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Producing low-caffeine tea through post-transcriptional silencing of caffeine synthase mRNA

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Abstract In this study, attempt has been made to produce a selected cultivar of tea with low-caffeine content using RNAi technology. The caffeine biosynthetic pathway in tea has been proposed to involve three N-methyltransferases such as xanthosine methyltransferase, 7-N-methylxanthine methyltransferase and 3, 7-dimethylxanthine methyltransferase. Last two steps of caffeine biosynthesis in tea have been known to be catalyzed by a bifunctional enzyme known as caffeine synthase. To suppress the caffeine synthesis in the selected tea [*Camellia sinensis* (L.) O. Kuntze] cv. Kangra jat, we isolated a partial fragment of caffeine synthase (*CS*) from the same cultivar and used to design RNAi construct (pFGC1008-CS). Somatic embryos were transformed with the developed construct using biolistic method. Transformed somatic embryos showed reduction in the levels of *CS* transcript expression as well as in caffeine content. Plants were regenerated from the transformed somatic embryos. Transgenic plants showed a significant suppression of *CS* transcript expression and also showed a reduction of 44–61% in caffeine and 46–67% in theobromine contents as compared to the controls. These results suggest that the RNAi construct developed here using a single partial fragment of *CS* gene reduced the expression of the targeted endogenous gene significantly. However, the reduction in theobromine content in addition to caffeine documented the involvement of this single CS in the catalysis of last two methyl transfer steps in caffeine biosynthesis of tea.

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Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is a perennial and most popular non-alcoholic caffeine-containing beverage crop. India occupies first rank in consumption and second rank in production of tea after China. Tea is grown in about 30 countries and is the most widely consumed beverage next to water worldwide. Tea is consumed by over two-third of the world population in the form of black, green, and oolong tea (Graham 1992; Yukiaki and Yukihiko 1999). The commercial value of tea is due to its tender shoots which contain huge amount of antioxidants in addition to caffeine (2–6% on dry weight basis). It is well known that caffeine is a psychostimulant and is widely used to enhance alertness and improve performance. But in recent years, tea quality has gained considerable significance as consumers have become more health conscious. However, the quality is a composite character depends on various constituents of tea in addition to caffeine such as polyphenols, catechins, proteins, enzymes, carbohydrates and inorganics (Marimuthu and Muraleedharan 2004). Along with other methylxanthines including theobromine (3, 7-dimethylxanthine), paraxanthine (1, 7-dimethylxanthine), and methyluric acids, caffeine (1, 3, 7-trimethylxanthine) is a natural purine alkaloid found in coffee, tea, mate, guarana, cola, and cocoa (Ashihara and Crozier 2001).

The major caffeine biosynthesis pathway in tea involved the conversion of xanthosine to caffeine via 7-methylxanthine and theobromine which are catalyzed by S-adenosyl-L-methionine (SAM) dependent *N*-methyltransferases

including caffeine synthase (Ashihara and Crozier 1999). Xanthosine is the initial substrate for caffeine synthesis. Xanthosine is derived from adenine and guanine nucleotide pools which are ultimately produced by de novo and salvage pathways. The conversion of xanthosine via adenosine monophosphate, inosine monophosphate and xanthosine monophosphate is likely predominant. SAM cycle also produces adenosine which is converted to AMP directly/or indirectly via adenine (Koshiishi et al. 2001; Li et al. 2008). In addition to this, purine nucleotides are also synthesized de novo from non-purine precursors such as CO_2, 10-formyl-tetrahydrofolate, 5-phosphoribosyl-1-pyrophosphate and the amino acids glycine, glutamine, and aspartate (Ashihara et al. 2008). The caffeine synthase is a bifunctional enzyme, encoded by a single caffeine synthase gene (*CS*). Caffeine synthase catalyzes final two steps of the caffeine biosynthesis pathway in tea (Kato et al. 1999). The probable pathway of caffeine biosynthesis in tea is shown in Fig. 1.

Tea contains various polyphenolic compounds such as catechins and flavonoids (Wang et al. 2000). These compounds act as strong antioxidants, contributing substantially to the promotion of health and prevent from various chronic diseases such as cancer (Jankun et al. 1997), type 2 diabetes (Van Dieren et al. 2009), and cardiovascular diseases (Sharma and Rao 2009). But high intake of tea can cause some adverse effects associated with high caffeine consumption at least in sensitive peoples such as palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure, insomnia (Chou and Benowitz 1994; Nurminen et al. 1999; Smith 2002; Riksen et al. 2009), reduction in bone mass, decrease in calcium absorption and birth defects (Chen and Whitford 1999; Massey 2001; Borse et al. 2002; Pan et al. 2003; Heckman et al. 2010). The reported anticancerous as well as stroke preventive effects of drinking tea, especially green tea may be amplified by the absence of caffeine (Fujiki 1999; Lopez-Garcia et al. 2009). Today, approximately 80% of the world's population consumes caffeinated products every day (Ogawa and Ueki 2007). The exact amount of caffeine necessary to produce an adverse effect varies from person to person depending on their weight and sensitivity to caffeine (Higdon and Frei 2006). The total daily intake as well as the leading source of caffeine also varies throughout the world. The caffeine has been considered to have generally regarded as safe (GRAS) status (FDA 2003). Caffeine was absent in the "Prohibited List-International Standard" published during 2008 by the World Anti-Doping agency (WADA). In addition, it is not present in the "substance dependence" category classification. But moderate consumption (<400 mg/day) of caffeine is only considered as safe by numerous regulatory agencies to prevent from its significant health risks to most consumers (Nawrot et al. 2003; Heckman et al. 2010). Thus, the caffeine addition up to 350 mg/l level is generally authorized. Caffeine is consumed most frequently in beverages such as coffee (71%), soft drinks (16%) and tea (12%) (Channel Check 2008). So, the demand for low-caffeine/decaffeinated beverages like tea and coffee has increased for the last few years.

