been investigated mainly in *Camellia sinensis* (tea) and *Coffea arabica* (coffee) [1, 2] and involves the sequential action of *S*-adenosyl-L-methionine (SAM)-dependent 7-, 3- and 1-*N*-methyltransferases (NMTs) which catalyse the three key methylation steps in the pathway [3]. The three NMT activities from tea leaves were not resolved by purification with anion-exchange and gel-filtration chromatography [4]. Chromatofocusing has recently facilitated the separation of 7-NMT activity from the 3-NMT and 1-NMT activities in extracts from young coffee leaves [5]. Although it is believed that caffeine-chlorogenic acid complexes accumulate in cell vacuoles [6], nothing is known about the sub-cellular compartmentation of the NMT activities. In this paper, we report on a study with preparations from young tea leaves, in which assays for 3-NMT activity have shown that the enzyme is associated primarily with chloroplasts.

RESULTS AND DISCUSSION

The homogenate from young tea leaves was centrifuged at 1000 *g* for 10 min, and chloroplasts in the pellet purified using a discontinuous Percoll density gradient. A single green band was observed and 3-NMT activity was associated with this band. The specific activity of 3-NMT in the chloroplast fraction was 60–85 pkat mg^{-1} protein. In order to examine the subcellular distribution of the NMT, the homogenate was layered onto a discontinuous Percoll density gradient and the 3-NMT and triose phosphate isomerase (TPI) activity assayed after centrifugation. The enzyme profiles that were obtained are shown in Fig. 1A and B. Chlorophyll and protein levels were also determined and their distributions are illustrated in Fig. 1C and D. The vast majority of the 3-NMT appeared as a sharp peak corresponding with the distribution of chlorophyll although a small amount of activity was also present in the upper part of the Percoll gradient. The distribution of TPI activity, which is a marker for chloroplasts and cytosol, indicates that little or no 3-NMT activity was present in the top factions of the gradient containing cytosol enzymes. These results suggest that 3-NMT activity is associated with chloroplasts.

Caffeine is synthesized from purine nucleotides via xanthosine [1] and there are only limited data on the sub-cellular compartmentation of purine nucleotide biosynthesis in higher plants. Most of the available information originates from studies on ureide synthesis in nodules of tropical legumes [7]. It has been shown that 5-phosphoribosyl-1-pyrophosphate amidotransferase (PRAT), a key enzyme of purine nucleotide biosynthesis *de novo*, which is located in proplastids of nodules of soybean [8] and cowpea [9], pea chloroplasts [10] and nodule plastids can produce purines from glycine in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP), glutamine, aspartate, ATP, bicarbonate, methyltetrahydrofolate, $MgCl_2$, and KCl [11]. PRPP synthetase, which cat-

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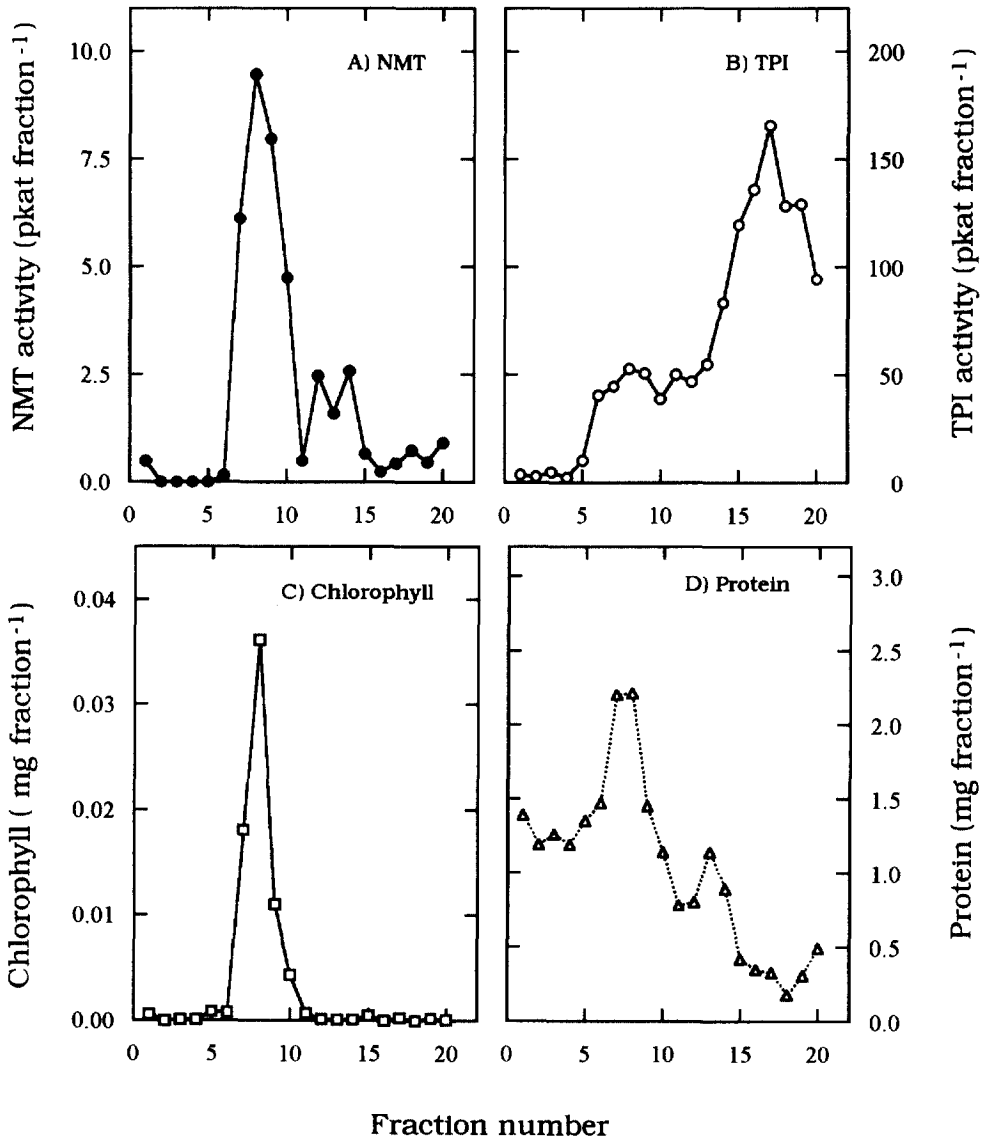

