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MATERIALS AND METHODS
MATERIALS AND METHODS

The present study was carried out at Division of Biotechnology, Institute of Himalayan Bioresource Technology (CSIR), Palampur (Himachal Pradesh).

3.1 Plant materials

Tea [Camellia sinensis (L.) O. Kuntze] was included for the scope of present study. Four tea cultivars, i.e., Kangra jat (KJ) and UPASI-9 (U9) belong to Chinary, Tocklai variety-23 (TV) belongs to Assamica, and Tocklai germplasm-270/2/13 (TG) belongs to Cambod tea varieties were selected for the present study. These cultivars were maintained under uniform environmental conditions at the Tea Experimental Farm, IHBT Palampur located at 1290 msl, 32°N and 76°E.

3.2 Isolation and identification of a partial fragment of caffeine synthase cDNA

Based upon the sequence homology from various genes encoding N-methyltransferases of caffeine biosynthesis pathway in tea and coffee available in GenBank at National Center for Biotechnology Information (NCBI), a set of gene specific primers was designed to amplify a partial fragment of caffeine synthase gene (CS) from tea (Camellia sinensis L.).

3.2.1 RNA isolation

Total RNA was isolated from young leaves of tea cultivars belonging to Chinary (KJ), Assamica (TV), and Cambod (TG) varieties using QIAGEN RNeasy Plant Mini Kit as described below:

1. For RNA isolation all the plastic wares and glass wares were treated with 0.1% DEPC for overnight and autoclaved for 20 min at 1.1 kg/cm² and 121°C. The pestle-mortar was baked at 300°C for 4 h incubation before its use for grinding the sample.
2. One hundred mg plant tissue was ground thoroughly into mortar and pestle in liquid nitrogen.
3. The tissue powder was put into a 2 ml microcentrifuge tube containing 450 µl of Buffer RLT (contains guanidine thiocyanate) and 5 µl of β-Mercaptoethanol. It was vortexed vigorously and incubated for 3 min at 56°C.
4. The lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 ml microcentrifuge tube and centrifugation was done for 2 min at 13000 rpm.
5. The supematant of the flow-through was carefully transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in microcentrifuge tube.

6. The 0.5 volume of ethanol (100%) was added to the cleared lysate and mixed immediately.

7. The above sample was transferred to an RNeasy spin column (pink) placed in a 2 ml microcentrifuge tube and centrifuged for 15 s at 10000 rpm.

8. After removal of flow-through, 700 µl of Buffer RW1 (contains ethanol) was added to the RNeasy spin column and centrifuged as described in step 7.

9. After removal of flow-through, 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged as described in step 7.

10. The above step was repeated once more except for 2 min time period. After removal of flow-through, empty spin was done at 13000 rpm for 1 min.

11. RNeasy spin column was transferred to a fresh collection tube. 30 µl of RNase-free water was added to the centre of the membrane and incubated for 1 min. Purified RNA sample was obtained by centrifugation at 10000 rpm for 1 min.

12. The isolated RNA sample was stored at -70°C until its use.

13. The isolated total RNA was resolved on formaldehyde agarose gel [containing 1 part formaldehyde solution, 3.5 part agarose (1%) in DEPC water, and 1.1 part 5x formaldehyde gel running buffer (5x FRB; Appendix-I)] using electrophoresis unit (Amersham Biosciences, USA). The electrophoresis was carried out at 70 V for 2-3 h. The gel was stained in ethidium bromide solution.

14. The quality of total RNA was checked by visualization of ribosomal genes bands in UV light. The isolated RNA was quantified in ND-1000 UV/Vis Spectrophotometer (NanoDrop Technologies, USA).

3.2.2 First strand cDNA synthesis

The cDNA was prepared in 20 µl reaction volume using 2 µg of total RNA from each sample as described below:

1. Two µg of total RNA was added with 250 ng of OligodT$_{12-18}$ and 1 µl of 10 mM dNTPs to make total volume of 14.5 µl.

2. The mixture was incubated at 65°C for 5 min. It was then chilled on ice for 1 min.

3. The above mixture was added with 4 µl of 5x First-Strand Buffer, 1 µl of 0.1 M DTT, and 100 units of SuperScript III Reverse Transcriptase (Invitrogen, USA).
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4. All components were gently mixed and incubated at 50°C for 60 min for cDNA synthesis.

5. After cDNA synthesis, the enzyme was heat killed by incubating the reaction mixture at 70°C for 15 min. The reaction mixture was incubated at ice for 2 min and then it was stored at -20°C.

3.2.3 PCR amplification

Equal quantity of cDNA from each sample was subjected to PCR using QIAGEN Taq DNA polymerase for amplification of the gene(s) of interest as described below:

1. Various components of PCR were pipetted in 1.5 ml microcentrifuge tube in the following sequence: autoclaved ddH₂O-19.8 µl, 10x Taq buffer-2.5 µl, forward primer (10 µM)-0.5 µl, reverse primer (10 µM)-0.5 µl, dNTPs mixture (2.5 mM each)-1.0 µl, Taq DNA polymerase (5 U/µl)-0.1 µl, Template-1.0 µl (cDNA).

2. For amplifying caffeine synthase cDNA fragment(s), the gene specific primers (forward primer: 5'-CACAAACAGTGACCTCAATGAC-3' and reverse primer: 5'-CAGGAATGAACCTAAATGCAAGC-3') were synthesized from Sigma-Aldrich, USA.

3. Amplification of caffeine synthase cDNA was carried out in a thermo cycler (BIO-RAD). 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 72°C for 7 min.

4. The amplified product was separated on 1% agarose gel in 1x TAE (Tris-acetate buffer (10x TAE; Appendix-I) and visualized with ethidium bromide staining (0.5 µg/ml) under ultraviolet light and photograph was taken in Alpha DigiDoc™ (Alpha Innotech Corporation, USA).

3.2.4 Caffeine synthase cDNA fragment elution

Using caffeine synthase gene specific primers a single 376 bp gene fragment was purified from the agarose gel using GenElute™ Gel Extraction Kit (Sigma-Aldrich, USA) as described below:

1. Gel slice containing DNA fragment of interest was excised from the agarose gel with a clean, sharp scalpel.
3. Three gel volume of Gel Solubilization Solution was added to the gel slice. The gel mixture was incubated at 55°C until the gel slice was completely dissolved. It was vortexed briefly in every 2-3 min during incubation.

3. GenElute binding column was prepared by adding 500 μl of Column Preparation Solution. It was centrifuged for 1 min. Flow-through was discarded.

4. The 0.6 volume of isopropanol was mixed in the above dissolved gel mixture. This mixture was loaded into the prepared binding column and centrifugation was done for 1 min. Flow-through was discarded.

5. DNA bound column was washed by adding 700 μl of Wash Solution and centrifugation was done for 1 min.

6. DNA binding column was transferred to a fresh collection tube. 30 μl of Elute Solution was added to the centre of the membrane and incubated for 1 min. Purified DNA was obtained by centrifugation for 1 min.

7. The eluted DNA was checked on 1% agarose gel and quantified in ND-1000 UV/Vis Spectrophotometer.

3.3 Cloning of CS cDNA fragment into sequencing vector and its sequence analysis

The gel purified CS cDNA fragment was ligated into pGEM-T Easy (Promega, USA) as sequencing vector and the ligated product was transformed into E. coli competent cells. Recombinant clones were isolated and their confirmation was done through sequencing.

3.3.1 Ligation

The isolated CS cDNA fragment was purified after PCR amplification and ligated in 3:1 ratio with the pGEM-T Easy vector. The ligation reaction was set up in a 10 μl volume containing 3 μl of PCR product, 1 μl of pGEM-T Easy vector (50 ng), 5 μl of 2x Rapid Ligation Buffer, and 1 μl of T4 DNA Ligase (3 Weiss units). The ligation reaction was carried out overnight at 4°C.