Tea is a woody perennial crop having long life cycle, self-incompatibility, and high inbreeding depression. These characteristics usually limit its genetic improvements by conventional breeding methods. To produce low-caffeine tea, conventional breeding may take more than 25 years. While the present industrial decaffeination process is expensive and also affecting the flavor of the product (Ashihara and Crozier 2001). Only after the year 2000, several caffeine biosynthesis pathway gene(s) from tea, coffee, and cocoa have been successfully reported (Kato et al. 2000; Uefuji et al. 2003; Mizuno et al. 2003a; b; Yoneyama et al. 2006). This has facilitated the use of genetic engineering towards the production of low-caffeine tea. Tea can become a most useful source of beneficial compounds upon reduction or elimination of caffeine from the plant. This can be achieved by silencing caffeine biosynthesis pathway gene(s) (Yadav and Ahuja 2007; Mohanpuria et al. 2010). Transgenic coffee plants have already been produced with reduced caffeine content using gene silencing approach (Ogita et al. 2003, 2004). Tea showed low competence for transformation as well as regeneration because of the presence of high levels of

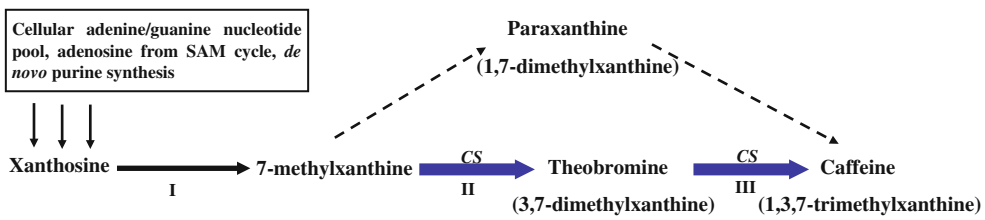

Fig. 1 The caffeine biosynthetic pathway in tea plants. Last two steps of the pathway are catalyzed by a bifunctional enzyme caffeine synthase. Broken arrows indicate the minor route for caffeine synthesis. Xanthosine is the initial substrate for three step methyltransferases in caffeine synthesis, which is derived from adenine and guanine nucleotide pools. Later are produced by de novo and salvage pathways. For more details Ashihara et al. (2008) can be referred

polyphenols with germicidal properties (Mondal et al. 2004). So, optimization of transformation efficiency, reproducibility, and regeneration is very critical. The somatic embryogenesis was considered as one of the most worked out regeneration system in tea (Jain and Newton 1990). It was used successfully in the production of first healthy transgenic tea plants via *Agrobacterium*-mediated transformation (Mondal et al. 2001a). Further, tea transformations and production of transgenics have been attempted via *Agrobacterium* or biolistic mediated approach by several workers using different explants such as embryogenic tissues, somatic embryos, and in vitro leaves (Lopez et al. 2004; Jeyaramraja and Meenakshi 2005; Bhattacharya et al. 2006; Sandal et al. 2007).

In the present study, attempt has been made to reduce the caffeine level of a specific China hybrid tea clone (*Camellia sinensis* cv. Kangra jat) through RNA interference (RNAi). This selected tea clone is commercially utilized for making 'Kangra Tea' and is very popular in Himachal Pradesh state of India. For this, a partial cDNA fragment of caffeine synthase (CS) encoding gene was isolated. The isolated fragment of *CS* was employed to design a RNAi construct (pFGC1008-CS). The construct was used for transformation of somatic embryos of the same tea clone with biolistic approach. The transformed embryos were analyzed initially for *CS* expression as well as caffeine content and later regenerated transgenic tea plants were analyzed for caffeine and theobromine contents in addition to *CS* expression.

Materials and methods

Plant materials

Mature green fruits were collected from a China hybrid tea [*Camellia sinensis* (L.) O. Kuntze] cv. Kangra jat, growing in the Banuri Tea Experimental Farm of IHBT at Palampur (Himachal Pradesh, India). Farm is situated at 32°N and 76°E, and 1,230 m above sea level. After removal of fruit coats, seeds were dipped in water for overnight as described earlier (Mondal et al. 2001a). Only sinkers were used for removal of seed coat. Cotyledons from such seeds were washed with Tween-20 for 5 min and were rinsed for 2–3 times with double-distilled water. Washed cotyledons were treated with tetracycline (0.02%), ampicillin (0.02%), streptomycin (0.04%) and 2–3 drops of Tween-20 for 25 min and then rinsed for 5–6 times with double-distilled water. Thereafter, washed cotyledons were surface-sterilized with 4% calcium hypochlorite solution for 10 min under sterile conditions. These were then washed with sterile double-distilled water, dried on sterile filter paper and used as explant for somatic embryos development.

Production of tea somatic embryos

Surface-sterilized cotyledons were inoculated in 1/2MS (Murashige and Skoog) media for proliferation of embryonal axis. Their embryonal axis was removed and de-embryonated cotyledons were used for aseptic inoculation on same media for the induction of primary somatic embryos. Secondary somatic embryos were produced from the primary embryos by inoculating them in betaine (1 g l^{-1}) and ABA (7.5 mg l^{-1}) containing media (Akula et al. 2000). However, synchronous and normal development of secondary embryos was obtained on secondary embryogenesis media (SEM). In SEM, optimal concentrations of plant growth regulators such as 2 mg l^{-1} of 6-BAP and 0.2 mg l^{-1} of IBA, L-glutamine (1 g l^{-1}), nitrates, and salts of potassium sulphate (300 mg l^{-1}) were used together (Mondal et al. 2001b). These secondary somatic embryos were maintained in culture laboratory conditions of 25 ± 2°C and 16 h light photoperiod. Also, the light intensity of 70 µmol (photons) m^{-2} s^{-1} and air humidity of 55 ± 5% was maintained. Tea somatic embryos were regularly subcultured after every 30 days for their maintenance. The globular somatic embryos having high potential for repetitive embryogenesis and conversion were used as explants for transformation.

RNAi construct preparation

A partial 376 bp cDNA fragment of caffeine synthase gene (Accession number: FJ554589) was fished out from Kangra jat tea cultivar using forward primer 5′-CACAAACAGT GACCTCAATGAC-3′ and reverse primer 5′-CAGGAAT GAACTAAATGCAAGC-3′. This fragment was used in designing RNAi construct for silencing caffeine synthase gene. It was amplified using primers containing restriction sites for *Asc*I at 5′ and *Swa*I at 3′ end to clone it in sense direction and using primers containing restriction sites *Spe*I at 5′ and *Bam*HI at 3′ end to clone it in antisense direction into pFGC1008 vector. The resulting pFGC1008-*CS* construct was confirmed through PCR. A 360 bp Gus-intron is harbored between these sense and antisense fragments as a spacer making it an intron-containing hairpin RNA (hpRNA) construct. The pFGC1008-*CS* construct contained CaMV 35S promoter and octopine synthase (OCS) terminator. The construct also contained hygromycin resistance gene as a plant selectable marker.

