

Caffeine Biosynthesis and Degradation in Tea [*Camellia sinensis* (L.) O. Kuntze] is under Developmental and Seasonal Regulation

Prashant Mohanpuria · Vinay Kumar ·
Robin Joshi · Ashu Gulati · Paramvir Singh Ahuja ·
Sudesh Kumar Yadav

Published online: 29 May 2009
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Abstract To study caffeine biosynthesis and degradation, here we monitored caffeine synthase gene expression and caffeine and allantoin content in various tissues of four *Camellia sinensis* (L.) O. Kuntze cultivars during non-dormant (ND) and dormant (D) growth phases. Caffeine synthase expression as well as caffeine content was found to be higher in commercially utilized tissues like apical bud, 1st leaf, 2nd leaf, young stem, and was lower in old leaf during ND compared to D growth phase. Among fruit parts, fruit coats have higher caffeine synthase expression, caffeine content, and allantoin content. On contrary, allantoin content was found lower in the commercially utilized tissues and higher in old leaf. Results suggested that caffeine synthesis and degradation in tea appears to be under developmental and seasonal regulation.

Keywords Caffeine synthase · Caffeine · Allantoin ·
Camellia sinensis

Prashant Mohanpuria and Vinay Kumar have contributed equally to this work.

P. Mohanpuria · V. Kumar · P. S. Ahuja · S. K. Yadav (✉)
Biotechnology Division, Institute of Himalayan Bioresource
Technology, CSIR, Palampur, HP 176061, India
e-mail: skyt@rediffmail.com; sudeshkumar@ihbt.res.in

R. Joshi · A. Gulati
Hill Area Tea Science, Institute of Himalayan Bioresource
Technology, CSIR, Palampur, HP 176061, India

Present Address:

S. K. Yadav
Department of Botany and Plant Sciences,
Institute for Integrative Genome Biology,
University of California Riverside,
Riverside, CA 92507, USA

Introduction

Caffeine (1,3,7-trimethylxanthine) is one of the member of group of compounds known as purine alkaloids. It is found in more than 60 different plant species including tea, coffee, mate, guarana, cola, and cocoa [1]. Caffeine accumulates in young leaves, fruits, seeds, and cotyledons. Caffeine biosynthesis involves xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine as a major and 7-methylxanthine → paraxanthine → caffeine pathway as one of the numbers of minor pathways operating in tea leaves [2]. The first, third, and fourth steps in the major pathway are catalyzed by *N*-methyltransferases that use *S*-adenosyl-*L*-methionine (SAM) as methyl donor [3]. The two final steps are catalyzed by caffeine synthase (CS), a bifunctional enzyme in tea comprising two SAM-dependent *N*-methyltransferase activities [4]. A gene encoding CS has been cloned from young tea leaves of a Japanese cultivar [5]. Also, cDNAs encoding *N*-methyltransferases (*CaXMT1*, *CaMXMT2*, and *CaDXMT1*) that are catalyzing the formations of 7-methylxanthosine from xanthosine, theobromine, and caffeine, respectively, have been cloned from immature fruits of coffee [6].

A high caffeine biosynthetic activity has been found in stamens and petals of tea prior to the opening of flowers [7]. Caffeine biosynthesis has also been detected in pericarp and seeds of tea fruits [8]. In addition, seasonal variation studies of caffeine biosynthetic activity have been investigated to a limited extent in tea. During spring, caffeine biosynthesis was restricted to young leaves of new shoots [9]. Recent study on expression of caffeine synthase (*TCSI*) gene in young and mature leaves, stems, and roots of tea seedlings has shown its higher expression in young leaves and proposed that caffeine biosynthesis could be dependent on expression of *TCSI* in young leaves [10].

Caffeine catabolism in coffee and tea has been investigated through tracer experiments using ^{14}C -labeled purine alkaloids. The main caffeine degradation pathway was observed via theophylline [11, 12]. Conversion of caffeine to theophylline has been assumed to be catalyzed by *N7*-demethylase [13] and considered to be a major rate-limiting step in caffeine catabolism in tissues of *Coffea sinensis* and *Coffea arabica* [11, 12, 14]. Further, conversion of theophylline to xanthine via 3-methylxanthine has been expected to be catalyzed by *N1*-demethylase and *N3*-demethylase. Hence, it is expected that demethylases might be present in coffee and tea plants. Catabolism of theobromine to xanthine via 3-methylxanthine has also been reported in *Theobroma cacao* [15].

Allantoin and allantoate are the metabolites of caffeine degradation in coffee [16], tea, and other caffeine containing plants [17, 18]. Therefore, relative allantoin content measurement in plant tissues containing caffeine alkaloid would reflect the caffeine degradation. However, caffeine synthase expression in different tissues of tea during dormancy and non-dormancy has not been studied. In this paper, we studied caffeine synthase expression in various tissues, presenting different developmental stages, of four tea cultivars during dormant and non-dormant growth phases. Further, we monitored the caffeine and allantoin contents in the same tissues and during the same growth phases.