3.3.2 Transformation

The ligated product was transformed in Escherichia coli (E. coli) (DH5α) cells for further storage and multiplication. The transformation was performed as described below:

1. The frozen E. coli competent cells were thawed on ice for 5-10 min. The ligated product was mixed with competent cells. The mixture was incubated on ice for 30 min.
2. Heat shock treatment was given to the above mixture at 42°C for 1 min and it was shifted immediately on ice for 2 min.

3. One ml of Luria broth (LB) was added to the above mixture under sterile conditions and it was kept on shaking with 199 rpm at 37°C for 2 h.

4. The bacterial pellet was made by centrifugation at 4000 rpm for 10 min and spreaded on LB plate containing ampicillin (100 μg/ml). This plate was incubated at 37°C for overnight.

3.3.3 Confirmation of positive bacterial colonies

The transformed bacterial colonies were selected on ampicillin. The positive bacterial colonies were screened through colony PCR using the similar primers and PCR conditions as described in section 3.2.3 except the bacterial colony as template. The colony PCR products were separated on 1% agarose gel and stained with ethidium bromide. Gel photograph was documented.

3.3.4 Plasmid isolation

The plasmids were isolated from the positive colonies using GenElute HP Plasmid miniprep Kit (Sigma-Aldrich, USA) as described below:

1. An overnight grown bacterial culture of 5 ml was pelleted at 12000 rpm for 1 min. Supernatant was discarded.

2. The cells were resuspended in 200 μl of Resuspension Solution. It was mixed by pipette up and down.

3. Two hundred μl of Lysis Buffer was added to the above mixture and the microcentrifuge tube containing these mixtures was gently inverted to mix them. The mixture was kept for 5 min.

4. Three fifty μl of Neutralization Buffer was added to the above suspension and the mixture was gently inverted.

5. The cell debris was pelleted by centrifugation at 13000 rpm for 10 min.

6. Five hundred μl of Column Preparation Solution was added to Miniprep Binding Column kept in a microcentrifuge tube. It was centrifuged at 12000 rpm for 1 min. Flow-through liquid was discarded.

7. The cleared lysate from step 5 was transferred to the prepared column and centrifuged at 12000 rpm for 1 min. Flow-through was discarded.
8. Five hundred μl of Wash Solution 1 was added to the above column and centrifugation was done at 12000 rpm for 1 min. Flow-through was discarded.

9. Seven fifty μl of Wash Solution 2 was added to the above column and centrifugation was done at 12000 rpm for 1 min. Flow-through was removed.

10. Centrifugation was done at 12000 rpm for 1 min additional period to remove excess ethanol.

11. Binding column was transferred to fresh microcentrifuge tube and 50 μl of Elution Solution was added to the centre of the column. It was centrifuged at 12000 rpm for 1 min.

12. The isolated recombinant plasmid was checked on 0.8% agarose gel and quantified in ND-1000 UV/Vis Spectrophotometer.

3.3.5 Sequence analysis
Sequencing of CS cDNA fragment cloned into pGEM-T Easy vector was carried out using automated DNA sequencing system (Applied Biosystems model ABI Prism 310). The sequencing procedure followed is described below:

1. Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, USA) was used for sequencing reactions. The reaction was contained with 4 μl of Terminator ready reaction mix (containing Ampli Taq DNA Polymerase-FS), 200-500 ng of recombinant pGEM-T Easy, 1.66 pmol of M13 forward primer (5’-GTAAACGACGCTATGAC-3’) or M13 reverse primer (5’-CAGGAAACACGCTATGAC-3’).

2. Volume of reaction mix was adjusted to 10 μl with deionised H2O. The thin walled PCR tubes (0.2 ml) containing the reaction mix was placed in a thermo cycler (BIO-RAD) and PCR was carried out under the conditions of 94°C-10 s, 50°C-40 s, and 60°C-4 min for 25 cycles.

3. After the completion of above PCR reaction, the volume of reaction mix was raised to 100 μl with deionised water. To this 10 μl sodium acetate (3 M, pH 4.6) and 250 μl ethanol were added and centrifuged at maximum speed for 20 min. The pellet thus obtained was washed twice with 250 μl of 70% ethanol and air dried.

4. The pellet was dissolved in 15 μl of template suppression reagent (TSR, Applied Biosystems) thereafter it was denatured by heating at 95°C for 4 min and placed on ice before loading in the sequencer.
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5. The isolated 376 bp cDNA sequence of caffeine synthase from Chinary, Assamica, and Cambod tea varieties were confirmed through Basic Local Alignment Search Tool (BLAST) at NCBI.

3.4 Caffeine biosynthesis in different tea cultivars

The caffeine synthase transcript expression was analyzed in KJ and U9 belongs to Chinary, TV belongs to Assamica, and TG belongs to Cambod varieties of *Camellia sinensis* (L.) O. Kuntze. The study was conducted during the years 2006-2008. Various vegetative tissues *i.e.*, apical bud or 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) were collected from these tea cultivars during non-dormant (ND; Apr-Sep) and dormant (D; Oct-Mar) 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 CS gene expression as well as caffeine and allantoin contents. In addition, fruits of these tea 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 occur once in a year.

3.4.1 RNA isolation and caffeine synthase transcript expression analysis

One hundred milligrams of each tissue was ground in liquid nitrogen and total RNA was isolated as described in section 3.2.1. The cDNA was prepared according to manufacturer's protocol (Invitrogen, USA) as described in section 3.2.2. Equal quantity of cDNA prepared from 2 µg of total RNA was used as template in PCR with caffeine synthase gene-specific primer set as described in section 3.2.3 to see the expression of endogenous CS gene. Since primer sequences do not differ for four cultivars, the same set of primers was used to see the expression of caffeine synthase in all the four tea cultivars. 26S *rRNA* based gene primers; 5'-CACAATGATAGGAAGAGCCGAC-3' and 5'-CAAGGGAACGGGCTTGGCAGAATC-3' amplification was used as internal control for gene expression studies (Singh *et al.*, 2004).

3.4.2 Caffeine content measurement

Caffeine was extracted following the HPLC method described (Sharma *et al.*, 2005). Each of the samples of all tea cultivars was dried constantly at 80°C. The 0.2 g of dried sample
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was used for caffeine extraction with 5 ml of 70% methanol with intermittent shaking. The sample was then centrifuged at 3538 rpm for 10 min at 16°C. The supernatant was taken and extraction steps were repeated three times and final volume was reached to 10 ml. The extract was filtered through a 0.5 μm Millipore filter before the injection was made. Based on the specific retention time of commercial caffeine as standard, caffeine content was estimated by Merck Hitachi HPLC (Darmstadt Germany) using C18 Lichrocart column (250 x 4 mm x 5 μm) and the absorbance was read at 210 nm. Caffeine content was calculated from standard curve prepared from pure caffeine (Sigma, USA).

3.5 Allantoin content measurement

Allantoin content as a mean for caffeine degradation was also estimated in all tea cultivars which were used for studying caffeine biosynthesis. Allantoin content in various tissues of tea was estimated using method described (Fink et al., 1963; Vrbaški et al., 1978). Ehrlich’s reagent was prepared by dissolving 1 g of p-dimethylaminobenzaldehyde (Fluka, USA) 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 formed 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 UV/Vis Spectrophotometer. Pure allantoin (Fluka, USA) was used as standard in the assay.

3.6 Statistical analysis of the data

Means and standard deviations were calculated according to the standard methods in the above caffeine and allantoin content data. One-way ANOVA statistical test was used to determine the differences between means of the caffeine and allantoin contents accepting the significance level at \( P \leq 0.05 \). Correlation between caffeine content and allantoin contents of all vegetative tissues of four tea cultivars during non-dormant and dormant growth phases was also calculated accepting the significance level at \( P \leq 0.05 \).