Transformation of tea somatic embryos with RNAi construct and their regeneration

For transformation, microprojectile bombardment of tea somatic embryos was conducted. The pFGC1008-*CS*

construct and pFGC1008 vector (as control) were used for microprojectile bombardment. The plasmid DNA was isolated using the QIAfilter Plasmid Maxi Kit (QIAGEN, Germany) as per manufacturer instructions. The unbombarded somatic embryos and embryos bombarded with naked gold particles served as negative controls. Gold particles (1.0 μm; Bio-Rad, Hercules, California, USA) were coated with 10 μg of pure plasmid DNA and used for particle bombardment of the globular tea somatic embryos using biolistic PDS-1000/He System (Bio-Rad). The somatic embryos placed on agar-solidified SEM were bombarded using a burst pressure of 1,100 psi, 9 cm target distance, 1/4 + 3/8 inches gap distance, and a constant macrocarrier flight distance of 16 mm. In addition, a constant vacuum of 28 inches of mercury within biolistic chamber was selected as speed of microprojectiles. After bombardment, plates containing somatic embryos were turned at 180° and bombarded again with the same parameters for better work efficiency. The bombarded somatic embryos were left undisturbed under diffused light for healing effect. After 7–10 days, these were cultured on SEM augmented with hygromycin at different concentrations (20, 30, 40, 50, and 60 mg l^{-1}). The tea transformants including positive control (somatic embryos transformed with pFGC1008 vector only) were selected continuously on 40 mg l^{-1} hygromycin containing media. For their maturation, the globular-shaped secondary somatic embryos were maintained on maltose (4%) and trans-cinnamic acid (3 mg l^{-1}) containing MS medium for 8 weeks period. After maturation, somatic embryos were shifted to 1.5 mg l^{-1} GA_3 containing MS medium for 6 weeks period for their germination. After germination of tea transformants, they were maintained on SEM augmented with selection agent continuously up to their regeneration into tea plants. Nine months old transgenic plants were used for various analysis. Topmost two leaves and a bud of tea plants were used for expression studies and caffeine and theobromine content estimations.

Semiquantitative-PCR for caffeine synthase transcript expression analysis

One hundred mg tissue from transformed tea somatic embryos as well as regenerated plants was ground in liquid nitrogen for total RNA isolation using RNeasy Plant Mini Kit (QIAGEN, Germany). The cDNA was prepared according to manufacturers protocol (Invitrogen, USA) using 1 μg of total RNA from each sample, 250 ng Oligod$T_{12–18}$, 200U of Superscript III RT and 10 mM dNTPs in a 20 μl reaction volume. Equal quantity of cDNA was used as template in PCR with caffeine synthase gene specific primer set (forward 5′-CACAAACAGTGACCTCA ATGAC-3′ and reverse 5′-CAGGAATGAACTAAATGC AAGC-3′) to check the transcript expression levels in transformed somatic embryos and regenerated microshoots. After standardization, 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- 25 s for 25 cycles and amplified product was separated on 1% agarose gel. Level of 26S rRNA expression in each sample amplified with standard primers was used as an internal control (Singh et al. 2004). The mean values in the presented data were calculated from at least three independent measurements per sample.

Detection of siRNAs in tea plants

Total RNA (60 μg) including small RNAs was isolated from young tissues (topmost two leaves and a bud) of two putative transgenic plants and a wild tea plant with miRNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. High molecular weight RNAs were removed from the total RNA by precipitation with 20% PEG-8000 in 2 M NaCl. Small RNAs in the supernatant were precipitated using isopropanol as described earlier (Goto et al. 2003). These small RNAs were resolved on 18% polyacrylamide/7 M urea gel and transferred onto Hybond N^+ nylon membrane (Ambion) by semidry electroblotting. The digoxigenin-labeled antisense *CS* riboprobe was prepared and used for hybridization with small RNAs on the blot at 37°C in the DIG Easy Hyb solution (Roche). The membrane was washed twice with 0.2× SSC and 0.1% SDS at room temperature and then twice at 50°C as described earlier (Tomita et al. 2004). The chemiluminescent detection of signals was performed according to the manufacture's instructions (Dig Northern Starter Kit, Roche).

Quantification of caffeine and theobromine contents

The caffeine and theobromine were extracted and measured following the HPLC method (Sharma et al. 2005). The tissue (topmost two leaves and a bud) from tea transformants was dried constantly at 80°C. Three g of each dried sample was used for caffeine and theobromine extraction with 70% methanol. The caffeine and theobromine contents were estimated by Merck Hitachi HPLC (Darmstad, Germany) using C18 Lichrocart column (250 × 4 mm × 5 μm). The absorbance was read at 210 and 280 nm, respectively for caffeine and theobromine. Caffeine and theobromine contents were calculated from standard curves prepared with pure caffeine and theobromine (Sigma). The mean values in the presented data were calculated from at least three independent measurements per sample.

