Profiles of Purine and Pyrimidine Nucleotides in Fresh and Manufactured Tea Leaves

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Profiles of nucleotide levels in two varieties of Japanese green teas (cv. Yabukita and Saemidori), a Chinese green tea (Longjing), and two Japanese black teas (cv. Benifuuki and Benihikari) were determined and compared with that of fresh tea leaves. The concentration of 5′-nucleotides in green tea was much higher than in black tea. Nucleoside diphosphates were present in larger amounts than nucleoside triphosphates in manufactured green and black teas, whereas the triphosphates predominated in fresh tea leaves. Low levels of 3′-nucleotides were found in green and black teas. Inosine 5′-monophosphate, which is utilized as a seasoning component, was found in all manufactured teas in concentrations ranging from 50 to 200 nmol/g of dry weight. The levels of both inosine 5′-monophosphate and guanosine 5′-monophosphate were high in Chinese Longjing green tea. The unique profiles of nucleotides in manufactured teas may be a consequence of the action of degradation enzymes, such as ribonuclease, apyrase, phosphatase, nucleotidase, and adenosine 5′-monophsphate deaminase during the commercial processing of the young leaves.

Keywords: *Purine nucleotide; pyrimidine nucleotide; inosinate; tea; Camellia sinensis*

INTRODUCTION

Free nucleotides are important precursors for the biosynthesis of nucleic acids and caffeine in tea leaves (*1*). From a food chemistry viewpoint, some of these nucleotides, such as inosine 5′-monophosphate (5′-IMP) and guanosine 5′-monophosphate (5′-GMP), are compounds with a unique taste quality called "umami", which is quite distinct from the four basic tastes, sweetness, sourness, saltiness, and bitterness (*2*). The occurrence of nucleotides in manufactured green and black tea leaves was first demonstrated almost 30 years ago following analysis by anion-exchange (Dowex 1-X8) column chromatography (*3*, *4*). This pioneering work indicated that adenosine 5′-monophosphate (5′-AMP) and uridine 5′-monophosphate (5′-UMP) were the major nucleotides in green tea, whereas 2′- and 3′-isomers of adenosine monophosphate (AMP), cytidine monophosphate (CMP), guanosine monophosphate (GMP), and uridine monophosphate (UMP) were detected together with their 5′-isomers in black tea. To the best of our knowledge, there have been no subsequent publications on the general nucleotide profiles of either fresh tea leaves or manufactured tea products. Since the mid-1980s, high-performance liquid chromatography (HPLC) has replaced the traditional ion-exchange column chromatography as the method of choice for the analysis of nucleotides (*5*−*7*). In the present study, we determined the nucleotide profiles of fresh tea leaves and manufactured green and black tea products using HPLC. The profile of purine and pyrimidine nucleotides in fresh tea leaves is similar to that of other plant species, whereas the profiles in green and black tea products have undergone major modifications during manufacture. Possible conversions of nucleotides during the commercial processing of tea leaves are discussed.

MATERIALS AND METHODS

Plant Materials. Fresh leaves of tea (*Camellia sinensis* cv. Theaceae) were collected at the Tokyo Metropolitan Agricultural Experimental Station, Tachikawa, Tokyo, Japan. Manufactured green and black tea samples were supplied by the National Research Institute of Vegetables, Ornamental Plants and Tea, Makurazaki, Kagoshima, Japan, and the Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China.

Biochemicals. All nucleotide and nucleoside standards and 5′-nucleotidase from *Crotalus atrox* venom were obtained from the Sigma Chemical Co., St. Louis, MO.

Nucleotide Analysis. For the HPLC of nucleotides, a Shimadzu LC-10A HPLC system (Shimadzu Corp., Kyoto, Japan) was used. This comprised two LC-10A pumps, an SPD-10A absorbance monitor operating at 260 nm, a CTO-10A column oven, and an SCL-10A computing integrator. Separations were carried out using a 3 μm (100-Å pore size) Shimpack WAX-1 anion-exchange column (50 mm × 4 mm i.d.) (Shimadzu) and an Asahipack GS-320H poly(vinyl alcohol) gel column (250 mm × 7.6 mm i.d.) (Showa Denko, Tokyo, Japan).

