CHAPTER 3
MATERIALS AND METHODS
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3.1 RNAi mediated down-regulation of caffeine synthase in *Camellia assamica* using *Agrobacterium tumefaciens* based genetic transformation

3.1.1 Plant material

Developing and mature seeds were collected from S3.A/3 (a garden series quality clone) available at Top replacement plot, Tocklai campus, TRA, Jorhat. S3.A/3 is a standard Assam hybrid with ovate, medium sized leaves having good flavor and quality with average yield potential.

3.1.2 Explant preparation

The seeds were first dipped in water for 12 hr after which the seed coats were removed. Cotyledons obtained from the seeds were rinsed with sterile distilled water 4-6 times for 15 min. The washed cotyledons were treated with 0.3% blitox (Rallis, India) and kept in shaking condition for 30 min at room temperature. This was followed by rinsing with sterile distilled water for 5-6 times. The washed cotyledons were again dipped in 0.1% HgCl₂ and kept in shaking condition for 10 min. Subsequently, the cotyledons were washed with sterile distilled water for another 5-6 times and dried on sterile filter paper for 1hr. All the steps were performed under laminar air flow allowing the axenic cotyledons to be taken as an explant for development of somatic embryogenesis.
3.1.3 Induction of somatic embryogenesis from S3.A/3 for transformation

Surface sterilized cotyledons were separated from the embryonal axis and placed in culture tubes containing various media compositions. At different stages of culture, the following media compositions were used: Induction (IM): $\frac{1}{2}$MS salts and vitamins, 0.5mg/l BA, 2.5mg/l NAA, sucrose 20g/l; pH 5.6; Secondary Embryogenesis (SEM): $\frac{1}{2}$MS, sucrose 20g/l, 0.5mg/l BA, 0.5mg/l NAA, 1mg/l L-glutamine; pH 5.6; Germination (GM): $\frac{1}{2}$ MS salts and vitamins, 30g/l sucrose, 250 mg/l ascorbic acid, 0.5mg/l IBA, 2mg/l GA$_3$, pH 5.8; Propagation & Maintenance (PM): $\frac{1}{2}$ MS and vitamins, 0.5mg/l IBA, 2mg/l BA, 30g/l sucrose, 1mg/l GA$_3$. After inoculation, cultures were kept in the culture room having a light intensity of 37.403550 μmole$^2$sec$^{-1}$ maintained by using Photosynthetically Active Radiation (PAR) light sources and fluorescent tubes for 16 hr photoperiod and a temperature of 25±2 °C. For regular maintenance of tea somatic embryos sub culturing was done every 25 days and highly repetitive globular shaped embryos were taken for transformation work.

3.1.4 Protocol for DNA isolation

Genomic DNA was extracted from fully developed expanded leaf samples of S3.A/3 clone using cetyl Trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1987). 100 mg of leaf tissue is taken and grinded into a pre-chilled mortar in liquid nitrogen to obtain a fine powder. The powder is then transferred to an Eppendorf tube and 700μl of fresh 2X CTAB buffer and β mercaptoethanol is added and gently vortexed (Appendix 1-2). The tube is then placed in a 65 °C water bath (Pharmacia, USA) for 45 min, shaking the
tubes every 15 min. The samples are then cooled at room temperature and 700μl of chloroform: isoamyl alcohol (24: 1) is added to each tube and vortexed briefly and gently to avoid shearing the DNA. Then the tubes are inverted several times and spinned at 14,000 rpm for 5 min in a micro centrifuge. The aqueous top layer is removed and transferred to a new, labelled Eppendorf tube. To the upper aqueous layer 50μl of 10% CTAB (in 0.7 M NaCl) is added, vortexed gently, and mixed thoroughly. Then 700 μl of phenol: (chloroform: isoamylalcohol) (1:1) is added to the tubes and gently inverted several times and spinned at 14,000 rpm for 5 min. The upper aqueous layer an equal volume of 4° C isopropanol (400– 500μl) is added. The tubes are inverted several times and allowed to sit at -20° C for 30 min. The tube is then spinned at 14,000 rpm for 20 min. The supernatant is carefully discarded to avoid losing the DNA pellet. The DNA pellet is then washed with 1 ml of 70% EtOH (for 3 min) and spinned at 14,000 rpm for 30 min. The EtOH is decanted carefully and the pellet is again washed in 1 ml of 90% EtOH and spinned for 30 min at 14,000 rpm. The EtOH is decanted and the tubes are inverted and dried using a using a vacuum pump for 15 min. 10. The DNA is dissolved in 100pl of T10E1 or distilled H2O per sample and 2 μl of DNAase free RNAase A (10mg/ ml) is added and incubated at 37° C for 1 hr. The DNA is then stored at 4° C (or at -20° C, for long term storage). Fresh leaf samples of S3.A/3 clone was also taken for preparation of cDNA for which the procedure described in 3.2.4 was followed.