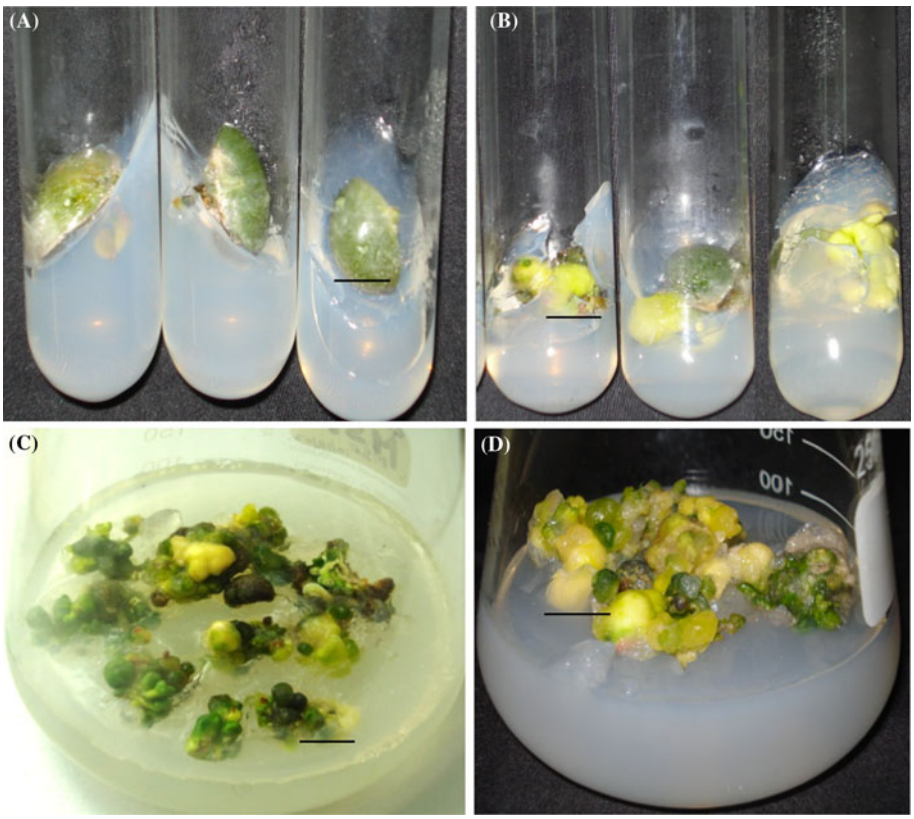

Fig. 2 Production of somatic embryos. **a** Inoculation of *Camellia sinensis* (L.) O. Kuntze cv. Kangra jat cotyledons in 1/2MS media for the proliferation of embryonal axis. **b** and **c** Production of somatic embryos from de-embryonated cotyledons in betaine (1 g/l) and ABA (7.5 mg/l) containing media. **d** Somatic embryos maintained on the somatic embryogenesis medium. *Scale bars* indicate 1 cm

Results

Establishment of tea somatic embryos for transformation

We followed the similar protocol, which was developed earlier for the establishment of tea somatic embryos and regeneration system with little modifications. For this, surface-sterilized fresh tea cotyledons were inoculated in 1/2MS media for the proliferation of embryonal axis (Fig. 2a). After 20 days, embryonal axis was removed. The de-embryonated cotyledons were employed in the same media for the induction of primary somatic embryos (Fig. 2b). These embryos were transferred to the media containing high concentrations of betaine and ABA for secondary somatic embryos formation. In this media, after 3–4 months secondary somatic embryos were directly formed from the epidermal tissues of the initial embryos without callus formation (Fig. 2c). These somatic embryos were further multiplied and maintained on the somatic embryogenesis media (SEM) (Fig. 2d).

Transformation of tea somatic embryos with hpRNAi construct (pFGC1008-CS) and their regeneration

A 376 bp cDNA fragment specific to caffeine synthase (*CS*) gene was isolated from tea (*Camellia sinensis* (L.) O. Kuntze cv. Kangra jat). Sequence of this partial fragment is shown in Fig. 3a. This cDNA fragment was used to make a hpRNAi construct as described under materials and methods (Fig. 3b). The developed pFGC1008-*CS* construct and pFGC1008 vector alone as a control were used to transform tea somatic embryos through biolistic method.

Somatic embryos were cultured on SEM augmented with hygromycin. For plant selection, it was observed that a hygromycin concentration of 50 mg/l or more was lethal to tea somatic embryos (data not shown). Therefore, 40 mg/l hygromycin dose was used for the selection during tea somatic embryos transformation and regeneration studies. Within 3 months of culture, the untransformed calluses were turned brown and died. However, putative transformants were able to proliferate and survived further on applied selection dose (Fig. 4a). The hygromycin resistant tea transformants were matured and germinated on maturation media and germination media combinations respectively as described in materials and methods (Fig. 4b and c). After 5 months of transformation, tea microshoots were started to emerge from the epidermal surface of each of the putative transformant somatic embryos on the same media SEM (Fig. 4d and e). On the same media combination, small tea plants were developed from these transformants (Fig. 4f). Seventeen PCR confirmed transgenic tea plants were obtained in this study. However, six plants could not produce rooting and thus could not survive. The

(A) CAC AAA CAG TGA CCT CAA TGA CAA TGCCAG TGC TAG AAA CTG CAG TTG AAA CCC TCT TCT CCA AAG ATT TCC ACC TTC ATC AAG CTC TTA ATG CAG TGG ACT TGG GTT GTG CAG CAG GTC CAA CCA CGT TCA CAG TGA TTT CTA CAA TCA AGA GAA TGA TGG AAA AGA AAT GCA GGG AAT TGA ATT GCC AAA CAC TGG AAC TTC AGG TTT ACT TGA ATG ATC TTC CTG GAA ACG ATT TCA ATA CCC TCT TCA AAG GCC TGC AAT CTA AGG TTG TTG GTA ACA AAT GTG AGG AAG TTT CTT GTT ATG TGG TGG GAG TAC CGG GGT CTT TCC ATG GCC GGC TTT TTC CTC GTA ACA GCT TGC ATT TAG TTC ATT CCT G

(B)

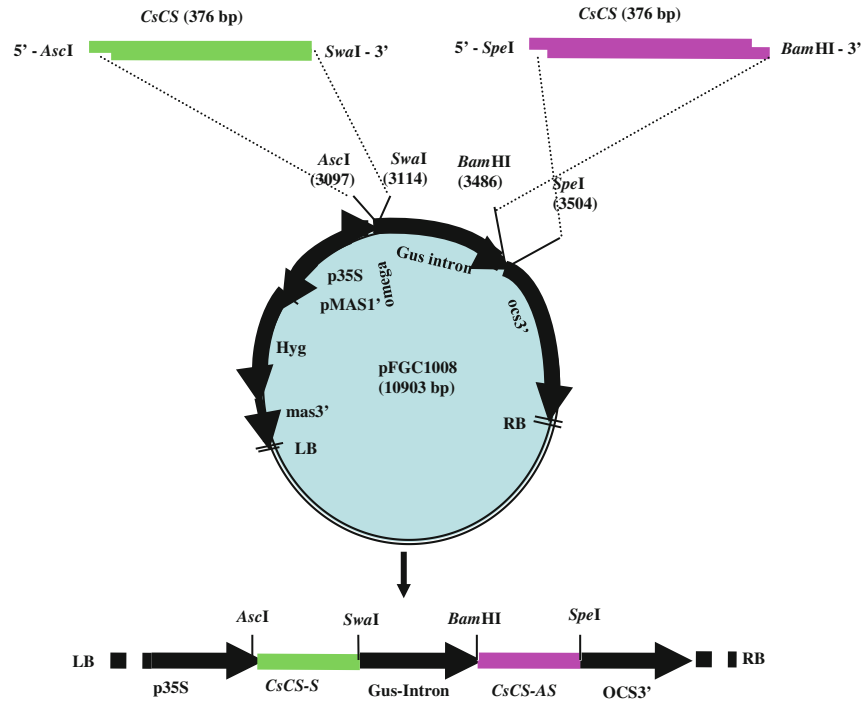

Fig. 3 **a** Sequence of a partial fragment of *CS* cDNA used for RNAi construct preparation. **b** Schematic representation of a RNAi construct prepared for silencing *Camellia sinensis* caffeine synthase (*CS*). A partial fragment of *caffeine synthase* (376 bp) was cloned in sense (CS-S) at *Asc*I and *Swa*I restriction sites and in antisense (CS-AS) at *Bam*HI and *Spe*I restriction sites of pFGC1008 vector. Resulting RNAi vector harbored Gus-intron in between sense and antisense fragments of *CS*

11 PCR confirmed tea plants (transformed with RNAi construct) along with control tea plants (untransformed) were shifted to green house conditions (Fig. 4g and h). Morphologically, the transgenic tea plants were similar to the control plants.