Various nucleotides were separated with the Wax-1 column, at 40 °C and a flow rate of 1.0 mL/min, using gradients of the phosphate buffer as described previously (*7*). Solvent A was 20 mM KH_2PO_4−Na_2HPO_4 buffer (pH 7.00), and solvent B comprised 480 mM KH_2PO_4−Na_2HPO_4 buffer (pH 6.85). The elution program gradient was as follows: 0−10 min, 0−50% B; 10−15 min, 50−60% B; 15−20 min, 60−100% B; 20−26 min, 100% B; 26−60 min 0% B.

Analysis of AMP and IMP was carried out after treatment of the nucleotides with 5′-nucleotidase. The resultant nucleo-

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Table 1. Retention Time of Standard Mixture of 5′- and 3′-Nucleotides by HPLC Using a Shim-pack WAX-1 Anion-Exchange Column

nucleotide[a]	retention time (min)	nucleotide[a]	retention time (min)
5′-UMP	3.4	CDP	10.3
3′-UMP	3.8	5′-XMP	11.7
5′-CMP	4.0	ADP	12.4
3′-CMP	4.9	UTP	14.9
3′-AMP	5.5	GDP	15.2
5′-AMP + 5′-IMP	5.7	CTP	16.4
3′-GMP	7.0	ATP	20.3
5′-GMP	7.9	GTP	23.5
UDP	9.5		

[a] All nucleoside di- and triphosphates are 5′-nucleotides.

sides, adenosine and inosine, were separated at 30 °C on the Asahipack GS-320H column using a mobile phase of 10 mM NaH_2PO_4 (pH 4.6) at a flow rate of 1.0 mL/min (*8*). Prior to HPLC, samples and solvents were filtered through a cellulose nitrate membrane filter with 0.45-μm pore size (Tokyo Roshi Kaisha Ltd., Tokyo. Japan).

Manufactured green and black tea samples (200 mg, dry weight) and fresh tea leaves (500 mg of fresh weight) were homogenized with 0.4 M perchloric acid in a chilled mortar and pestle. The homogenate was centrifuged at 20000*g* for 15 min at 4 °C, and the supernatant (0.5 mL) was applied to an SPE-Phenyl disposable extraction cartridge (J. T. Baker, Phillipsburg, NJ), which had been washed with methanol before being equilibrated with 0.4 M perchloric acid. The cartridge was eluted with ~4.5 mL of 0.4 M perchloric acid, the eluate was neutralized with 20% KOH, and the potassium perchlorate produced was removed by brief centrifugation. After lyophilization, the dried extract was redissolved in ~300 μL of 20 mM KH_2PO_4−Na_2HPO_4 buffer (pH 7.00) and centrifuged at 18000*g* for 10 min at 4 °C prior to analysis on the WAX-1 HPLC column as described above. The 5′-AMP plus 5′-IMP fraction, which eluted at 5.7−6.0 min, was collected and freeze-dried. The residue was dissolved in 40 mM glycine buffer (pH 9.0) and treated with 30 nkat of the snake venom 5′-nucleotidase at 30 °C for 20 min. The resultant nucleosides were analyzed on the Asahipack GS 320-H HPLC column as outlined above. In some instances, tea extracts were directly treated with the snake venom 5′-nucleotidase prior to analysis of the nucleosides.