3.7 RNAi vector used

An RNAi vector employed for the genetic transformation of Kangra jat tea cultivar to reduce its caffeine content was based on binary vector pCAMBIA1200. It has the omega leader sequence of Tobacco Mosaic Virus at the end of the promoter sequence. This sequence is thought to increase translation efficiency and is not expected to influence
RNAi. The RNAi vector (pFGC1008) [Appendix-II] contains a chloramphenicol resistance gene (Cm) as bacterial selection and a hygromycin resistance gene (HYG) as plant selection marker. A gene fragment was directionally cloned in sense and antisense orientations into pFGC1008 and a 360 bp fragment of GUS (E. coli beta-glucuronidase gene) was cloned as an intron in between sense and antisense gene fragments. This has resulted in making an intron-containing hairpin RNA (ihpRNA) construct. The ihpRNA producing cassette was driven by a strong constitutive, viral CaMV 35S promoter and an OCS terminator.

3.7.1 Preparation of RNAi construct (pFGC1008-CS)
A 376 bp cDNA fragment of caffeine synthase (CS) was cloned in sense (CS-S) at Ascl and Swal restriction sites and antisense (CS-AS) orientations at SpeI and BamHI restriction sites of pFGC1008 vector one after another.

3.7.2 PCR amplification of CS cDNA fragment
For cloning into sense orientation, CS cDNA fragment was amplified through PCR using the primers (PF1 5’-GGCGCGCCCAAAACAGTGACCTCAATGAC-3’ and PR1 5’-ATTATAATCAGGAAATGAACTAATGCAAGC-3’) containing Ascl and Swal restriction sites. For cloning into antisense orientation, CS cDNA fragment was amplified using the primers (PF2 5’-ACTAGTCAGGAACTAATGCAAGC-3’ and PR2 5’-GGATCCCAAAACAGTGACCTCAATGAC-3’) containing SpeI and BamHI restriction sites. PCR amplifications using the above sets of primers were carried out in a thermo cycler under similar condition of 94°C-4 min for 1 cycle, 94°C-30 s, 55°C-30 s, 72°C-30 s for 35 cycles, and 72°C for 7 min.

3.7.3 Cloning of CS cDNA fragment into pGEM-T Easy
PCR amplified products using the primer sets described in section 3.8.4.1 were separated on 1% agarose gel in 1x TAE buffer and purified from the gel as described in section 3.2.4. Firstly, these purified products were cloned into pGEM-T Easy vector as described in section 3.3 and confirmation was done through colony PCR using the primer sets described in section 3.7.2. The recombinant pGEM-T Easy plasmids were extracted from positive colonies as described in section 3.3.4.

3.7.4 Restrict digestion of CS cDNA fragment from recombinant pGEM-T Easy
Restrict digestion of CS cDNA fragment from recombinant pGEM-T Easy vector was as described below:

1. In one set, the recombinant pGEM-T Easy vector was double digested with SpeI and BamHI restriction enzymes (New England Biolabs Incorporation, USA). The double digestion reaction was set up in 50 μl volume: Template-25 μl, NEB#2-5 μl, SpeI- 1 μl (10 U), BamHI- 1 μl (10 U), 10x BSA-5 μl and remaining volume was made up with autoclaved ddH2O. The reaction was carried out at 37°C for overnight. Double digested product was heat killed at 65°C for 20 min. The SpeI and BamHI digested CS was resolved in 1% agarose gel along with a DNA ladder and desired digested fragment was purified from gel as described in section 3.2.4.

2. In another set, the recombinant pGEM-T Easy vector was double digested with Ascl and SwaI restriction enzymes (New England Biolabs Incorporation, USA). Because the SwaI restriction enzyme was blunt-ended cutter, double-digestion with these restriction enzymes was conducted separately. The double digestion reaction was set up in 50 μl volume: Template-30 μl (5 μg), NEB#3-5 μl, SwaI-1 μl (10 U), 10x BSA-5 μl and remaining volume was made up with autoclaved ddH2O. The reaction was carried out at 25°C for overnight. The reaction mixture was then heat killed at 65°C for 20 min. The SwaI digested product was purified after resolving in 0.8% agarose gel and second digestion was set up again in 50 μl volume: Template-30 μl, NEB#4-5 μl, Ascl-1 μl (10 U) and remaining volume was made up with autoclaved ddH2O. The reaction was carried out at 37°C for overnight. The Ascl and SwaI digested CS fragment was resolved in 1% agarose gel along with a DNA ladder and desired digested fragment was purified from gel as described in section 3.2.4.

3.7.5 Cloning of CS cDNA fragment in sense orientation into RNAi vector
The cloning of CS cDNA fragment in sense orientation into RNAi vector was as follows:

1. The pFGC1008 plasmid was digested first with SwaI and then with Ascl restriction enzymes as described in section 3.7.4.

2. Thus, the Ascl and SwaI digested pFGC1008 was ligated with the similar double-digested CS fragment (described in section 3.7.4) as insert in 1:3 ratio using T4 DNA ligase (1 Weiss unit) (Fermentas, GBH Germany). The ligation reaction was carried out at 12°C.
3. The ligated product was transformed in *E. coli* as described in section 3.3.2 and colonies were selected on chloramphenicol containing LB plate.

4. Colonies produced in above step were screened through colony PCR using the vector specific primers (AS F 5’-CAATCCCACTATCCTTCGCAAG-3’ and AS R 5’-GACAGCAGCAGTTTCATTCAATCAC-3’). PCR amplification was carried out in a thermo cycler under conditions of 94°C-5 min for 1 cycle, 94°C-1 min, 55°C-1 min, 72°C-30 s for 35 cycles, and 72°C for 7 min. The amplified product was separated on 1% agarose gel in 1x TAE and visualized in ethidium bromide.

5. As a positive control, PCR amplification of 225 bp was obtained with the primers (AS F and AS R) using pFGC1008 plasmid as template.

### 3.7.6 Cloning of *CS* cDNA fragment in antisense orientation into RNAi vector

The cloning of *CS* cDNA fragment in antisense orientation into RNAi vector was as follows:

1. The pFGC1008 plasmid containing sense caffeine synthase fragment (CS-S) was isolated from the positive colony as described in section 3.3.4 and it was digested with *SpeI* and *BamHl* restriction enzymes and the digested vector was resolved in 0.8% agarose gel and purified from gel as described in section 3.2.4.

2. Similar double-digested (as in step 1) RNAi vector and *CS* fragment (described in section 3.7.4) were ligated in 1:3 ratio using T4 DNA ligase (1 Weiss unit) (Fermentas, GBH Germany). The ligation reaction was carried out at 22°C.

3. The ligated product was transformed in *E. coli* as described in section 3.3.2 and colonies were selected on chloramphenicol containing LB plate.

4. Colonies produced in above step were screened through colony PCR using the vector specific primers (BS F 5’-TGTGGAGTATTGCCAACGAAC-3’ and BS R 5’-GTAATCAGTCTGTTAGGTTTGACCG-3’). PCR amplification was carried out in a thermo cycler under conditions of 94°C-5 min for 1 cycle, 94°C-1 min, 55°C-1 min, 72°C-40 s for 35 cycles, and 72°C for 7 min. The amplified product was separated on 1% agarose gel in 1x TAE and visualized in ethidium bromide.

5. As a positive control, PCR amplification of 565 bp was obtained with the primers (BS F and BS R) using pFGC1008 plasmid as template.
3.7.7 Final confirmation of prepared RNAi construct (pFGC1008-CS)
The pFGC1008-CS plasmid was isolated from the positive colony from step 4 of section 3.7.6. The final confirmation of this RNAi construct (pFGC1008-CS) was done through PCR using pFGC1008-CS as template, with the two sets of vector specific primers as described in section 3.7.5 and 3.7.6.