Materials and Methods

Plant Material

Four tea (*Camellia sinensis* (L.) O. Kuntze) cultivars, i.e., Kangra jat (KJ), Upasi-9 (U-9), Tocklai germplasm-270/2/13 (TG), and Tocklai variety-23 (TV) used in the present study were maintained at our Institute experimental tea farm. We collected various tissues, i.e., apical bud (AB), 1st leaf (IL), 2nd leaf (IIL), young stem (YS; stem portion up to third leaf), and old leaf (OL; fourth leaf from top) of four different tea cultivars during non-dormant (ND; April–September) and dormant (D; October–March) growth phases. Specifically, ND samples were collected in the month of July and D samples were collected during December. Leaf positions represent different developmental stages. During D growth phase, AB of these cultivars undergoes dormancy and is not available for making tea extract. These tissues were used to study the endogenous caffeine synthase (*CS*) gene expression as well as caffeine and allantoin contents. In addition, fruits of these cultivars were also collected to carry out similar experiments in fruit coat and cotyledons. Fruits have no such growth phases as their formation and maturation in tea occurs once in a year.

Isolation of Caffeine Synthase Partial cDNA

To monitor the expression of caffeine synthase in tea, we have isolated partial fragments of caffeine synthase cDNAs using reverse transcriptase-PCR approach. The isolated 376 bp partial fragments of caffeine synthase cDNA from Kangra jat (KJ), Upasi-9 (U-9), Tocklai germplasm (TG), and Tocklai variety (TV) show very high sequence similarity. Four cDNA sequences differ by only 1–2 nucleotides and a representative sequence of partial cDNA of caffeine synthase from KJ cultivar has been submitted to GeneBank (Accession number: FJ554589). Also the isolated fragments show close sequence homology with the reported *TCSI* from tea [5].

RNA Isolation and Caffeine Synthase Transcript Expression Analysis

One hundred milligrams of each tissue was ground in liquid nitrogen and total RNA was isolated using Qiagen RNeasy Plant Mini Kit. cDNA was prepared according to manufacturer's protocol (Invitrogen, USA) using 2 μg of total RNA from each sample, 250 ng OligodT_{12–18}, 200 U of superscript III RT, and 10 mM dNTPs in a 15 μl reaction volume. Equal quantity of cDNA was used as template in PCR with caffeine synthase gene-specific primer set (forward 5'-CACAAACAGTGACCTCAATGAC-3' and reverse 5'-CAGGAATGAACTAAATGCAAGC-3') to see the expression of endogenous caffeine synthase (*CS*) gene. Since primer sequences do not differ for four cultivars, we used the same set of primers to see the expression of caffeine synthase in all the four tea cultivars. Linearity between the amount of input RNA and the final PCR products was verified and confirmed. After standardizing the optimal amplification at exponential phase, PCR was carried out under the conditions of 94°C—4 min for 1 cycle, 94°C—30 s, 55°C—30 s, 72°C—30 s for 25 cycles, and amplified product was separated on 1% agarose gel and visualized with ethidium bromide staining. The 26S rRNA-based gene primers were used as internal control for gene expression studies [19].

Caffeine and Allantoin Content Measurement

Caffeine content was estimated following the HPLC method described earlier [20]. Briefly, 3 gm of each tissue was dried at 80°C and used for caffeine extraction with 70% methanol. The caffeine content was estimated by Merck Hitachi HPLC (Darmstadt Germany) using C18 Lichrocart column (250 \times 4 mm \times 5 μm) and absorbance was read at 210 nm. Pure caffeine (Sigma) was used as standard in the assay. Allantoin content in various tissues of tea was estimated using method described earlier with

some modifications [21, 22]. Ehrlich's reagent was prepared by dissolving 1 gm of *p*-dimethylaminobenzaldehyde (Fluka) in a mixture of 1:3 HCl and methanol and was refrigerated in a dark bottle. The 100 mg tissue was ground in 1 ml of Ehrlich's reagent. Allantoin present in the sample forms a colored complex with Ehrlich's reagent and was stable at 15–40°C temperature up to 30 min. After standing at room temperature for 10 min, absorbance was optimized and read at 440 nm on an ND-1000 (Nanodrop spectrophotometer). Pure allantoin (Fluka) was used as standard in the assay.

Analysis of Data

Means and standard deviations were calculated according to the standard methods. One-way ANOVA statistical test