CS expression and caffeine content analysis in somatic embryos after transformation with hpRNAi construct

To check the effectiveness of silencing at an early stage, transformed somatic embryos on the selection medium were analyzed for *CS* expression and caffeine content after 7, 9 and 11 days of transformation (Fig. 5). Somatic embryos transformed with pFGC1008 vector alone, having no *CS* gene fragment as insert, were used as control for the analysis. Control sample was analyzed for *CS* expression and caffeine content only after 11 days of transformation. A significant decrease of 75% in *CS* transcript levels was observed after 7 days in pFGC1008-CS transformed somatic embryos compared to that of transformed with pFGC1008 alone (Fig. 5a and b). After 9 and 11 days of transformation, the *CS* transcript expression level was decreased up to 90%. Results documented that the partial fragment of *CS* gene used in making gene silencing vector and employed for somatic embryos could successfully reduce the *CS* expression levels.

The caffeine content was also monitored in transformed embryos. Caffeine content was measured as 6.24 mg g^{-1} DW (41% reduction) in somatic embryos after 7 days of transformation with pFGC1008-*CS* construct compared to embryos transformed with pFGC1008 vector alone. Caffeine content was further decreased to 3.64 mg g^{-1} DW (65% reduction) and 3.12 mg g^{-1} DW (70% reduction) in somatic embryos after 9 and 11 days of transformation, respectively (Fig. 5c). However, the caffeine content in control was measured as 10.41 mg g^{-1} DW. This has suggested that low level of *CS* transcript expression due to its silencing reduced the caffeine content in somatic embryos.

CS transcript expression analysis in transgenic plants

Out of 11 tea transgenic lines, three confirmed transgenic lines (K5b, K7b and K8a) and one positive control (C–I; transformed with pFGC1008 vector alone) and an untransformed tea plant (C–II) were finally used to analyze

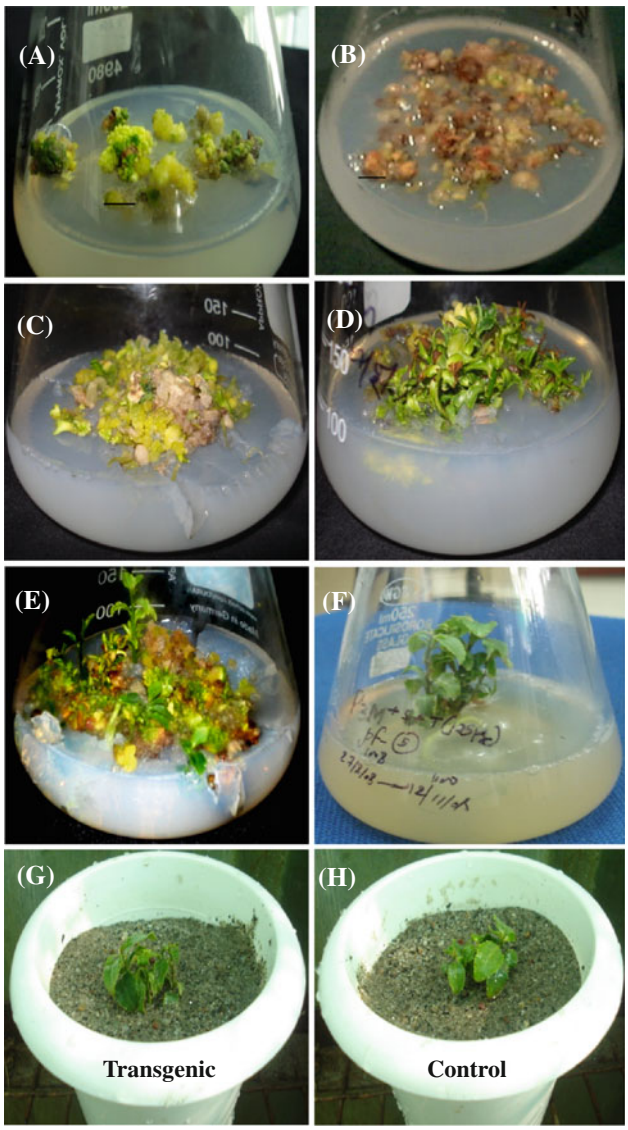

Fig. 4 Transformation of tea somatic embryos and their regeneration. **a** Globular tea somatic embryos were transformed with RNAi construct (pFGC1008-*CS*) and pFGC1008 vector (as control) via particle bombardment. The transformed somatic embryos were selected on hygromycin (40 μg ml^{-1}) containing somatic embryogenesis media. **b** Maturation of transformed tea somatic embryos on maltose containing media. **c** Transformed mature tea somatic embryos were shifted to GA_3 containing media. **d**, **e** Germination of transformed tea somatic embryos into microshoots. **f** Putative transformants were regenerated into tea plantlets. **g** Tea plantlet (regenerated from somatic embryos transformed with pFGC1008-*CS*) produced were grown under green house conditions. **h** Tea plantlet (regenerated from somatic embryos transformed with pFGC1008) produced were grown under green house conditions. *Scale bars* in (**a**) and (**b**) indicate 1 cm, respectively