RESULTS

Separation of Purine and Pyrimidine Nucleotides. In addition to common 5′-nucleotides, 5′-IMP, xanthosine 5′-monophosphate (5′-XMP), and various nucleoside 3′-monophosphates were separated using the WAX-1 HPLC system. The retention times of these nucleotides are shown in Table 1. Although almost all nucleotides were separated, 5′-IMP and 5′-AMP cochromatographed with a retention time of 5.7 min. To identify and quantify these two nucleotides, the 5.6−5.9 min fraction was collected and treated with 5′-nucleotidase. The inosine and adenosine produced were then analyzed on the Asahipack GS 320-H column, which readily separated inosine and adenosine with retention times of 12.0 and 24.5 min, respectively. In some experiments, inosine and IMP levels were determined by analyzing tea extracts before and after treatment with snake venom 5′-nucleotidase.

Concentration of Nucleotides in Fresh Tea Leaves. Nucleotide profiles in fresh samples, which consist of an apical bud and two young leaves of tea shoots, cv. Yabukita, are shown in Table 2. Nucleotide triphosphates were present in much higher amounts than nucleoside mono- and diphosphates. The adenine nucleotide pool was the largest, followed by uracil and cytosine nucleotides, with the cytidine nucleotide pools being the smallest. These profiles are similar to those found in young tobacco leaves (*9*). It may be noteworthy that 5′-XMP, a possible intermediate of caffeine biosynthesis, was detected in the fresh leaf extracts, whereas 5′-IMP was not.

Table 2. Concentration of 5′-Nucleotides in Fresh Tea Leaves[a]

nucleotide	concentration	nucleotide	concentration
AMP	19 ± 2	XMP	145 ± 5
ADP	131 ± 11	UMP	100 ± 36
ATP	701 ± 44	UDP	103 ± 7
ΣAN	851 ± 58	UTP	285 ± 19
GMP	6 ± 2	ΣUN	488 ± 63
GDP	11 ± 0.5	CMP	110 ± 10
GTP	132 ± 16	CDP	3 ± 3
ΣGN	149 ± 18	CTP	100 ± 3
IMP	nd	ΣCN	213 ± 16

[a] Values are expressed as nmol/g of dry weight ± standard deviation (n = 3). nd, not detected. ΣAN, ΣGN, ΣUN and ΣCN mean total adenine, guanine, uridine, and cytidine nucleotides, respectively.

Table 3. Concentration of 5′-Nucleotides and 3′-Nucleoside Monophosphates in Tea Beverages[a]

	green tea			black tea	
nucleotide	Yabukita[b]	Saemidori[b]	Longjin[c]	Benifuuki[d]	Benihikari[d]
AMP	21 ± 2	18 ± 1	57 ± 7	15 ± 2	25 ± 1
ADP	314 ± 1	401 ± 13	211 ± 12	61 ± 7	87 ± 1
ATP	227 ± 9	347 ± 24	14 ± 4	11 ± 1	6 ± 0.5
ΣAN	561 ± 10	766 ± 38	281 ± 22	87 ± 7	118 ± 1
GMP	13 ± 1	13 ± 1	83 ± 12	39 ± 3	40 ± 1
GDP	104 ± 10	139 ± 20	79 ± 1	tr	tr
GTP	90 ± 8	114 ± 6	26 ± 6	5 ± 1	15 ± 5
ΣGN	208 ± 15	266 ± 28	188 ± 22	44 ± 2	55 ± 6
UMP	21 ± 3	70 ± 22	68 ± 9	8 ± 0.2	32 ± 3
UDP	143 ± 8	147 ± 15	158 ± 13	61 ± 11	81 ± 2
UTP	82 ± 6	85 ± 1	20 ± 0	nd	nd
ΣUN	246 ± 12	302 ± 37	247 ± 22	69 ± 11	113 ± 5
CMP	nd	nd	45 ± 5	tr	tr
CDP	36 ± 9	22 ± 2	70 ± 1	11 ± 1	tr
CTP	35 ± 9	33 ± 2	nd	nd	nd
ΣCN	71 ± 18	55 ± 4	115 ± 4	11 ± 1	tr
IMP	73 ± 10	65 ± 0	199 ± 21	53 ± 5	86 ± 3
XMP	13 ± 9	3 ± 0.3	5 ± 0.5	3 ± 0.4	5 ± 1
3′-AMP	nd	nd	68 ± 29	16 ± 6	34 ± 0.1
3′-GMP	38 ± 8	29 ± 7	58 ± 5	63 ± 7	70 ± 1
3-′UMP	131 ± 4	124 ± 6	279 ± 35	44 ± 6	28 ± 1
3′-CMP	nd	nd	nd	7 ± 2	8 ± 3
Σ3′-NMP	169 ± 4	152 ± 1	406 ± 68	130 ± 7	133 ± 1