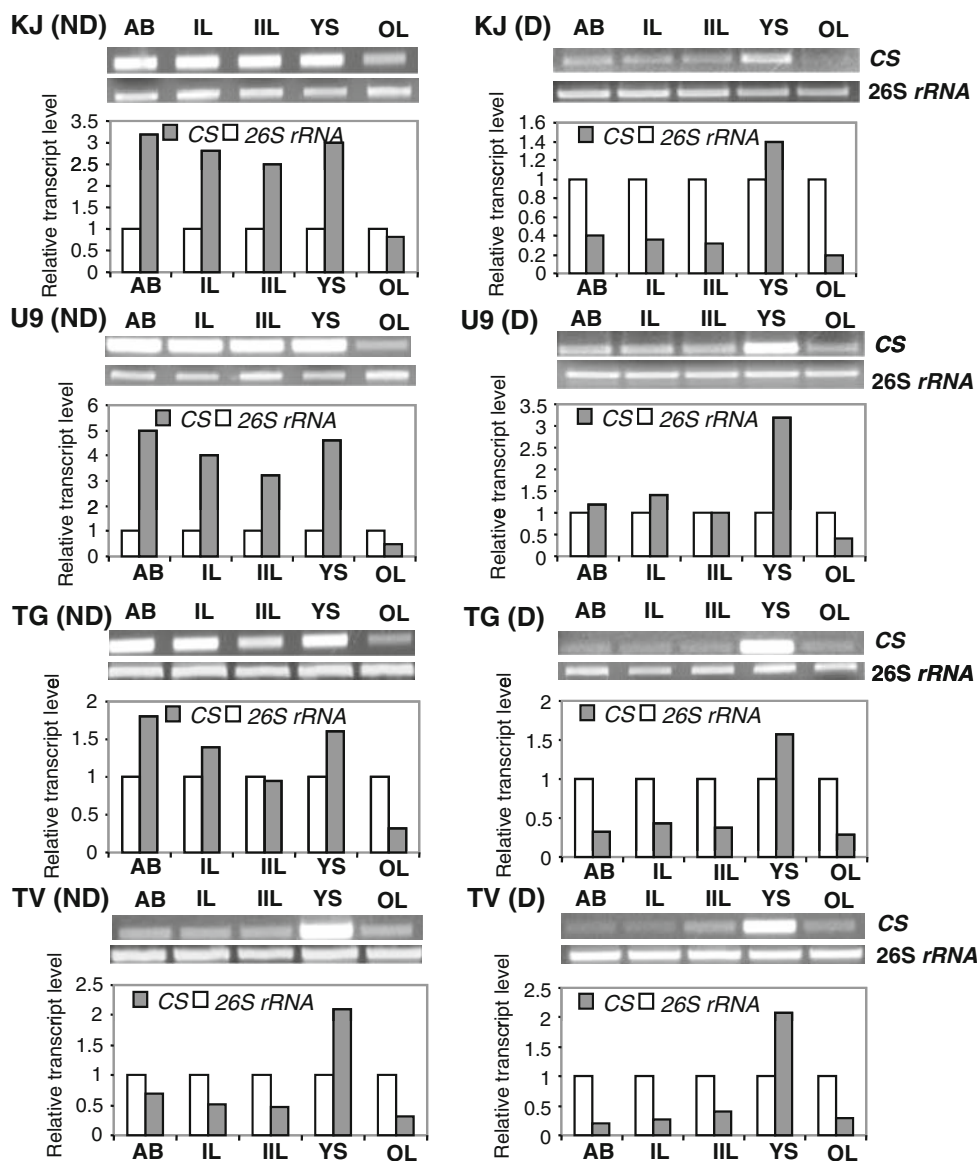
was used to determine the differences between means of the caffeine and allantoin contents accepting the significance level at $P \leq 0.05$.

Results

Caffeine Synthase Expression in Various Tissues of Tea Cultivars

Caffeine synthase (CS) transcript expression analysis was carried out in various tissues such as AB, IL, IIL, YS, OL in Kangra jat (KJ), UPASI9 (U9), Tocklai germplasm (TG), and Tocklai variety (TV) tea cultivars during dormant (D) and non-dormant (ND) growth phases, while in fruit coat and cotyledons only for one season. Leaf

Fig. 1 Caffeine synthase transcript expression in various tissues of different tea cultivars. Expression levels in apical bud (AB), 1st Leaf (IL), 2nd leaf (IIL), young stem (YS), and old leaf (OL) during non-dormant (ND) and dormant (D) growth phases in four tea cultivars Kangra jat (KJ), UPASI9 (U9), Toklai germplasm (TG), and Toklai variety (TV). Lower panel in gel pictures shows expression of 26S rRNA as internal control. Bar diagram represents relative levels of transcript expression



positions are representing different developmental stages. Tissues collected during D and ND growth phase represent two seasons.

The *CS* transcript expression level was observed to be higher during ND growth phase compared to D growth phase in all tissues of four tea cultivars. During ND growth phase, the expression of *CS* was higher in AB, IL, IIL, and YS compared to OL in all four tea cultivars. The *CS* expression was highest in AB of KJ, U9, and TG cultivars during ND growth phase. While, in TV cultivar *CS* expression was highest in YS during ND growth phase compared to other tissues. During D growth phase, *CS* expression was decreased in AB, IL, IIL, and OL of all four tea cultivars compared to ND growth phase. In YS, the decrease in *CS* expression during D growth phase was less in KJ and U9 compared to other tissues. While no effect was observed on the *CS* expression in YS of TV and TG cultivars during D growth phase compared to ND growth phase (Fig. 1).

Further, *CS* expression was monitored in fruit coat and cotyledons of fruit in all four tea cultivars (Fig. 2a). The *CS* expression was found to be higher in fruit coat compared to cotyledons in all tea cultivars. The expression of *CS* gene was highest in fruit coat of KJ compared to U9, TV, and TG tea cultivars (Fig. 2b).

Caffeine Content in Various Tissues of Tea Cultivars

Similar to *CS* expression, caffeine content was also observed to be higher in all tissues of these four tea cultivars during ND growth phase compared to D growth phase (Fig. 3a). Caffeine content was higher in AB, IL, IIL, and YS of all the cultivars compared to OL. Note the highest caffeine content in YS of TV that also showed highest *CS* expression level in this cultivar. Caffeine content decreased significantly during D growth phase in all the tissues of these four tea cultivars. Even in YS of TG and TV cultivars caffeine content was decreased to the similar extent as in other tissues where *CS* expression was unaffected. Caffeine content was highest (4.7%) in U9 during ND growth phase and lowest (0.1%) during D growth phase in KJ tea cultivar. From the data, effect of dormancy on caffeine content was quite prominent in KJ as compared to other tea cultivars. Therefore, on the basis of *CS* expression and caffeine contents, these tea cultivars can be ranked as U9 \gg KJ \gg TV > TG.