3.8 Bacterial strains used
The RNAi vector was maintained and stored in *E. coli* (DH5α). A partial fragment of *CS* was directionally cloned in this RNAi vector to make it pFGC1008-CS construct. This RNAi construct was transferred into *Agrobacterium tumefaciens* strains LBA4404 and GV3101 using triparental mating, where *E. coli* (strain PRK2013) served as helper was employed. In the present study, *Agrobacterium tumefaciens* strain LBA4404 harboring RNAi construct was employed for tea somatic embryos transformation studies. While *Agrobacterium tumefaciens* strain GV3101 harboring RNAi construct was employed for tea root transformation studies. The RNAi construct was also used for biolistic-mediated tea somatic embryos transformation.

3.9 Triparental mating
Triparental mating involved the mating between three parental strains such as *Agrobacterium*, *E. coli* (helper strain) and *E. coli* containing construct. By this procedure the RNAi construct was transferred into *Agrobacterium tumefaciens* for tea transformations studies. The procedure for triparental mating was as follows:

1. *Agrobacterium tumefaciens* strains LBA4404 and GV3101 were streaked freshly on yeast extract mannitol (YEM; Appendix-II) plate containing streptomycin sulfate (50 µg/ml) and rifampicin (25 µg/ml) respectively. These plates were incubated at 28°C for 48 h.
2. *E. coli* Helper strain was streaked freshly on Luria broth (LB, pH 7.0) plate containing kanamycin (50 µg/ml). It was incubated at 37°C for overnight.
3. *E. coli* (DH5α) containing RNAi construct was streaked freshly on LB, pH 7.0 plate containing chloramphenicol (50 µg/ml).
4. The above freshly grown *Agrobacterium* was resuspended in YEM medium (1 ml), while the freshly grown *E. coli* helper strain and *E. coli* containing RNAi construct were resuspended in LB liquid medium.
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5. The above suspension of each parental strain was vortexed and was centrifuged at 6000 rpm for 5 min.
6. Each of the supernatants was discarded and the resulting pellet was resuspended with 100 µl autoclaved double-distilled water.
7. Each of the suspensions of 25 µl helper, 50 µl E. coli containing RNAi construct, and 100 µl Agrobacterium was mixed together. The mixture was placed on sterile piece of a nylon membrane kept at centre of yeast extract (YE) plate which contained YEM except mannitol. It was incubated at 28°C for 24-48 h for the growth of colonies.
8. A loopful of each of the colonies was resuspended with autoclaved water to make different dilutions (1:10, 1:4, and 1:1 etc.).
9. Each dilution was spreaded under sterile condition on YEM plate containing rifampicin and chloramphenicol. These YEM plates were incubated at 28°C for 48 h.
10. The colonies produced on the selection medium were further screened through colony PCR for their confirmation using vector specific primers.

3.10 Tea regeneration system
Based upon the optimized and established tea somatic embryogenesis and regeneration system in ‘Kangra jat’ tea cultivar, similar tea regeneration protocol was followed.

3.10.1 Production of tea somatic embryos
Based on report of Mondal et al. (2001a), somatic embryos were produced from mature tea cotyledons as described below:
1. Mature green fruits were collected from ‘Kangra jat’ tea cultivar during Oct-Nov months of the year. After removal of fruit coats, seeds were dipped in water for overnight. Only sinkers were used after removal of seed coat.
2. Collected cotyledons were washed in Tween-20 for 10 min and were rinsed (2-3 times) in double-distilled water.
3. Washed cotyledons were given treatment with tetracycline (0.02%), ampicillin (0.02%), streptomycin sulfate (0.04%) and 2-3 drops of Tween-20 for 25 min and were rinsed (5-6 times) in double-distilled water.
4. Further, washed cotyledons were surface-sterilized with 4% Calcium hypochlorite solution for 10 min under sterile condition. These were then washed with sterile double-distilled water, dried on sterile filter paper and used as explant.

5. Surface-sterilized cotyledons were inoculated in half-strength MS medium (MS; Appendix-I) containing 20 g/l sucrose, 2.5 mg/l NAA and 0.2 mg/l BAP and solidified with 0.7% (w/v) agar (Sigma-Aldrich) at pH 5.7 for proliferation of embryonal axis and it took 30 days.

6. Embryonal axis was removed and the de-embryonated cotyledons were used for aseptic inoculation on same medium for the induction of primary somatic embryos.

7. Secondary somatic embryos were produced from the primary embryos by inoculating them on embryo induction medium (EIM; Appendix-III) containing high concentration of betaine (1 g/l) and ABA (7.5 mg/l) as mentioned by Akula et al. (2000).

3.10.2 Maintenance of tea somatic embryos
A synchronous and normal development of secondary embryos was obtained on somatic embryogenesis medium (SEM; Appendix-II) containing full strength MS basal salts modified with half-strength nitrates of potassium (950 mg/l) and ammonium (825 mg/l), potassium sulphate (300 mg/l), 6-BAP (2 mg/l), IBA (0.2 mg/l), L-glutamine (1 g/l), 30 g/l sucrose and 0.75% agar as described by Mondal et al. (2001b). The pH of medium was adjusted to 5.6 and dispensed 100 ml medium into 250 ml of Erlenmeyer flask (Borosil Ltd, Mumbai). The medium was autoclaved for 20 min at 1.1 kg/cm² and 121°C. These secondary somatic embryos were maintained in culture laboratory conditions of 25±2°C and cool fluorescent light of 70 μmol (photons) m⁻² s⁻¹ with a 16 h light photoperiod, and 55±5% air humidity. Regular subculturing of tea somatic embryos was done in every 21 days for their maintenance.

3.10.3 Regeneration of tea somatic embryos
The globular somatic embryos having high potential for repetitive embryogenesis and conversion were used as explants for transformation studies and also for regeneration into tea microshoots. For their maturation, the globular-shaped secondary somatic embryos were maintained on full strength basal MS medium containing with maltose (40 g/l), trans-cinnamic acid (3 mg/l), and agar (0.75%) with pH 5.6 for 8 weeks period. The medium was autoclaved for 20 min at 1.1 kg/cm² and 121°C. After maturation, somatic embryos
were shifted to 1.5 mg/l GA<sub>3</sub> containing MS medium for 12 weeks period for the regeneration of microshoots.

3.11 Tea transformation
Genetic transformation of selected tea cultivar (Kangra jat) of *Camellia sinensis* (L.) O. Kuntze was attempted using *Agrobacterium* as well as biolistic-mediated methods for the purpose of reducing its caffeine content.

3.11.1 Tea explants used
Globular somatic embryos of Kangra jat tea cultivar with high potential for repetitive embryogenesis and regeneration were used as explants for transformation through *Agrobacterium* as well as biolistic-mediated approaches. In addition, roots of one month old tea seedlings belonging to Kangra jat cultivar were also used for developing a novel tea transformation protocol.

3.11.2 Transformation methods employed
*Agrobacterium* and biolistic-mediated methods were employed for transforming tea somatic embryos. In addition, *Agrobacterium*-mediated method was employed for developing the tea root transformation protocol.