Fig. 1. Discontinuous Percoll density gradient profiles of (A) 3-*N*-methyltransferase, (B) triose phosphate isomerase, (C) chlorophyll and (D) soluble protein in a preparation from young leaves of *Camellia sinensis*.

alyses the formation of PRPP, the donor of ribose phosphate for nucleotide synthesis, has been found in chloroplasts from a green alga, *Briopsis* sp. [12], and the presence of adenine phosphoribosyltransferase and adenosine kinase, the salvage enzymes of adenine and adenosine, have been detected in spinach chloroplasts [13]. Xanthosine formation from IMP, an intermediate of purine nucleotide biosynthesis *de novo*, appears to be catalysed by IMP dehydrogenase and 5′-nucleotidase [1]. These enzymes have been detected in a plastid fraction from soybean nodules [11]. Localization of 5′-nucleotidase in the stroma of *Beta vulgaris* chloroplasts has also been reported [14].

The localization of 3-NMT activity in tea chloroplasts suggests that caffeine is synthesized in chloroplasts although it remains to be determined if the 1- and 7-NMTs involved in caffeine biosynthesis are similarly compartmentalized. One can speculate, in view of the results from nodule proplastids, that the *de novo* purine biosynthesis pathway is operative in tea chloroplasts with the initial step being the formation of IMP from ribose-5-P, an intermediate of the reductive pentose phosphate pathway. In such circumstances, it is feasible that caffeine is produced in chloroplasts from purine nucleotide precursors. At present, there is no information on whether SAM required for purine alkaloid biosynthesis is generated within chloroplasts or elsewhere. In barley leaves, most SAM synthase activity is associated with the cytosol [15].

EXPERIMENTAL

Young leaves from flush shoots of tea (*Camellia sinensis* L.) were collected at the experimental farm of

the National Research Institute of Vegetables, Ornamental Plants and Tea, Makurazaki, Japan. The most recently emerged young leaves (up to 3 cm long, ca 50 mg fr. wt) were collected. De-ribbed leaves (2 g fresh weight) were cut into ca 5 mm square segments and immersed immediately in 10 ml of buffer A (50 mM HEPES-NaOH buffer, pH 6.8 that contained 0.3 M sucrose, 2 mM NaEDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.5% (v/v) Na ascorbate). These were homogenized for 5 s (×2) using a Polytron homogenizer. These procedures were repeated 3 times. The homogenate obtained from 6 g leaves was filtered through 4 layers of nylon gauze and a layer of Miracloth. A portion of homogenate was centrifuged at 1000 *g* for 10 min at 2°. The surface of the pellet was first washed gently with buffer A, which was discarded, before being suspended in buffer A. This crude chloroplast prepn was further purified by Percoll gradient centrifugation. The chloroplast suspension (4 ml) was layered onto a discontinuous gradient consisting of 4 steps of 5, 28, 45 and 60% (v/v) Percoll (10 ml each) containing 25 mM HEPES buffer (pH 7.6), 0.25 mM sucrose, 5 mM $MgSO_4$, 5 mM KCl and 1 mM dithiothreitol. In some experiments, filtered homogenate was directly layered on to the Percoll gradient mentioned above. The gradients were placed in an angle rotor and centrifuged at 10,000 *g* for 20 min. After sepn, 2 ml frs were collected, frozen with liquid N_2 and stored at −80° overnight. The thawed samples were centrifuged at 10,000 *g* for 10 min and supernatant was used for determination of the enzyme activities.

Activity of 3-NMT was determined radiochemically as in Ref. [4]. The reaction mixture (100 μl) contained enzyme preparation in 100 mM Tris-HCl buffer, pH 8.5, 0.2 mM paraxanthine and 5 μM [methyl-^{14}C]SAM (sp. act. 1.96 GBq mmol^{-1}) (Amersham). The reactions were carried out at 27° for 10 min. TPI activity was assayed spectrophotometrically [16] while protein and chlorophyll were determined by the methods of [17] and [18], respectively.

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