Caffeine content in fruits of these four tea cultivars also showed similar trend to that of *CS* expression. Caffeine content was estimated in fruit coat and cotyledons of all four tea cultivars and was found to be higher in fruit coat as compared to cotyledons. Similar to *CS* expression, caffeine content was highest in fruit coat of KJ compared to U9, TV, and TG tea cultivars (Fig. 3b).

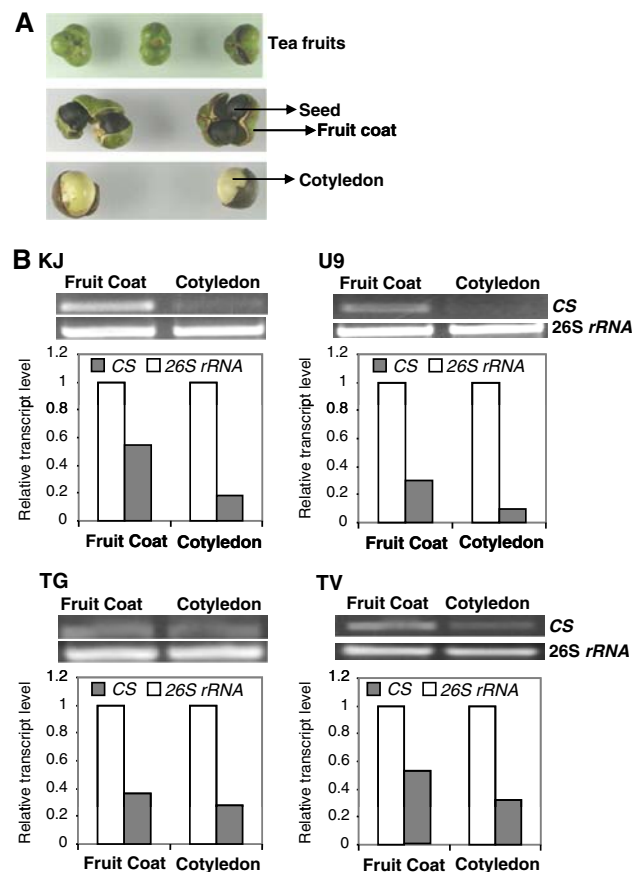


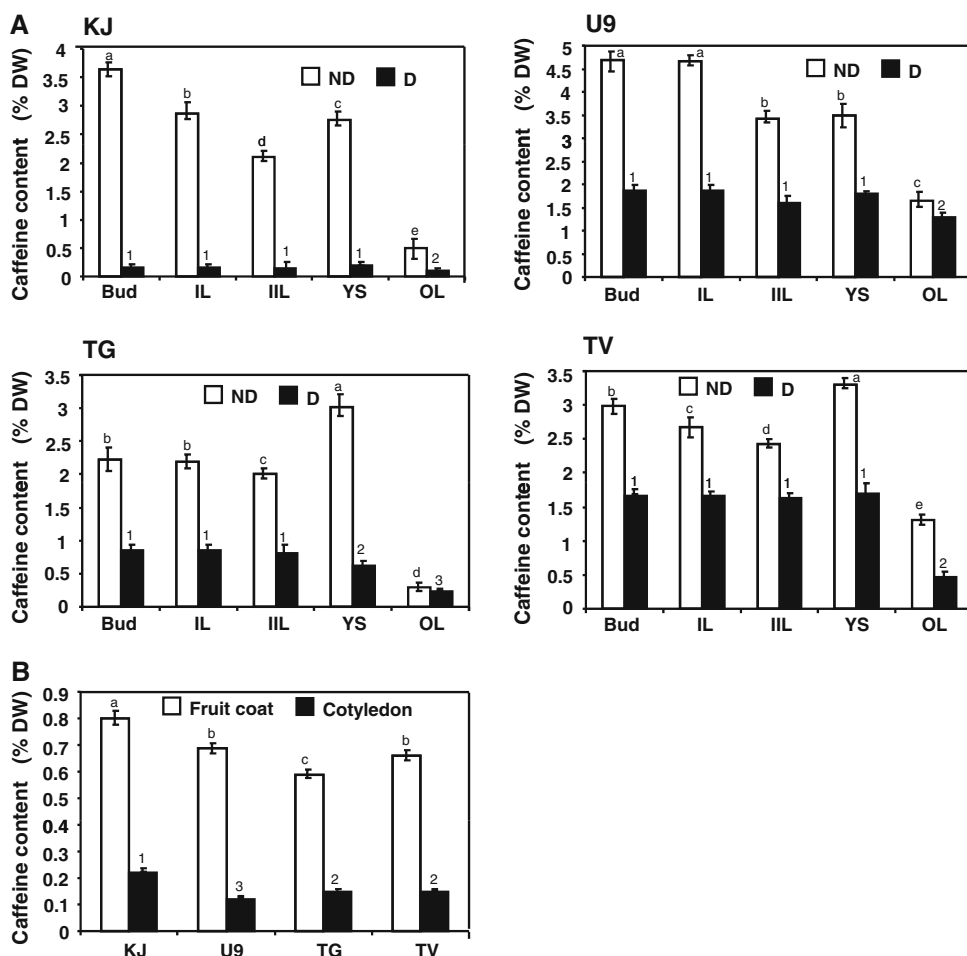
Fig. 2 Caffeine synthase transcript expression in fruit parts of different tea cultivars. **a** Different parts of tea fruit. **b** Gel pictures represent expression levels of caffeine synthase in fruit coat and cotyledons of all the four tea cultivars. Lower panel in gel pictures shows expression of 26S rRNA as internal control. Bar diagram represents relative levels of transcript expression