3.11.3 *Agrobacterium*-mediated transformation of somatic embryos
*Agrobacterium* harboring pFGC1008-CS construct and pFGC1008 alone as a control were used to transform tea somatic embryos using the established protocol of Mondal *et al.* (2001a). Tea somatic embryos were maintained on somatic embryogenesis medium (SEM) prior to transformation. The procedure of *Agrobacterium*-mediated transformation was as follows:

1. To prepare the inoculation medium, 50 ml of YEM liquid medium was inoculated with overnight grown stock culture of *Agrobacterium* strain LBA4404 harboring pFGC1008-CS. YEM was supplemented with the appropriate antibiotic (pFGC1008: chloramphenicol 50 mg/l, streptomycin 50 mg/l). Cultures were grown in dark at 28°C for 16-18 h with shaking at 199 rpm.
2. *Agrobacterium* cells corresponding to OD<sub>600</sub>=0.6-0.8 were harvested by centrifugation at 6000 rpm for 10 min at room temperature, followed by twice
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washing with liquid YEM medium. The final cell density was adjusted to $10^9$ cells/ml with the liquid SEM using the formula:

$$x \times 3 \times \text{O.D. obtained} \times 10^9 \text{cells/ml} = 1 \times 10^9 \text{cells/ml} \times y \text{ml}$$

where, $x$ = amount of bacterial solution to be taken from the overnight grown bacterial culture in order to obtained an optimal density of $10^9$ cells/ml; $y$ = correction factor; $y$ = the total volume of culture required for genetic transformation.

3. Globular somatic embryos were submerged in the bacterial suspension for 20 min, blot-dried on sterile filter paper and finally transferred to agar-solidified SEM.

4. Following five days of dark co-cultivation, the somatic embryos were washed with autoclaved distilled water, then subjected to two additional rinses with liquid SEM containing sporidex (300 mg/l), blot-dried and transferred to SEM containing two bactericidal antibiotics (sporidex and carbenicillin, 250 mg/l each). To remove further bacterial growth, repeated washing and transferring to fresh medium was done.

5. Following 15 days of culture, somatic embryos were transferred to the same medium augmented with one bactericidal antibiotic (sporidex 400 mg/l) as well as the phytotoxic antibiotic hygromycin (40 mg/l).

6. Following 12- to 16-week culture period, with subculturting to fresh medium at 21 days interval, hygromycin resistant somatic embryos were kept on maturation medium containing maltose (4%), trans-cinnamic acid (3 mg/l) and hygromycin (40 mg/l) for 8 weeks period.

7. After maturation, transformants were shifted to germination medium containing 1.5 mg/l GA$_3$ and hygromycin (40 mg/l) for 12 weeks period.

3.11.3.1 Semiquantitative-PCR based analysis of transformants

Tea transformants were analyzed for reduction in the caffeine synthase transcript level through reverse transcription-PCR. For this, total RNA was isolated from transformed and untransformed tissues as described in section 3.2.1. Using 2 $\mu$g of total RNA from each of the samples, cDNA was prepared as described in section 3.2.2. Equal quantity of cDNA was used as template in PCR with caffeine synthase gene specific primers to check the transcript expression levels. After standardization, PCR was carried out as described earlier and amplified product was separated on 1% agarose gel and visualized with ethidium bromide staining. Level of 26S rRNA in samples amplified with standard primers
was used as an internal control (Singh et al., 2004) for the gene expression studies. The relative level of expression from reverse transcription-PCR was also estimated densitometrically.

3.11.3.2 Quantification of caffeine content
Caffeine was extracted following the HPLC method described (Sharma et al., 2005). Each of the samples of tea transformants were dried constantly at 80°C and used for caffeine extraction as described in section 3.4.2. Caffeine content was calculated from standard curve prepared from pure caffeine (Sigma).

3.11.4 Biolistic-mediated transformation of somatic embryos
Globular tea somatic embryos were also transformed with biolistic-mediated method. For this, established standard protocol of microprojectile bombardment of tea somatic embryos was employed (Saini, 2007; Saini et al., 2010). The pFGC1008-CS and pFGC1008 vector (as positive control) were used for microprojectile bombardment of tea somatic embryos. Before transformation, the lethal dose of hygromycin to explants was decided by culturing the somatic embryos on different concentrations (20, 30, 40, 50, and 60 mg/l) of hygromycin containing SEM. This antibiotic was filter sterilized and added to the autoclaved medium at 45°C before it solidified. Subculturing of somatic embryos was done in the same medium at 21 days interval until the tissue became necrotic. Before bombardment, several pre-bombardment preparations were done which are as follows:

3.11.4.1 Plasmid isolation
The plasmid DNA was isolated using the QIAfilter Plasmid Maxi Kit (QIAGEN, Germany) as described below:

1. Overnight grown 300 ml of bacterial culture was centrifuged at 6000 rpm for 35 min to make pellet.
2. Supernatant was removed and pellet was resuspended in 10 ml of Buffer P1 (Resuspension buffer: 50 mM TrisHcl, pH 8.0; 10 mM EDTA, 100 μg/ml RNaseA, store at 4°C) by vortexing.
3. The resuspended pellet was added with 10 ml of Buffer P2 (Lysis buffer: 200 mM NaOH, 1% SDS (w/v)). It was gently mixed and incubated for 5 min.
4. The above suspension was added with 10 ml of chilled Buffer P3 (Neutralization buffer: 3 M potassium acetate, pH 5.5). After gentle mixing, it was incubated for 20 min on ice.

5. Centrifugation was done at 12000 rpm for 35 min at 4°C and supernatant was collected. This step was repeated for three times and finally transparent supernatant was collected.

6. The Qiagen Tip 100 (column) was equilibrated by applying 10 ml of QBT buffer (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% (v/v) isopropanol). It was allowed to empty by gravity.

7. The transparent supernatant was applied to column.

8. The column was washed two times with 30 ml of Buffer QC.

9. The DNA was eluted with 15 ml of Buffer QF (Elution buffer: 1.25 M NaCl; 50 mM TrisHCl, pH 8.0; 15% (v/v) Isopropanol). It was precipitated by adding with 0.7 volume of isopropanol. The pellet was made by centrifugation at 14000 rpm for 30 min at 4°C.

10. The pellet was washed with 5 ml of 70% ethanol.

11. The pellet was dried and dissolved in 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

12. The isolated plasmid was checked on 0.8% agarose gel.

3.11.4.2 Preparation of microcarriers

The following procedure prepared gold microcarriers for 120 bombardments, where 3 mg of the microcarriers were used per bombardment. The microcarriers were prepared as described below:

1. Weighed 60 mg of gold particles (1.0 µm; Bio-Rad, Hercules, California, USA) were resuspended in 1 ml of 70% ethanol (Fluka).

2. The above resuspended suspension was vortexed for 5 min and incubated for 15 min.

3. Centrifugation was done at 9000 rpm to make pellet of gold particles. The supernatant was discarded. This step was repeated thrice.

4. The pellet was resuspended in 1 ml of 70% ethanol. Brief spin was done at 9000 rpm for 20 s. After removal of supernatant, 1 ml of sterile water was added to the washed gold particles.
5. Different aliquots each of 50 µl were made during vortexing and were stored at 4 °C.

3.11.4.3 Sterilization of rupture discs, stopping screens, macrocarriers, and particle gun machine
Before the bombardment of microcarriers, the rupture discs, stopping screen, and macrocarriers were sterilized by dipping in absolute ethanol for 3 min. These were dried on a sterile filter paper in laminar air flow cabinet. The Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) was sterilized thoroughly with absolute ethanol before its use. This particle gun machine was also given UV-light treatment for 15 min.

3.11.4.4 Coating of microcarriers with plasmid DNA
The following procedure was sufficient for six bombardments. Continuous vigorous vortexing of the microcarriers was conducted for uniform DNA preparation onto microcarriers. The coating of washed microcarriers with plasmid DNA was as follows:

1. Fifty µl of prepared microcarriers was added with 10 µl (1 µg/µl) of pure plasmid.
2. Fifty µl of 2.5 M filter sterilized CaCl₂ was added to help in binding of plasmid DNA with gold particles.
3. Twenty µl of 0.1 M filter sterilized spermidine (free base, tissue culture grade) was added to prevent agglutination of gold particles.
4. All the above components were slowly vortexed for 3 min. The centrifugation was done at 9000 rpm for 1 min.
5. The supernatant was discarded and the pellet was resuspended in 250 µl of absolute ethanol. The step 4 was repeated again.
6. Supernatant was discarded and finally the pellet was resuspended in 60 µl of absolute ethanol.
7. Ten µl of resuspended gold particles were pipetted out for coating onto the macrocarrier. The coated macrocarriers were dried before particle bombardment.