[a] Values are expressed as nmol/g of dry weight ± standard deviation (n = 3). tr, trace; nd, not detected. All nucleotides not specified are 5′-nucleotides. Σ indicates total nucleotides. 3′-NMP, nucleoside 3′-monophosphate. [b] Japanese green tea (Sencha) made of *C. sinensis* var. *sinensis*. [c] Chinese green tea (pan-fired tea) made of *C. sinensis* var. *sinensis*. [d] Black tea (fermented tea) made of *C. sinensis* var. *assamica*.

Concentration of Nucleotides in Manufactured Green and Black Teas. Profiles of nucleotide concentration in different kinds of manufactured tea products were determined using (i) two samples of a Japanese green tea (cv. Yabukita and Saemidori), both of which are nonfermented teas produced by steaming immediately after plucking; (ii) a Chinese tea (cv. Longjing), which is a nonfermented green tea that is stored for a few hours after plucking before pan firing; and (iii) two samples of fermented black tea (cv. Benifuuki and Benihikari). The data obtained are presented in Table 3. The concentration of total 5′-nucleotides in manufactured tea was always lower than in fresh tea leaves. The levels of 5′-nucleotides in manufactured green tea were much higher than those in black tea. Adenine

nucleotides were present in highest concentrations followed by uracil nucleotides. Cytosine nucleotides were found in the lowest concentrations. In most instances, nucleoside diphosphate levels were higher than those of nucleoside triphosphates. In contrast to the concentration of 5′-nucleotides, there were no great differences in the levels of 3′-nucleotides in green and black teas. The proportion of 3′-nucleotides in the total nucleotide pool in Longjing tea (28.2%) and two black tea samples (26.1 and 32.7%) was much higher than in Japanese green tea (9.5 and 12.6%).

5′-IMP, an important "umami" compound, was found in all of the manufactured tea samples in concentrations ranging from 50 and 200 nmol/g of dry weight. Highest amounts were detected in the Longjing tea. 5′-IMP accounted for ~20% of the total 5′-nucleotide pool in Longjing and black tea but for only 4−6% in the green tea samples. The concentration of 5′-GMP, another possible "umami" compound in tea, was also higher in Longjing (83 nmol/g of dry wt) and black tea (39−40 nmol/g of dry wt) than in Japanese green tea (13 nmol/g of dry wt).

The concentration of total 5′-nucleotides in fresh leaves (1850 nmol/g of dry wt) was ~1.6 times higher than in manufactured tea of the same cultivar (1170 nmol/g of dry wt).

DISCUSSION

Although Takino et al. (*3*) demonstrated the presence of 5′-AMP, 5′-UMP, 5′-GMP, and adenosine 5′-diphosphate (ADP) in green tea, there are no reports on the general profile on nucleotides in manufactured tea and fresh tea shoots. In the present study, profiles of various purine and pyrimidine nucleotides in manufactured tea and in fresh tea leaves were investigated. The nucleotide pools in tea leaves are similar to those in plants such as tobacco, which does not produce caffeine (*9*−*13*). The adenylate energy, defined as [ATP] + 0.5 [ADP])/([ATP] + [ADP] + [AMP] (*14*), was 0.90 in the fresh Yabukita tea leaves but decreased to 0.65 in commercial tea manufactured from the same cultivar. This was mainly due to the loss of the ATP-generating system during the processing of the tea as the concentration of ADP was always higher than that of ATP in both green and black teas. Similarly, concentrations of GTP, UTP, and CTP were lower than those of their respective nucleoside diphosphates in most manufactured teas.