Allantoin Content in Various Tissues of Tea Cultivars

Allantoin content measurement in different tissues could be an important aspect in measuring caffeine degradation as it has been reported as one of the important products of caffeine catabolism in coffee and tea [22]. To check caffeine degradation, we estimated allantoin content in all tissues which were used for *CS* expression and caffeine content study. Higher allantoin content was observed in AB, IL, IIL, YS, and OL during D growth phase as compared to ND growth phase. Allantoin content was highest in OL (3.3 mg/ml) of KJ cultivar during D growth phase and was lowest in YS (0.30 mg/ml) of TV cultivar during ND growth phase. In tea manufacturing tissues, AB, IL, and IIL, lower allantoin content was measured as compared to OL during both ND and D growth phases in all the four tea cultivars (Fig. 4a).

Interestingly, among fruits the same tissue fruit coat that contains higher levels of *CS* expression and caffeine content showed higher levels of allantoin compared to

Fig. 3 Caffeine content in various tissues of different tea cultivars. **a** Caffeine content monitored in apical bud (AB), 1st Leaf (IL), 2nd leaf (IIL), young stem (YS), and old leaf (OL) during non-dormant (ND) and dormant (D) growth phases in four tea cultivars Kangra jat (KJ), UPASI9 (U9), Toklai germplasm (TG), and Toklai variety (TV). **b** Caffeine content in fruit coat and cotyledons of all the four tea cultivars. Values are the mean of three replications \pm SD. Data with same alphabet and numeric above SD bar in each column are not significantly different from each other at $P \leq 0.05$. Data with alphabet are significantly different from the data with numeric above SD bar



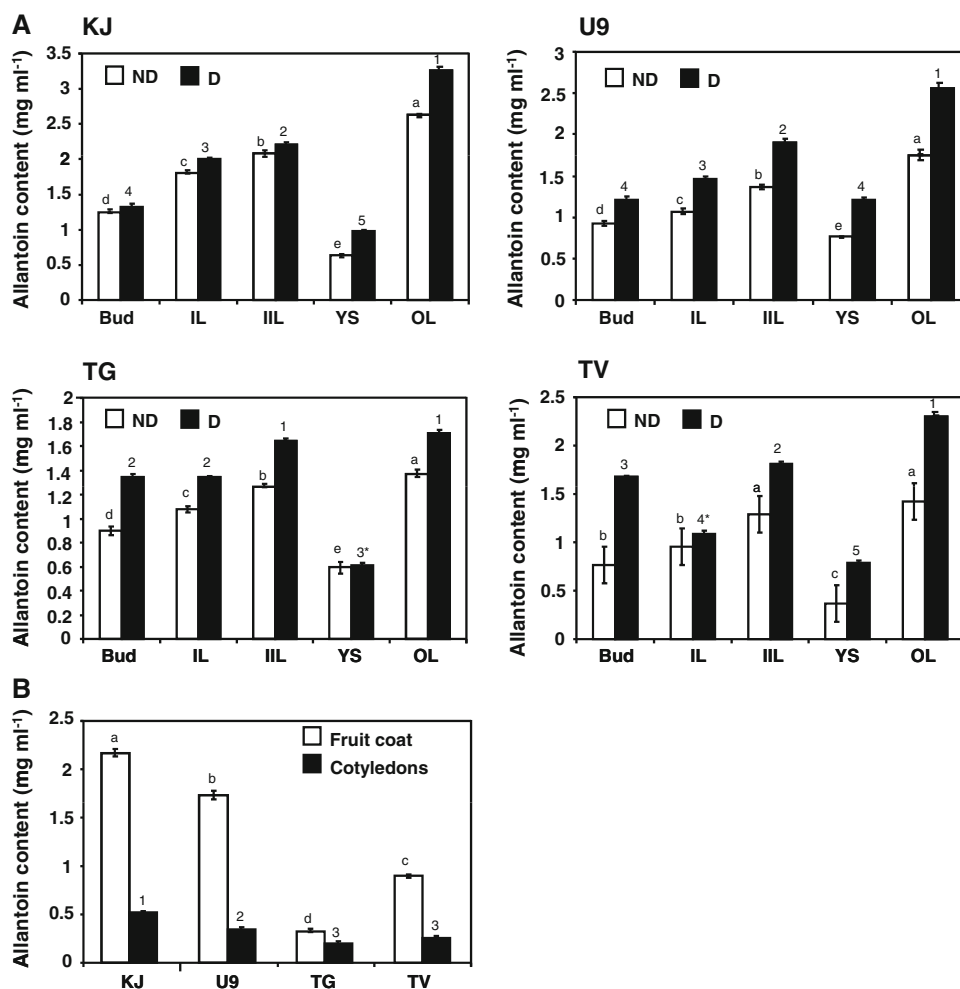
cotyledons. KJ fruit coat showed highest level of allantoin followed by U9 > TV > TG (Fig. 4b).