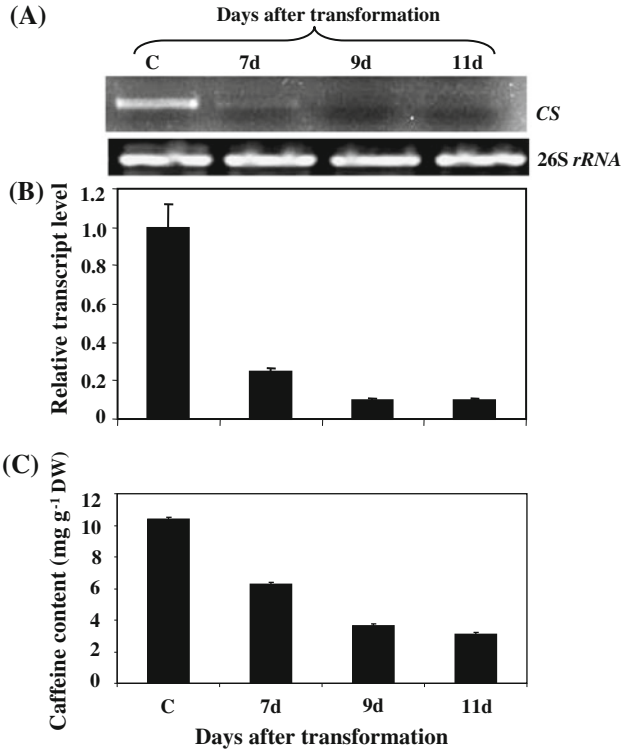

Fig. 5 Characterization of tea somatic embryos transformed with RNAi construct. **a** Semiquantitative-PCR indicating the decrease in expression levels of *Camellia sinensis* caffeine synthase (*CsCS*) transcript. Tea somatic embryo transformed with pFGC1008 vector alone was used as control (C). As an internal control, transcripts expression of 26S *rRNA* was simultaneously measured. **b** *Bar diagram* represents the relative *CS* transcript levels quantified densitometrically. **c** Caffeine content measured after 7, 9 and 11 days of transformation in somatic embryos

the primary effects of *CS* gene silencing. The expression analysis was performed in tea plants after 9 months of their regeneration. The obtained transgenic plants showed similar morphological characteristics as that of a wild type tea plant. Three selected transgenic plants were examined for the efficacy of gene silencing by estimating the levels of *CS* transcript. Reduction in the level of *CS* transcript expression was observed in all three transgenic plants as compared to control tea plants (C–I and C–II) (Fig. 6a). The expression of *CS* transcript was equal in both the control, a vector alone transformed and untransformed tea plants. However, PCR confirmed transgenic microshoots showed a differential reduction of *CS* transcript expression. Out of three transgenic plants, K7b plant showed highest reduction (Fig. 6b). The reduction in *CS* expression of K7b plant was 78% compared to control. The other two plants K5b and K8a showed 55% and 51% reduction in *CS* transcript expression, respectively. The corresponding bands in the gels indicating reduction in the expression levels were sequence confirmed and found to be specific to *CS*. These results suggest that a partial *CS* gene sequence used for caffeine synthase silencing is effective in post-transcriptional down regulation of its endogenous expression in transgenic plants.

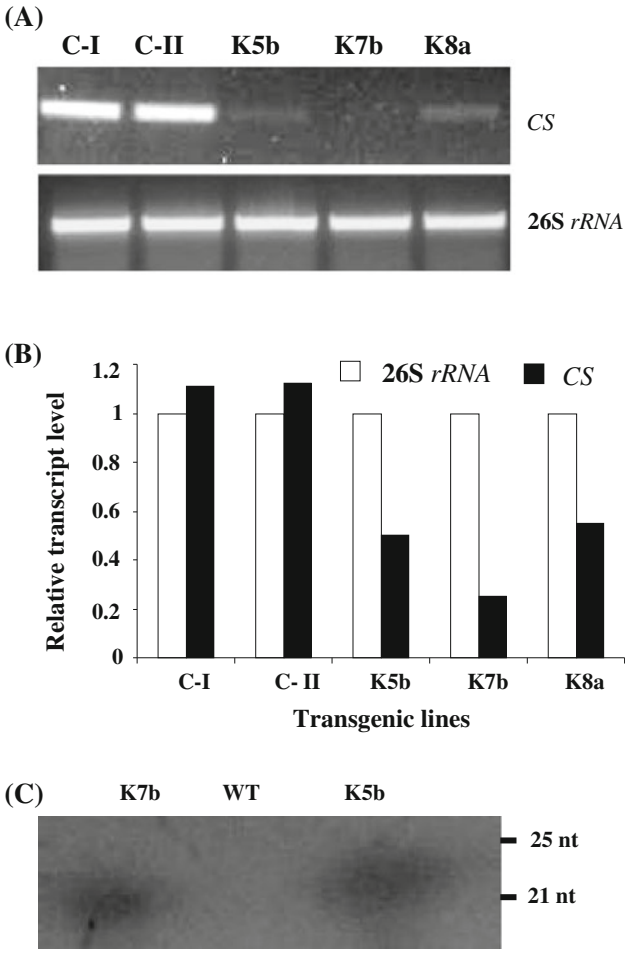

Fig. 6 Caffeine synthase (CS) transcript expression and siRNA accumulation in transgenic plants. **a** CS transcript expression levels analyzed through semiquantitative-PCR in tea plants. Three different lines K5b, K7b and K8a transformed with pFGC1008-*CS* and C–I (transformed with pFGC1008) and C–II (untransformed wild) as controls were analyzed. As an internal control, transcripts expression of 26S *rRNA* was simultaneously measured. **b** *Bar diagram* represents the relative CS transcript levels quantified densitometrically in transgenic microshoots as well as control tea plants. **c** Small interfering RNAs (siRNAs) in transgenic plants (K7b and K5b) and wild tea plants were analyzed through northern blot using *CS* gene specific probe. Transgenic plants showed siRNAs at size between 21–25 nt

Detection of siRNAs specific to CS in transgenic plants

To validate the hypothesis that reduction in *CS* expression was through small interfering RNA (siRNA) mediated gene silencing, siRNA specific to *CS* was determined in confirmed transgenic tea plants. siRNAs specific to *CS* were monitored in two transgenic plants K7b and K8a vis a vis untransformed wild type tea plant. Small RNA fraction was extracted from young tissues of each of the two transgenic plants and a wild type plant. It was separated by denaturing PAGE, blotted to the nylon membrane and hybridized with a Dig labeled *CS* probe. Hybridization signals in the size range of 21-25 bp were detected in the silenced plants and not in the wild-type plant (Fig. 6c). This result documented the formation of siRNA from the cloned *CS* gene fragment after transformation in regenerated transgenic plants. The reduction in *CS* transcript expression further suggests the involvement of siRNA in posttranscriptional silencing in the developed transgenic plants.