The decline in the nucleotide pool was more evident in black tea than in green tea. This is understandable as more nucleotides would degrade during the fermentation process that is involved in the manufacture of black tea. Possible degradation pathways of nucleotides in tea leaves are shown in Figure 1. Not all of these pathways are necessarily always functional in intact tea leaves. However, various enzymes, such as ribonuclease, phosphatase, nucleotidase, and apyrase (ATP-diphosphohydrolase), released from vacuoles during fermentation, probably catalyzed the degradation of the nucleotides. There are a number of reports of very high, nonspecific nucleotidase and/or phosphatase activities, possibly derived from vacuoles, being detected in extracts from young tea leaves (*1*, *15*).

As the net purine and pyrimidine nucleotide levels were reduced in manufactured green and black teas, a similar breakdown of these nucleotides may have occurred. Most of these nucleotides are probably degraded by the conventional purine and pyrimidine catabolism

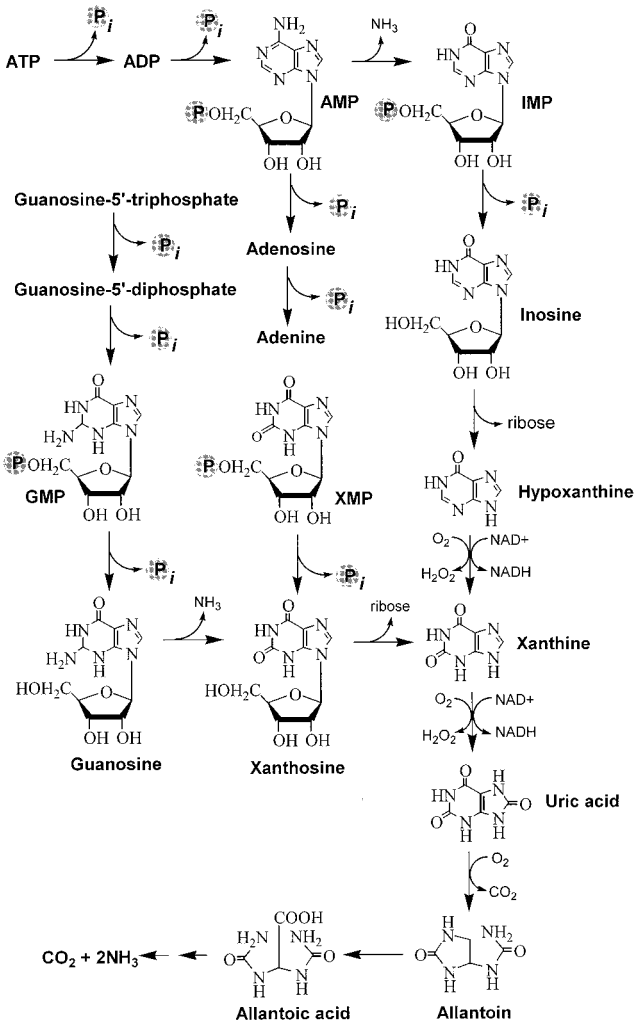

Figure 1. Possible biodegradation pathways of purine 5′-ribonucleotides in tea leaves during manufacture. In addition to the normal catabolic pathways in tea leaves, various degradation enzymes distributed in other cellular compartments may be functional. Removal of P_i from nucleotides may be catalyzed by phosphatases, 5′-nucleotidases, apyrase plus pyrophosphatase, and many nucleoside tri- and/or diphosphate-utilizing enzymes. Removal of ribose from nucleosides may be catalyzed by nonspecific nucleosidases and/or specific adenosine nucleosidase and/or inosine−guanosine nucleosidase. Deamination is carried out by AMP deaminase, guanosine deaminase, and possibly guanine deaminase. Oxidation of purine bases to uric acid may be catalyzed by xanthine oxidase and/or xanthine dehydrogenase. Catabolism of uric acid to allantoic acid may be catalyzed by the sequential reactions of uricase and allantoinase. The degradation pathway of allantoic acid to CO_2 plus NH_3 in tea plants has not yet been determined.