Discussion

Tea is essentially an out-breeding crop and is found in three major races Chinaria, Assamica, and Cambod. Morphologically, Chinaria tea plant is shorter and its leaves are smaller compared to Assamica. Cambod tea has morphological characteristics in between the Chinaria and Assamica. These cultivars are highly interfertile and therefore, the present tea populations are highly heterogeneous [23]. Kangra jat (KJ) is a Chinaria type of tea clone possessing small serrated leaves and multiple stems at bottom. UPASI-9 (U9) is a Chinaria hybrid possessing more Assamica type of morphological and systematic characteristics. Toklai variety-23 (TV) is an Assamica type of tea clone possessing single stem at bottom and broad leaves. While, Toklai germplasm-270/2/13 (TG) is a Cambod type of tea clone. In the present study, these four tea cultivars belonging to different races were used for developmental and seasonal regulation of caffeine metabolism.

Tea (*Camellia sinensis* (L.) O. Kuntze) is a perennial and the cheapest non-alcoholic caffeine containing beverage crop grown in the world. Its young shoots including two leaves and a bud are commercially utilized for making tea drink. However, during winter (October to March), tea undergoes a dormancy period. During this, growth of apical bud almost ceases, severely reducing the commercial yield of tea [24]. Rate of caffeine biosynthesis depends upon the amount and activity of enzymes, availability of substrates such as xanthosine and SAM, stage of development, tissue specific, and environmental growth period. In tea, single *N*-methyltransferase (NMT) catalyses the methylation steps of caffeine biosynthetic pathway. High expression level of gene encoding NMT (*TCSI*) has been reported to be responsible for the production of high caffeine content in tea leaves [5]. Lower expression of *CS* in OL of tea compared to other younger tissues documented developmental effect on its expression. Constitutive production of caffeine in relevant tissues has also been reported in coffee, where transcript accumulation for *CS* was detected predominantly in immature fruits. However, other *N*-methyltransferase genes of the caffeine biosynthesis pathway accumulated in leaves, floral buds, and immature fruits [6]. Higher *CS*

Fig. 4 Allantoin content in various tissues of different tea cultivars. **a** Allantoin content monitored in apical bud (AB), 1st Leaf (IL), 2nd leaf (IIL), young stem (YS), and old leaf (OL) during non-dormant (ND) and dormant (D) growth phases in four tea cultivars Kangra jat (KJ), UPASI9 (U9), Toklai germplasm (TG), and Toklai variety (TV). **b** Allantoin content in fruit coat and cotyledons of all the four tea cultivars. Values are the mean of three replications \pm SD. Data with same alphabet and numeric above SD bar in each column are not significantly different from each other at $P \leq 0.05$. Data with alphabet are significantly different from the data with numeric above SD bar. Numeric with asterisk data is not significantly different from the same tissue alphabet data



expression and caffeine content during ND and lower during D in various tea tissues documented the seasonal effect on caffeine biosynthesis.

Before this study, seasonal variation in capacity of caffeine biosynthesis in tea leaves has been studied by measuring the rates of incorporation of radiolabeled 8-¹⁴C-adenine in caffeine and has suggested the biosynthesis of caffeine occurs in tea young leaves during April to June month of the year [9]. Dormancy had a down regulation effect on the expression of *CS*. During dormancy, the *CS* expression was decreased significantly in the tea manufacturing tissues AB, IL, and IIL. However, the expression of *CS* in YS seems to be cultivar specific. There was no effect of dormancy on *CS* expression in YS of TG and TV cultivars. In contrast, expression of *CS* was decreased in YS of KJ and U9. Further, caffeine content and *CS* expression showed direct relation in various tissues except in YS of TG and TV, suggesting caffeine synthase as one of regulatory enzymes of caffeine biosynthesis. The decrease in caffeine content of YS of TG and TV cultivars during dormancy and had no effect on *CS* expression suggested that other caffeine biosynthetic pathway genes

might be regulating the caffeine synthesis in YS during D growth phase. Secondly, this could also be due to post-transcriptional regulation of *CS* in YS. The expressed *CS* transcript might not be translated to active caffeine synthase protein and resulted in lesser caffeine content during D growth phase in YS compared to ND growth phase. Tea fruits have lower levels of *CS* expression and caffeine contents compared to other tissues used in this study. Higher caffeine content in fruit coat compared to cotyledons was also in direct correlation with higher expression levels of *CS* in the same tissue.

In spite of caffeine biosynthesis, a degradation pathway has been documented in coffee which is a close relative of tea in terms of caffeine containing plants. In coffee, caffeine is catabolized to xanthine via theophylline and 3-methylxanthine and these are then degraded further by the conventional purine catabolism pathway to CO₂, NH₃, and urea via uric acid, allantoin, and allantoic acid [11]. Caffeine degradation pathways have also been well studied in lower organisms such as bacteria [25], fungi [26, 27], and yeast [28]. Demethylase(s) activity for caffeine degradation has been documented in smaller organisms but it has not

been elucidated in tea. However, based on tracer experiments several catabolites of caffeine degradation have been identified in *Coffea arabica* leaves and tea [29].