3.11.4.5 Particle bombardment with RNAi construct
Particle bombardment of tea somatic embryos with the RNAi construct (pFGC1008-CS plasmid) was described as below:

1. The wild somatic embryos (non-transgenic) and embryos bombarded with naked gold particles served as negative controls. Gold particles coated with pure plasmid
DNA, as stated in section 3.12.4 were used for particle bombardment of the globular tea somatic embryos, using the biolistic PDS-1000/He System. The somatic embryos were maintained on SEM prior to transformation.

2. The somatic embryos arranged at the centre of agar-solidified SEM plate were bombarded using a burst pressure of 77Kg/cm², 9 cm target distance, 1.56 cm 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.

3. After bombardment, the plates containing somatic embryos were turned at 180° and then bombarded again with the same parameters for better work efficiency. The bombarded somatic embryos were left undisturbed under diffuse light for healing the wounds.

4. After 10 days of bombardment, somatic embryos were transferred to SEM supplemented with hygromycin (40 mg/l) as plant selection agent. The somatic embryos were allowed to proliferate and mature on the same selection medium for 8-9 weeks and were sub-cultured at regular interval of 21 days. After this period, only somatic embryos that were resistant to selection agent were allowed to grow on the same medium supplemented with selection agent.

5. Transgenic microshoots were produced from the epidermal surface of the transformants after a minimum period of 5-7 months.

3.11.4.6 Semiquantitative-PCR based analysis of transformants

Total RNA was isolated from transformed and untransformed tissues as described in section 3.2.1. Using 2 μg of total RNA from each of the samples, cDNA was prepared as described in section 3.2.2. Equal quantity of cDNA was used as template in PCR with caffeine synthase gene specific primers to check the transcript expression levels. After standardization, PCR was carried out as described earlier and amplified product was separated on 1% agarose gel and visualized with ethidium bromide staining. Level of 26S rRNA in samples amplified with standard primers was used as an internal control (Singh et al., 2004) for the gene expression studies. The relative level of CS transcript expression was also estimated densitometrically.

3.11.4.7 Quantification of caffeine content

Caffeine was extracted following the HPLC method described (Sharma et al., 2005). Each of the samples of tea transformants were dried constantly at 80°C and used for caffeine
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3.4.2. Extraction as described in section 3.4.2. Caffeine content was calculated from standard curve prepared from pure caffeine (Sigma).

3.11.4.8 Quantification of theobromine content

Theobromine was extracted following the HPLC method described (Sharma et al., 2005). Each of the samples of tea transformants were dried constantly at 80°C. The 0.2 g of dried sample was used for theobromine extraction with 5 ml of 70% methanol with intermittent shaking. The sample was then centrifuged at 3538 rpm for 10 min at 16°C. The supernatant was taken and extraction steps were repeated three times and final volume was reached to 10 ml. The extract was filtered through a 0.5 μm Millipore filter before the injection was made. Based on the specific retention time of commercial theobromine as standard, theobromine content was estimated by Merck Hitachi HPLC (Darmstadt Germany) using C18 Lichrocart column (250 x 4 mm x 5 μm) and the absorbance was read at 280 nm. Theobromine content was calculated from standard curve prepared from pure theobromine (Sigma).

3.12 Hardening of transgenic microshoots

To stabilize and harden the low-caffeine transgenic shoots produced through Agrobacterium as well as biolistic-mediated methods, these were micrografted on tea seedlings of the same tea cultivar. Micrografting of transgenic tea microshoots was as follows:

1. Tea microshoots of 20-25 mm long with an average of four to six leaves produced from germinated putative transformants were used as scion. Four month old tea seedlings of the same cultivar, grown in nursery were used as root stock.
2. After decapitating the hypocotyl region of tea seedling at 2 cm above the soil, with a scalpel blade, a slanting cut was applied to expose the cambium and then a matching slanting cut at the lower end of the scion was made.
3. The decapitated end of the root stock and matching cut end of the scion was treated with BAP and NAA in combination (5.0 mg/l each) for 10 min. The root stock and scion were held together at the point of matching cuts with moist cotton wrapped in parafilm.
4. The grafted plants were kept for acclimatization in humid chambers enriched with CO₂ and 60-65% relative humidity, and 20-25°C temperature as standardized earlier in our institute (Prakash et al., 1999). Considering the nature of tea seed
biology and germination behaviour, tea micrografting was performed during Feb-Mar of the year.

3.13 Quantification of caffeine and theobromine contents in transgenic tea plants
After one year of micrografting, caffeine and theobromine were extracted from young shoots of transgenic tea plants as described in section 3.10.7 and 3.10.8.

3.14 Agrobacterium-mediated tea root transformation
Agrobacterium-mediated root transformation system for tea (*Camellia sinensis* L.) was developed. For this, *Agrobacterium* suspension was used to transform one month old tea seedlings by making fresh wounds with a sterile needle at elongation zone of roots. The feasibility of the developed protocol was documented through silencing caffeine biosynthesis. Several parameters were optimized for developing the present root transformation protocol.

3.14.1 Plant materials and growth conditions
Tea (*Camellia sinensis* (L.) O. Kuntze cv. Kangra jat] seedlings were raised in Banuri Tea Experimental Farm of our Institute IHB T at Palampur, Himachal Pradesh, India. The intact roots of one month old tea seedlings after seed break were washed thoroughly in double-distilled water and employed for developing the root transformation system.

3.14.2 RNAi construct and Agrobacterium strain used
An ihpRNA construct (pFGCI008-CS) described in section 3.8.3 was employed for developing this tea root transformation protocol. *Agrobacterium tumefaciens* strain GV3101, harboring pFGCI008-CS construct and pFGC1008 vector (used as control) was used as the host for tea root infiltration.

3.14.3 Preparation of Agrobacterium inoculum
To prepare the inoculum, 50 ml of yeast extract mannitol (YEM) liquid medium was inoculated with overnight grown stock culture of *Agrobacterium* strain GV3101 harboring either pFGC1008-CS or pFGC1008 vector. YEM was supplemented with the appropriate antibiotics chloramphenicol (50 μg/ml) and rifampicin (50 μg/ml). Cultures were grown in dark at 28°C for 16-18 h with shaking at 200 rpm. *Agrobacterium* cells corresponding to OD$_{600}$= 0.6-0.8 were harvested by centrifugation at 5000 rpm for 5 min at room
temperature followed by washing with liquid YEM. The final cell density was adjusted to $10^9$ cells/ml with Murashige and Skoog (MS) medium.

3.14.4 Standardization of parameters for root transformation

Based upon the reports of tea transformation (Mondal et al., 2001a), a number of other parameters such as age of the tea plants, site and extent of wounding roots, submerge time of bacterial suspension, and co-cultivation period were optimized for maximum transformation efficiency for developing the present root transformation protocol. To standardize each of parameters, 50 tea seedlings were employed.

3.14.4.1 Age of tea plants

To determine the optimal age of tea seedlings for development of root transformation protocol, tea seedlings of different age groups such as 1 month, 4 months, and 12 months after seed break were initially tested.

3.14.4.2 Site and extent of wounding tea roots

The elongation zone of intact tea roots was selected specifically for wounding the seedlings. Wounding beyond the root cortex and wounding up to root cortex were selected initially as extent of wounding tea roots for maximum root transformation efficiency.

3.14.4.3 Submerge time of bacterial suspension

To determine the optimal submerge time of bacterial suspension, freshly wounded roots of tea seedlings were left in Agrobacterium suspension for bacterial infection at $25 \pm 2^\circ C$ for 30, 45, 60, and 120 min.