Reduction in caffeine and theobromine content of transgenic plants

The silencing effect of *CS* was monitored on the caffeine and theobromine contents in the developed transgenic plants. The caffeine content in transgenic lines K5b, K7b and K8a was measured as 20.8 mg g^{-1} DW (47% reduction), 15.5 mg g^{-1} DW (61% reduction) and 23.1 mg g^{-1} DW (44% reduction) respectively, compared to that of controls (39.1 mg g^{-1} DW) (Fig. 7a). Similarly, theobromine content in transgenic lines K5b, K7b and K8a was measured as 0.128 mg g^{-1} DW (46% reduction), 0.079 mg g^{-1} DW (67% reduction) and 0.098 mg g^{-1} DW (60% reduction) respectively, compared to that of controls (0.238 mg g^{-1} DW) (Fig. 7b). Out of these three transgenic lines, K7b showed maximum reduction in caffeine and theobromine contents, documenting the correlation in reduction with *CS* transcript expression data (Fig. 6a).

Stable CS silencing as well as reduced caffeine and theobromine content in older transgenic plants

The stability of CS silencing was tested through stem cutting method. In this, tissue culture raised plants were cut into segments in such a way that each segment contained a node and internode. These segments were grown in medium to regenerate into full plants. After 1 year and 9 month, these plants were tested for caffeine and theobromine content. A total of eight lines were developed. This number included the previous three lines (K5b, K7b and K8a) and five new lines developed through stem cuttings (K2, K3, K4, K5 and K6). The caffeine content of K5b, K7b, K8a, K2, K3, K4, K5 and K6 lines was measured as 20.99 mg g^{-1} DW (46.3% reduction), 15.91 mg g^{-1} DW (59.3% reduction), 21.72 mg g^{-1} DW (44.43% reduction), 23.59 mg g^{-1} DW (39.66% reduction), 20.85 mg g^{-1} DW (46.67% reduction), 21.94 mg g^{-1} DW (43.87% reduction), 22.08 mg g^{-1} DW (43.52% reduction), and 23.35 mg g^{-1} DW (40.27% reduction) respectively, compared to that of controls (39.1 mg g^{-1} DW) (Fig. 8a). Similarly, theobromine content of K5b, K7b, K8a, K2, K3, K4, K5 and K6 lines was measured as 0.130 mg g^{-1} DW (45.2% reduction), 0.079 mg g^{-1} DW (66.4% reduction), 0.097 mg g^{-1} DW (59.31% reduction), 0.118 mg g^{-1} DW (50.22% reduction), 0.123 mg g^{-1} DW (48.06% reduction), 0.121 mg g^{-1} DW

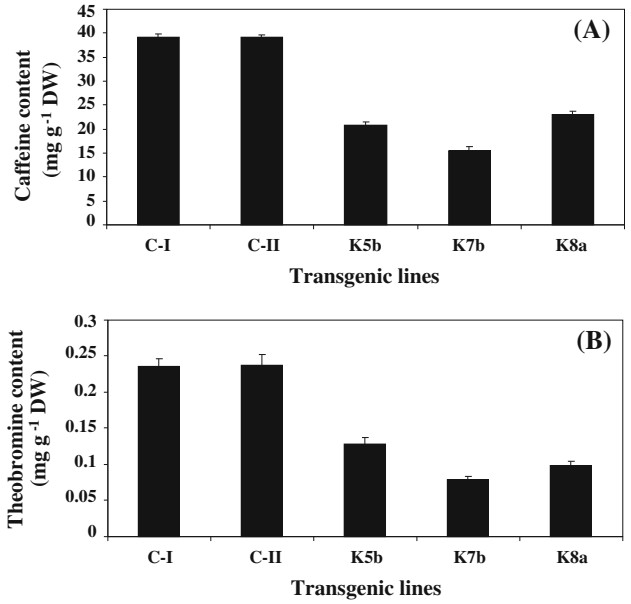

Fig. 7 Caffeine and theobromine contents in transgenic plants of *Camellia sinensis*. **a** Caffeine content was estimated through HPLC in K5b, K7b, and K8a transgenic plants transformed with pFGC1008-*CS*, whereas C–I (transformed with pFGC1008) and C–II (untransformed wild) were used as controls. **b** Theobromine content was also estimated through HPLC. The pure caffeine and theobromine were used as standards in the HPLC measurements

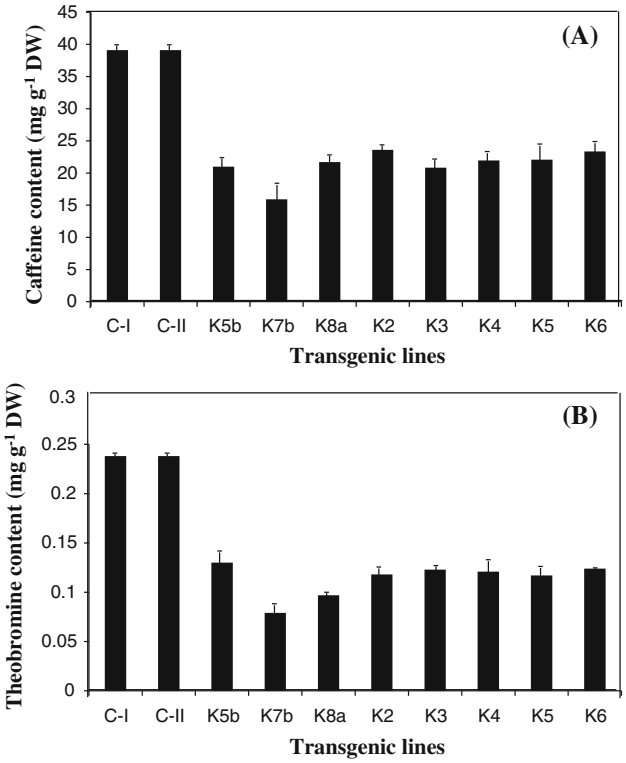

Fig. 8 Caffeine and theobromine contents in older transgenic plants of *Camellia sinensis* developed through stem cuttings. **a** Caffeine content was estimated through HPLC in 1 year and 9 month old K5b, K7b, K8a, K2, K3, K4, K5 and K6 transgenic plants transformed with pFGC1008-*CS*, whereas C–I (transformed with pFGC1008) and C–II (untransformed wild) were used as controls. **b** Theobromine content was also estimated through HPLC. The pure caffeine and theobromine were used as standards in the HPLC measurements

(48.92% reduction), 0.117 mg g^{-1} DW (50.65% reduction), and 0.124 mg g^{-1} DW (48.06% reduction) respectively, compared to that of controls (0.238 mg g^{-1} DW) (Fig. 8b). Lower levels of caffeine and theobromine content in these transgenic lines produced through stem cutting documented the stable *CS* silencing character.