Caffeine is mainly produced in young leaves of tea plants and it continuously accumulates during its maturation. It has also been observed that caffeine is slowly catabolized as the tissue ages [1]. From this, it is expected that demethylase(s) might be present in tea plants and is responsible for the removal of methyl groups from caffeine to convert it into xanthine. Caffeine degradation measured in terms of allantoin content was higher in OL during ND growth phase. Hence, low *CS* expression and caffeine content and high allantoin content in OL during ND growth phase document the faster degradation of caffeine in OL. In contrast, AB, IL, IIL, and YS have higher rate of caffeine biosynthesis than degradation during ND. Furthermore, the decrease in *CS* expression and caffeine content and increase in allantoin content in all tissues of all the four tea cultivars during D growth phase has suggested that dormancy might be activating caffeine degradation and inhibiting caffeine biosynthesis.

Interestingly, fruits show different behavior than other tissues. High level of allantoin along with *CS* expression and caffeine content in fruit coat compared to cotyledons suggested caffeine biosynthesis as well as caffeine degradation might be simultaneously operative at a competitive pace in fruit coat. Earlier reports based upon physiological and biochemical studies have documented that the ratio of caffeine biosynthesis and degradation in fruit and leaves determines the caffeine content. Furthermore, degradation of caffeine in fruits has been reported faster as compared to leaves [30–32]. Among fruits, fruit coat was found to be possessing higher rate of caffeine biosynthesis and degradation compared to cotyledons. However, a high rate of caffeine biosynthesis accompanied by a slow rate of caffeine degradation has been reported in endosperm of developing seed of *Coffea arabica*, while *Coffea dewevrei*, which is characterized by low caffeine content, has been reported to catabolize caffeine much more efficiently than *C. arabica* [32, 33].

The mature fruit and old leaves of tea have minimum amount of caffeine compared to young leaves [17]. The similar observations were concluded in our present study and this could be due to differential regulation of the biosynthetic and degradation pathways of caffeine in different tissues of tea. In conclusion, data shown here provide evidence for regulation of caffeine in different tissues of tea by caffeine synthase. Further results also document that caffeine biosynthesis and degradation in tea seems to be cultivar-specific, tissue-specific, and season-dependent.

Acknowledgments We would like to acknowledge the financial support from Department of Science and Technology (DST),

Government of India, grant GAP095 and Council of Scientific and Industrial Research (CSIR), Government of India, grant SIP003.

References

- Ashihara, H., & Crozier, A. (2001). Caffeine: A well known but little mentioned compound in plant science. *Trends in Plant Science*, 6, 407–413. doi:10.1016/S1360-1385(01)02055-6.
- Kato, M., Kanehara, T., Shimizu, H., Suzuki, T., Gillies, F. M., Crozier, A., et al. (1996). Caffeine biosynthesis in young leaves of *Camellia sinensis*: In vitro studies on *N*-methyltransferase activity involved in the conversion of xanthosine to caffeine. *Physiologia Plantarum*, 98, 629–636. doi:10.1111/j.1399-3054.1996.tb05720.x.
- Ashihara, H., & Crozier, A. (2000). Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. In J. R. Callow (Ed.), *Advances in botanical research* (Vol. 30, pp. 117–205). London: Academic press.
- Kato, M., Mizuno, K., Fujimura, T., Iwama, M., Irie, M., Crozier, A., et al. (1999). Purification and characterization of caffeine synthase from tea leaves. *Plant Physiology*, 120, 579–586. doi:10.1104/pp.120.2.579.
- Kato, M., Mizuno, K., Crozier, A., Fujimura, T., & Ashihara, H. (2000). Caffeine synthase gene from tea leaves. *Nature*, 406, 956–957. doi:10.1038/35023072.
- Uefuji, H., Ogita, S., Yamaguchi, Y., Koizumi, N., & Sano, H. (2003). Molecular cloning and functional characterization of three distinct *N*-methyltransferases involved in the caffeine biosynthetic pathway in coffee plants. *Plant Physiology*, 132, 372–380. doi:10.1104/pp.102.019679.
- Fujimori, N., & Ashihara, H. (1990). Adenine metabolism and the synthesis of purine alkaloids in flowers of *Camellia* plants. *Phytochemistry*, 29, 3513–3516. doi:10.1016/0031-9422(90)85266-I.
- Terasaki, Y., Suzuki, T., & Ashihara, H. (1994). Purine metabolism and the biosynthesis of purine alkaloids in tea fruits during development. *Plant Physiology*, 13, 135–142.
- Fujimori, N., Suzuki, T., & Ashihara, H. (1991). Seasonal variation in biosynthetic capacity for the synthesis of caffeine in tea leaves. *Phytochemistry*, 30, 2245–2248. doi:10.1016/0031-9422(91)83622-R.
- Li, Y., Ogita, S., Keya, C. A., & Ashihara, H. (2008). Expression of caffeine biosynthesis genes in tea (*Camellia sinensis*). *Journal of Biosciences*, 63, 267–270.
- Ashihara, H., Monteiro, A. M., Moritz, T., Gillies, F. M., & Crozier, A. (1996). Catabolism of caffeine and related purine alkaloids in leaves of *Coffea arabica* L. *Planta*, 198, 334–339. doi:10.1007/BF00620048.
- Ashihara, H., Kato, M., & Ye, C. X. (1998). Biosynthesis and metabolism of purine alkaloids in leaves of cocoa tea (*Camellia pitilophylla*). *Journal of Plant Research*, 111, 599–604. doi:10.1007/BF02507798.
- Ashihara, H., Zheng, X. Q., Katahira, R., Morimoto, M., Ogita, S., & Sano, H. (2006). Caffeine biosynthesis and adenine metabolism in transgenic *Coffea canephora* plants with reduced expression of *N*-methyltransferase genes. *Phytochemistry*, 67, 882–886. doi:10.1016/j.phytochem.2006.02.016.
- Ito, E., Crozier, A., & Ashihara, H. (1997). Theophylline metabolism in higher plants. *Biochimica et Biophysica Acta*, 1336, 323–330.
- Ashihara, H., Sano, H., & Crozier, A. (2008). Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. *Phytochemistry*, 69, 841–856. doi:10.1016/j.phytochem.2007.10.029.