3.14.4.4 Co-cultivation period

After bacterial infection, the excess inoculation medium was poured off. Roots were then incubated (co-cultivated) completely in the sterile 0.75% agar containing MS medium (pH 5.6) for 2, 3, 4, 5, 6, and 7 days in dark at $25 \pm 2^\circ C$.

3.14.5 Stabilization of transformed tea plants

The transformed tea plants were stabilized and hardened. For this, after co-cultivation roots of transformed plants were washed thoroughly in double-distilled water and potted in sand soil::1:1 under green house conditions for 1-2 months.
3.14.6 T-DNA integration in tea roots
To check the integration of T-DNA in transformed tea roots, PCR was conducted using various integration fragment specific primers with the root genomic DNA. The transformed lines were further confirmed through dot blot and southern blot analysis in tea roots.

3.14.6.1 Genomic DNA isolation from transformed roots
Total genomic DNA was isolated from roots of four transformed tea lines K6a, K25, K29a, and K31a, and CI as untransformed control tea root sample, and CII as transformed with pFGC1008 vector alone control tea root sample using DNeasy Plant Mini Kit (QIAGEN, Germany) as described below:

1. Hundred mg plant tissue was ground thoroughly in liquid nitrogen.
2. The tissue powder was put into a 1.5 ml microcentrifuge tube containing preheated (65°C) 400 µl of Buffer AP1 and 4 µl of RNaseA (stock 100 mg/ml). It was vortexed vigorously and incubated for 10 min at 65°C with intermittent mixing.
3. After incubation, 130 µl of Buffer AP2 was added to the above mixture and mixed immediately. The mixture was incubated for 5 min on ice.
4. The above lysate was centrifuged for 5 min at 14000 rpm.
5. The above lysate was pipetted into a QIAshredder Mini Spin Column in a 2 ml collection tube and centrifuged for 2 min at 14000 rpm.
6. The flow-through fraction was transferred carefully into a new microcentrifuge tube and was added 1.5 volume of Buffer AP3/E mixed by pipetting.
7. The above mixture was transferred into a DNeasy Mini Spin Column in a 2 ml microcentrifuge tube and centrifuged for 1 min at 8000 rpm.
8. The flow-through was discarded and 500 µl of Buffer AW was added into the above DNeasy Mini Spin Column and centrifuged as described in step 7.
9. The above step was repeated except for 2 min at 14000 rpm. The flow-through was discarded.
10. Fifty µl of Buffer AE was added to the above DNeasy Mini Spin Column and after incubation for 5 min, centrifuged for 1 min at 8000 rpm.
11. The isolated genomic DNA was checked on 0.8% agarose gel and visualized with ethidium bromide.
3.14.6.2 PCR confirmation of T-DNA region integration in transformed roots

Using the isolated genomic DNA from tea roots as template, PCR amplification was conducted with the various sets of ihpRNA construct (pFGC1008-CS) specific primers in four tea seedlings as described below:

1. To confirm the integration of sense caffeine synthase (CS-S) fragment in tea roots, PCR amplification was conducted with the primers (F1 5'-CAATCCCACTATCCTTCGCAAG-3' and R1 5'-GACAGCAGCAGTTTCATTCAATCAC-3'). PCR amplification was carried out in a thermo cycler under conditions of 94°C-5 min for 1 cycle, 94°C-1 min, 55°C-1 min, 72°C-30 s for 35 cycles, and 72°C for 7 min.

2. To confirm the integration of antisense caffeine synthase (CS-AS) fragment in tea roots, PCR amplification was conducted with the primers (F2 5'-TGTGGAGTATTGCCAACGAAC-3' and R2 5'-GTAATCAGTCTGGTTAGGTTTGACCG-3'). PCR amplification was carried out in a thermo cycler under conditions of 94°C-5 min for 1 cycle, 94°C-1 min, 55°C-1 min, 72°C-40 s for 35 cycles, and 72°C for 7 min.

3. The amplified products were separated on 1% agarose gel in 1x TAE and visualized in ethidium bromide.

4. To confirm the integration of whole RNAi cassette in transformed tea roots, PCR amplification was conducted with the primers (F1 and R2) described above.

5. In addition, genomic DNA-PCR amplification was also conducted with the hygromycin gene specific primers (PF 5'-CTTGTCGATCGACAGATCC-3' and PR 5'-CTTTCGCAGATCCCGGGG-3') to confirm the integration of T-DNA in tea roots. PCR amplification was carried out in a thermo cycler under conditions of 94°C-4 min for 1 cycle, 94°C-30 s, 54°C-30 s, 72°C-1 min for 35 cycles, and 72°C for 7 min.

6. After amplifications with the above described primers, the PCR products were separated on 1% agarose gel and visualized with ethidium bromide staining.

3.14.6.3 Dot blot analysis

Total genomic DNA (500 ng) isolated from roots of transformed tea lines (K6a and K31a) and control tea lines (C1 and CII) was digested with HindIII for dot blot analysis. Total DNA was transferred onto Hybond N+ nylon membrane (Ambion) and was fixed by UV cross-linking. The digoxigenin-labeled caffeine synthase cDNA probe was prepared and
used for hybridization with blotted genomic DNA at 37°C in the DIG Easy Hyb solution (Roche). The membrane was washed twice with 0.2 X SSC and 0.1% SDS at room temperature and then twice at 50°C. The chemiluminescent detection of signal was performed according to the manufacture’s instructions (Dig labeling and detection Kit Roche) as described below:

1. The membrane was washed briefly for 5 min in Washing Buffer.
2. The membrane was incubated in Blocking Solution for 30 min.
3. The membrane was incubated in Antibody Solution for 30 min.
4. The membrane was washed twice in Washing Buffer.
5. The washed membrane was equilibrated in Detection Buffer for 5 min.
6. The NBT/BCIP solution was spreaded evenly onto the membrane. It was kept in dark for the colour development.

3.14.6.4 Southern blot analysis
Total genomic DNA (5 μg) from tea roots of transformed tea line (K6a) and control tea line (CI as untransformed control tea root sample) were isolated and digested with HindIII, Ascl and Swal for southern blotting. Restriction digestion with Ascl and Swal enzymes separated out the caffeine synthase fragment cloned in sense orientation of pFGC1008-CS. The digested DNA was electrophoresed on 1% agarose gel and transferred onto Hybond N* nylon membrane (Ambion). The transferred DNA was fixed by UV cross-linking. The blot was developed as described in section 3.13.6.3.

3.14.7 Detection of RNAi signals
To determine the site of small interfering RNA (siRNA) formation and their spreading in other parts of the plant, presence of siRNAs specific to CS were detected in both the root and leaf tissues of transformed tea seedlings. For this, total RNA (50 μg) including small RNA fractions was isolated from root and leaf of tea transformants as well as control wild tea plants using miRNeasy Mini Kit (QIAGEN, Germany). The high-molecular-weight RNAs were removed by precipitation with 20% PEG-8000 in 2 M NaCl and small RNAs in the supernatant were precipitated with isopropanol as described (Goto et al., 2003). The small RNA-enriched nucleic acid produced was resolved on 18% polyacrylamide/7 M urea gel [4.2 g urea, 3 ml of RNase-free water, 4.5 ml (40% polyacrylamide stock), 0.5 ml of 5x TBE (5x TBE; Appendix-II), 100 μl of APS and 5 μl of TEMED] in 1xTBE at 200 V and 30 mA for 1-2 h and transferred onto Hybond N* nylon membrane (Ambion) by
semidry electroblotting. The electroblotting was attempted at 30 mA for 1 h in ATTO assembly. The transfer assembly was prepared by placing blotting sheets, membrane, gel, and again blotting sheets soaked with 20x SSC (20x SSC; Appendix-II) in sequence towards anode. After electroblotting, the membrane was removed and it was exposed to UV-light in UV crosslinker (Amersham Biosciences) for crosslinking of transferred RNA to the membrane. The digoxigenin-labeled antisense CS riboprobe was prepared as described below:

1. Two hundred ng of PCR product (antisense CS gene fragment) in 10 μl volume was added with 4 μl of 5x Labeling mix, 4 μl of Transcription buffer (5x), and 2 μl of RNA polymerase (SP6, T7).
2. All the components were mixed and centrifuged briefly. It was incubated at 42°C for 1 h.
3. Two μl of DNase I was added to the above mixture and it was incubated at 37°C for 15 min.
4. The reaction was stopped by adding 0.8 μl of 0.5 M EDTA (pH 8.0).