pathways shown in Figures 1 and 2, respectively, although participation of other catabolic routes cannot be excluded, especially in the fermented black teas.

Although 5′-IMP, which is an important nucleotide for taste (*2*), was not detected in fresh tea leaves (Table 1), it was present in all of the manufactured tea products (Table 3). It is probable, therefore, that 5′-IMP is produced from 5′-AMP by the action of AMP deaminase during the commercial processing of the leaves. Significant amounts of 3′-nucleotides found in the manufactured tea may be derived from RNA in fresh leaves as suggested by Takino and Imagawa (*4*).

The present research has shown that the nucleotide profiles of tea leaves are similar to those of other plant

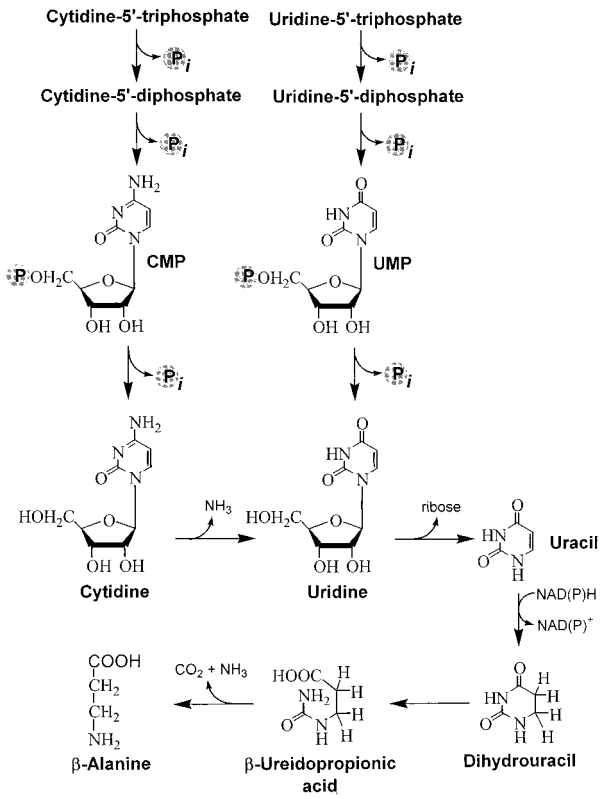

Figure 2. Possible biodegradation pathways of pyrimidine 5′-ribonucleotides in tea leaves during manufacture. Similar to the purine nucleotide catabolism, various enzymes shown in the caption for Figure 1 may be also included in the catabolism of pyrimidine nucleotides to pyrimidine bases. Deamination may be catalyzed by cytidine deaminase. Catabolism of uracil to β-alanine is probably catalyzed by uracil reductase and/or dihydrouracil dehydrogenase, dihydropyrimidinase, and β-ureidopropionase. The presence of these enzymes in tea leaves has not yet been reported.