16. Filippi, S. B., Azevedob, R. A., Sodeka, L., & Mazzafera, P. (2007). Allantoin has a limited role as nitrogen source in cultured coffee cells. *Journal of Plant Physiology*, *164*, 544–552. doi:10.1016/j.jplph.2006.03.005.
17. Ashihara, H., & Crozier, A. (1999). Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *Advances in Botanical Research*, *30*, 117–205. doi:10.1016/S0065-2296(08)60228-1.
18. Mazzafera, P. (2004). Catabolism of caffeine in plants and microorganisms. *Frontiers in Bioscience*, *9*, 1348–1359. doi:10.2741/1339.
19. Singh, K., Raizada, J., Bhardwaj, P., Ghawana, S., Rani, A., Singh, H., et al. (2004). 26S rRNA-based internal control gene primer pair for reverse transcription-polymerase chain reaction-based quantitative expression studies in diverse plant species. *Analytical Biochemistry*, *335*, 330–333. doi:10.1016/j.ab.2004.08.030.
20. Sharma, V., Gulati, A., Ravindranath, S. D., & Kumar, V. (2005). A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. *Journal of Food Composition and Analysis*, *18*, 583–594. doi:10.1016/j.jfca.2004.02.015.
21. Fink, K., Cline, R. E., & Fink, R. M. (1963). Paper chromatography of several classes of compounds: correlated R_f values in a variety of solvent systems. *Analytical Chemistry*, *35*, 389–398. doi:10.1021/ac60196a036.
22. Vrbaški, M. M., Grujić-Injac, B., & Gajić, D. (1978). A new method for allantoin determination and its application in allantoin determination in *Agrostemma githago* L. seed. *Analytical Biochemistry*, *91*, 304–308. doi:10.1016/0003-2697(78)90844-8.
23. Barua, D. N. (1989). *Science and practice in tea culture*. Calcutta, India: Tea Research Association.
24. Vyas, D., Kumar, S., & Ahuja, P. S. (2007). Tea (*Camellia sinensis*) clones with shorter periods of winter dormancy exhibit lower accumulation of reactive oxygen species. *Tree Physiology*, *27*, 1253–1259.
25. Blecher, R., & Lingens, F. (1997). The metabolism of caffeine by a *Pseudomonas putida* strain. *Hoppe-Seyler's Zeitschrift für Physiologische*, *358*, 807–817.
26. Asano, Y., Komeda, T., & Yamada, H. (1993). Microbial production of theobromine from caffeine. *Bioscience, Biotechnology, and Biochemistry*, *57*, 1286–1289.
27. Brand, D., Pandey, A., Roussos, S., & Soccol, C. R. (2000). Biological detoxification of coffee husk by filamentous fungi using a solid state fermentation system. *Enzyme and Microbial Technology*, *27*, 127–133. doi:10.1016/S0141-0229(00)00186-1.
28. Sauer, M. (1982). Comparison of the cytochrome P-450 containing monooxygenases originating from two different yeasts. *Developmental Biochemistry*, *23*, 452–457.
29. Kalberer, P. (1965). Breakdown of caffeine in the leaves of *Coffea arabica* L. *Nature*, *205*, 597–598. doi:10.1038/205597a0.
30. Mazzafera, P. (1991). A cafeína do café. *Documentos do Instituto Agronômico de Campinas*, *25*, 1–22.
31. Mazzafera, P. (1993). 7-methylxanthine is not involved in caffeine catabolism in *Coffea dewevrei*. *Journal of Agricultural and Food Chemistry*, *41*, 1541–1543. doi:10.1021/jf00034a002.
32. Mazzafera, P., Crozier, A., & Sandberg, G. (1994). Studies on the metabolic control of caffeine turnover in developing endosperms and leaves of *Coffea arabica* and *Coffea dewevrei*. *Journal of Agricultural and Food Chemistry*, *42*, 1423–1427. doi:10.1021/jf00043a007.
33. Mazzafera, P., Crozier, A., & Magalhães, A. (1991). Caffeine metabolism in *Coffea arabica* and other species of coffee. *Phytochemistry*, *30*, 3913–3916. doi:10.1016/0031-9422(91)83433-L.