The digoxigenin-labeled antisense CS riboprobe was hybridized to small RNAs at 37°C in the DIG Easy Hyb solution (Roche). The membrane was washed twice with 0.2 X SSC and 0.1% SDS at room temperature and then twice at 50°C as described (Tomita et al., 2004). The chemiluminescent detection of RNAi signal (siRNA specific to CS) was performed according to the manufacturer’s instructions (Dig Northern Starter Kit, Roche) as described in section 3.13.6.3.

3.14.8 Semiquantitative-PCR based analysis of caffeine synthase (CS) transcript

To determine the CS transcript expression, total RNA was isolated from young shoots of seven independent confirmed transformed tea lines (K6a, K31a, K25, K29a, K23b, K24b, and K26b) as well as two control tea lines (C-I as untransformed wild type and C-II as transformed with pFGC1008 vector alone) using RNeasy Plant Mini Kit (QIAGEN, Germany) as described in section 3.2.1. The cDNA was prepared according to manufacturers protocol (Invitrogen, USA) using 1 μg of total RNA as described in section 3.2.2. To check the CS transcript expression levels, equal quantity of cDNA was used as template in PCR with CS gene specific primer set as described in section 3.2.3. The PCR amplified product was separated on 1% agarose gel. Level of 26S rRNA in samples amplified with standard primers was used as an internal control for transcript expression.
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studies (Singh et al., 2004). The relative level of CS transcript expression was also estimated densitometrically in transformed and control tea lines.

3.14.9 Quantification of caffeine and theobromine contents

Caffeine and theobromine were extracted following the HPLC method described (Sharma et al., 2005). For this, young shoots from all the seven independent confirmed transformed tea lines (K6a, K31a, K25, K29a, K23b, K24b, and K26b) as well as two control tea lines (C-I as untransformed wild type and C-II as transformed with pFGC1008 vector alone) were dried constantly at 80°C and used for caffeine and theobromine extraction as described in section 3.4.2 and section 3.10.8 respectively. Caffeine and theobromine content were calculated from standard curves prepared from pure caffeine and theobromine respectively (Sigma).

3.14.10 Statistical analysis of the data

Means and standard deviations were calculated according to the standard methods in the above caffeine and theobromine content data. One-way ANOVA statistical test was used to determine the differences between means of the caffeine and theobromine contents accepting the significance level at P ≤ 0.05.

3.15 Biochemical and molecular analysis of caffeine induced growth inhibition in plants

To analyze the effect of caffeine in plants which are not producing caffeine, treatment of different caffeine concentrations was given to Arabidopsis and tobacco. The effect of caffeine treatment was analyzed on seedlings growth, total chlorophyll content, and Rubisco.

3.15.1 Plant materials, growth conditions, and caffeine treatments

Seeds of Arabidopsis thaliana and Nicotiana tabacum were surface sterilized with 70% alcohol for 30 s and 5% bleach (prepared by dissolving 5 ml of Tween-20 in 95 ml water) for 10 min. Subsequently the seeds were washed 4–5 times with autoclaved distilled water in a laminar air flow chamber. These surface sterilized seeds were germinated on MS medium (MS; Appendix-I) plates containing no caffeine (control), 1, and 5 mM caffeine. Plates were kept in tissue culture room at 25±2°C with 16 h light photoperiod. Light
intensity of tissue culture room was 70 μmol (photons) m\(^{-2}\) s\(^{-1}\) and air humidity was 55±5%.

3.15.2 Seedlings growth analysis
To assess whether caffeine exposure has any influence on seedling growth of plants, Arabidopsis and tobacco seeds were grown on MS medium containing no caffeine (control), 1 mM and 5 mM caffeine continuously for 17 days. After 17 days, seedlings were photographed and used for phenotypic observation, shoot and root length measurement.

3.15.3 Caffeine content estimation
Caffeine from Arabidopsis and tobacco seedlings was extracted. After 17 days exposure of Arabidopsis and tobacco seedlings to 1 and 5 mM caffeine on MS plates, tissues were dried constantly at 80°C and used for caffeine extraction as described in section 3.4.2.

3.15.4 Total chlorophyll content estimation
To check the influence of caffeine on total chlorophyll, seeds of Arabidopsis and tobacco were germinated on MS medium for 17 days and then treated with 1 and 5 mM caffeine for 96 h. Seedlings kept in water alone for 96 h were used as a control. Total chlorophyll was estimated following the method of Lichtenthaler (1987). For chlorophyll estimation, one hundred mg of fresh seedlings were ground with 80% chilled acetone and the mixture was centrifuged at 12000 rpm for 15 min. The supernatant was separated and absorbance was read at 648 nm and 664 nm for estimation of chlorophyll B and A respectively. Total chlorophyll content was estimated (in µg/g FW) using the following formula:

\[
\text{Chl (A+B)} = 5.24A_{664} + 22.24A_{648}
\]

3.15.5 Rubisco expression analysis
For Rubisco gene expression analysis, seeds of Arabidopsis and tobacco were germinated on MS medium for 17 days and then treated with 1 and 5 mM caffeine. Seedlings kept in water were used as a control. After 48, 72 and 96 h of caffeine treatment, total RNA was isolated from the seedlings. The cDNA was synthesized using 4 µg of RNA in the presence of 200 U reverse transcriptase Superscript™ III (Invitrogen), 0.001 cm\(^3\) of 10 mM dNTPs and 250 ng oligo (dT)\(_{12-18}\). Resulting cDNA was used to carry out PCR with Rubisco (large subunit) gene specific internal primers; PF 5'-
CACAATGATAGGAAGAGCCGAC-3’ and CAAGGGAACGGGCTTGGCAGAATC-3’. After standardizing the optimal amplification at exponential phase, PCR was carried out under the following conditions: 94°C, 4 min for 1 cycle; 94°C, 30 s; 60°C, 40 s; 72°C, 1 min for 25 cycles. α-tubulin based gene primers; 5’-GAGAGTTTCATTTCGATCCAC-3’ and 5’-CTGAGACGAGCCTGTTG-3’ were used as the internal control for expression studies.

3.1.5.6 Rubisco activity analysis

Rubisco activity was determined following the described methods (Rice and Pon, 1978; Yokota et al., 1996). One hundred mg caffeine treated (48, 72, and 96 h) and untreated (kept in water only) seedlings of Arabidopsis and tobacco were ground in ice cold activating mixture and after centrifugation at 4°C, clear supernatant was activated at 25°C for 30 min. One ml of the activating mixture contained 25 mM Hepes-KOH buffer (pH 8.3), 20 mM NaHCO₃ and 20 mM MgCl₂. One ml of the Rubisco assay mixture contained 25 mM Hepes-KOH buffer (pH 8.3), 1 mM RuBP, 10 mM MgCl₂, 20 mM NaHCO₃, 3 mM DPNH, 200 units of caronic anhydrase (Sigma) and 100 μl of activated solution. The change in absorbance was read at 280 nm and activity was calculated using the extinction factor of 50 M⁻¹ cm⁻¹.