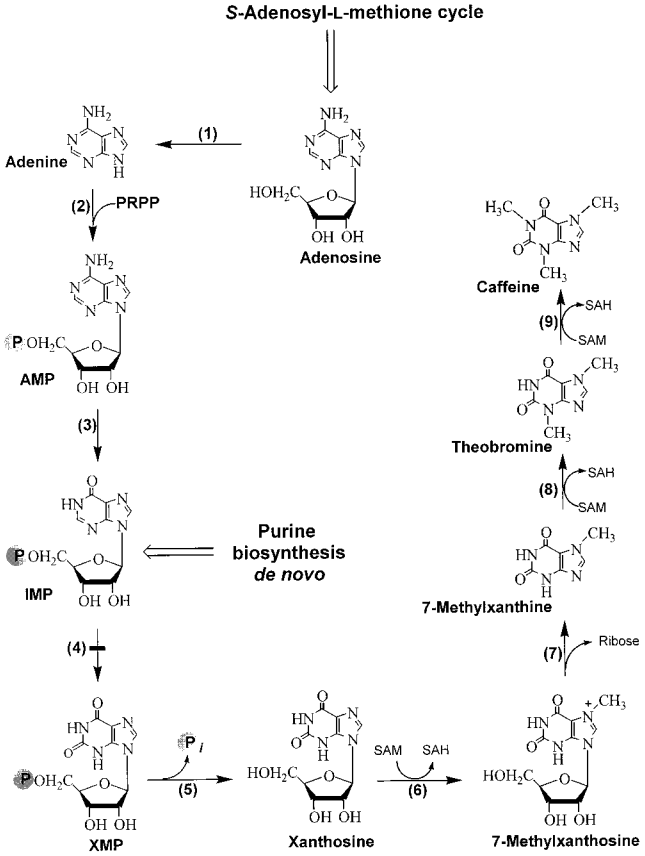

Figure 3. Metabolism of inosine 5′-monophosphate (IMP) as an intermediate of the caffeine biosynthetic pathway in young tea leaves: (**1**) adenosine nucleotidase; (**2**) adenine phosphoribosyl transferase; (**3**) adenosine 5′-monophosphate deaminase; (**4**) inosine 5′-monophoshate dehydrogenase; (**5**) 5′-nucleosidase; (**6**) xanthosine 7-methyltransferase; (**7**) 7-methylxanthine nucleosidase; (**8**) 7-methylxanthine 3-methyltransferase (caffeine synthase); (**9**) theobromine 1-methyltransferase (caffeine synthase). A solid bar shows the reduction of IMP dehydrogenase activity for IMP accumulation. PRPP, 5-phosphoribosyl-1-pyrophosphate.

species. Nucleotide levels are, however, reduced and their compositions modified during the manufacture of commercial teas. One of the notable modifications is the appearance of IMP in manufactured green and black teas. It is currently believed that the tea-specific amino acid, theanine, is an important factor contributing to "umami" taste. However, IMP may also participate as it has been shown that nucleotide seasonings have synergistic interactions with amino acid-based taste (*16*). If so, metabolic engineering to accumulate IMP may well be of value. Recent research has demonstrated that IMP, utilized as a precursor for caffeine biosynthesis, is produced from adenosine, released from the *S*-adenosyl-L-methionine cycle (*17*) and/or the purine nucleotide biosynthesis de novo (*18*). Therefore, if IMP dehydrogenase activity is blocked, caffeine synthesis will be reduced and IMP may accumulate (see Figure 3). In these circumstances, transgenic tea plants with reduced IMP dehydrogenase activity offer the intriguing prospect of yielding a beverage with a low caffeine content coupled with enhanced flavor quality.

ABBREVIATIONS USED

3′-AMP, adenosine 3′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; 3′-CMP, cytidine 3′-monophosphate; 5′-CMP, cytidine 5′-monophosphate; 3′-GMP, guanosine 3′-monophosphate; 5′-GMP, guanosine 5′-monophosphate; 5′-IMP, inosine 5′-monophosphate; 3′-UMP, uridine 3′-monophosphate; 5′-UMP, uridine 5′-monophosphate; 5′-XMP, xanthosine 5′-monophosphate; ΣAN, total adenine nucleotides; ΣCN, total cytidine nucleotides; ΣGN, total guanine nucleotides; ΣUN, total uridine nucleotides.

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