Discussion

Recently, we have revealed the possible strategies for caffeine reduction in tea (*Camellia sinensis* L.). Apart from industrial and breeding techniques, genetic engineering appears to be promising strategy. Later involved both either overexpression of caffeine degradation pathway genes or silencing of a regulatory step of caffeine biosynthesis pathway (Yadav and Ahuja 2007; Mohanpuria et al. 2010). These modern techniques of plant manipulation need the development of transgenic plants. In order to develop any transgenic plants, two requirements had to be fulfilled: isolation of a gene of interest and establishment of an efficient transformation and regeneration systems.

In tea, caffeine degradation pathway is not yet known. Therefore, we preferred to go with RNAi for the reduction of caffeine levels in tea. Further, the RNAi based on hpRNA strategy has been reported as more efficient for gene silencing (Smith et al. 2000). Also methods are available for transformation and regeneration in tea (Lopez et al. 2004; Bhattacharya et al. 2006; Sandal et al. 2007). Our recent study has documented the regulatory role of caffeine synthase (CS) in caffeine biosynthesis of tea (Mohanpuria et al. 2009). Therefore using the available information and known sequence for caffeine synthase from a tea clone (Kato et al. 1999), we successfully cloned a partial cDNA fragment for CS from the selected Kangra jat tea clone to suppress caffeine synthesis.

During the last one decade, tea improvement using *Agrobacterium* and biolistic-mediated approaches has gained a significant insight by several workers. Also, somatic embryogenesis was considered as one of the most worked out regeneration system. Later was considered as more efficient and uniform in tea as compared to other regeneration systems. Despite to the fact that tea is recalcitrant to the *Agrobacterium*-mediated transformation, first healthy transgenic tea plant was produced through this method only (Mondal et al. 2001a). After that, several workers have attempted *Agrobacterium*-mediated genetic transformation of tea using different explants (Lopez et al.

2004; Jeyaramraja and Meenakshi 2005; Bhattacharya et al. 2006; Sandal et al. 2007). Though tea transformation via *Agrobacterium* is a more specific to integrate the transgene into host genome compared to biolistic, but it is a very laborious and difficult method. The biolistic-mediated approach has also been optimized for the transformation of tea (Bhattacharya et al. 2006). Keeping in view the transformation protocols in tea, we have attempted here for the first time production of transgenic tea plants with suppressed caffeine synthesis using biolistic mediated approach.

An isolated partial *CS* gene specific fragment of 376 bp was used in making RNAi construct (pFGC1008-CS). The developed construct was used for transformation of somatic embryos of *Camellia sinensis* cv. Kangra jat following biolistic approach. Tea transformants were observed with suppressed endogenous caffeine synthase gene expression (maximum up to 90% reduction) and reduced caffeine contents (maximum up to 70% reduction) among initial stages. This has suggested that the partial cDNA sequence used in the present RNAi strategy for silencing caffeine synthase gene was found effective in regulating the caffeine production.

The regenerated transgenic plants from somatic embryos showed similar morphology to that of plants regenerated from untransformed somatic embryos. They were observed for reduced expression of *CS* and for reduction of 44–61% in caffeine and 46–67% in theobromine contents compared to controls. Thus, present study provides evidence for successful production of a low caffeine tea with 70% caffeine reduction in transformed somatic embryos and 61% in transgenic plants, respectively. Since tea takes 5–6 years to set seeds from the tissue culture raised plants, stability of *CS* silencing character was analyzed through stem cuttings. The reduction in caffeine (39.66–59.3%) and theobromine (45.2–66.4%) content of stem cutting raised plants documented the stable *CS* silencing character in tea plants. Importantly, the possible strategy to develop low-caffeine cultivars from the transgenic lines developed in this study could be through maintaining them in containment facility and selecting up to T2 generation for CS silencing character.

However, the caffeine content of coffee plants was reduced to 70% through employing the same technique of RNAi (Ogita et al. 2003). In transgenic plants, caffeine was not reduced completely. This could either be due to the efficiency of RNAi construct in tea or due to the possible existence of other minor or bypass pathway(s) of caffeine biosynthesis. Such minor pathways might be activated upon the blockage of major pathway. The production of caffeine from 7-methylxanthine via paraxanthine has been documented as one of the important minor pathway for caffeine synthesis. But the enzymes catalyzing steps of this minor pathway are not known (Kato et al. 1998). The limited synthesis of paraxanthine from 7-methylxathine in tea could be due to its less methyl acceptor potential (Kato et al. 1996). In addition, the formation of caffeine from paraxanthine is 10 times slower as compared to that of major caffeine biosynthesis pathway. In view of this, involvement of the minor pathways can not be ruled out.

This study of RNAi for the first time in tea delineated several important features of caffeine biosynthesis in tea through both transgenic embryogenic tissues and regenerated plants. The known dual functionality of CS was found to be relevant as the transgenic plants showed reduction of theobromine in addition to the final product, caffeine. This has documented the suppression of last two steps in caffeine biosynthesis. Therefore, similar to coffee theobromine could be the major intermediate in caffeine biosynthesis, as its reduction was generally proportional to the reduction of caffeine contents in tea. Caffeine and theobromine have some biological roles like allelopathy and chemical defense in the growing tea plants. Therefore, complete removal of such methylxanthines would not be acceptable in tea plant. This is justified by several facts; first, it would make the transgenic tea plants more susceptible to the herbivores; second, such tea plants might produce low quality tea drink for humans; and third, it would reduce the survival ability of transgenic tea plants among existing biodiversity on this earth. Therefore, some amount of these secondary metabolites should be present in the genetically modified crops like tea. Through different methods, the maximum reduction in caffeine levels to 4 mg g^{-1} has been reported for decaffeinated leaf teas and 10 mg g^{-1} for decaffeinated instant teas (Ye et al. 2007). However, in this study maximum reduction from 39.1 mg g^{-1} DW to 15.5 mg g^{-1} DW was observed in one of the three transgenic tea plants K7b.

In conclusion, data of this study provides evidence for the successful production of a low-caffeine phenotype in a Kangra jat tea (*Camellia sinensis*) clone. Both transformed somatic embryos and regenerated plants exhibited reduction in their *CS* expression and caffeine content. The produced plants were morphologically similar and at maturity, they are expected to produce essentially normal tea except for low theobromine and caffeine content. The degree of caffeine reduction and their effects on the tea production in terms of quality and yield will be evaluated upon maturity. The stability and heritability pattern of this low caffeine and theobromine trait will also be studied in future.

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