3.1.5 DNA gel electrophoresis

The extracted DNA sample was gel electrophoresed in 1% (w/v) agarose gel to check the quality and concentration by comparing the intensity of the bands with
100ng of uncut λ DNA size marker through a UV spectrophotometer. The concentration was also determined spectrophotometrically (Bio Photometer, Eppendorf, Germany) before storing in −20 °C. The requirements are mentioned in Appendix (3-5).

3.1.6 Designing of oligonucleotides

The primers were designed based on the sequence information of caffeine synthase gene of *Camellia sinensis* from publicly available database (ACCESSION EF526217) using PRIMER 3 OUTPUT software. The nucleotide sequence of the primers were designed having attB sequence in the 5’ direction followed by the gene specific sequence for cs in the 3’ direction as per the Gateway manual instructions is presented in table 3.1

Table 3.1- Primers used for cloning Caffeine synthase gene following Gateway Technology

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer(CSF)</th>
<th>Reverse primer(CSR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine synthase</td>
<td>5’GGGGACAAGTTTGTACA</td>
<td>5’GGGGACCACTTTGTACAAG</td>
</tr>
<tr>
<td></td>
<td>AAAAAGCAGGCTNNAAAG</td>
<td>AAAGCTGGGNTCCCCCTGT</td>
</tr>
<tr>
<td></td>
<td>GCCTGCCATCTAGTT3’</td>
<td>TTAATGCCAAG 3’</td>
</tr>
</tbody>
</table>

3.1.7 PCR amplification of partial cs gene fragment

The primers pairs used for amplification of cs were initially standardized in a number of temperatures of which the best annealing was found at 60°C giving
an amplification of product size of 800bp (genomic DNA) and 200bp (cDNA) in size. The PCR reaction was carried out for 30μl reaction volume by briefly mixing the following reaction components in a micro centrifuge tube. (Table 3.2)

Table 3.2 – Components of PCR reaction mixture

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>VOLUME(μl)</th>
<th>FINAL CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Reaction Buffer</td>
<td>3.0</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>2.1</td>
<td>1.75mM</td>
</tr>
<tr>
<td>Platinum <em>Taq</em> DNA polymerase(5U/μl)</td>
<td>0.3</td>
<td>0.05U/μl</td>
</tr>
<tr>
<td>Primer CSF(Forward)</td>
<td>1.2</td>
<td>0.2μM</td>
</tr>
<tr>
<td>Primer CSR(Reverse)</td>
<td>1.2</td>
<td>0.2μM</td>
</tr>
<tr>
<td>Template(~100ng)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>dNTPs mix (10mM)</td>
<td>0.75</td>
<td>0.25mM</td>
</tr>
<tr>
<td>Nuclease- free water</td>
<td>19.45</td>
<td></td>
</tr>
</tbody>
</table>

PCR amplification of cs containing the above contents was carried out in a Gradient Mastercycler (Eppendorf, Manheim, Germany) in a reaction condition of 94 °C for 2 min followed by 35 cycles of 3 temperature holds 1st at 94 °C for 30 sec, 2nd at 60 °C for 30 sec (annealing temp.) and 3rd at 72 °C for 1 min, the three holds were followed by a final extension at 72 °C for 10 min.
3.1.8 Purification of cs amplicon

PCR product of 30 μl was gel electrophoresed in a 1.3% agarose gel and the size of the partial fragment of cs was checked by comparing with a 100bp DNA ladder (New England BioLabs, Beverly, Mass). The amplicon was purified using GenElute PCR Clean Up Kit (Sigma, USA) following manufacturer's instructions.

3.1.9 RNAi construct preparation

Cloning of Caffeine synthase gene fragment

PCR generated partial fragment of cs amplicon was taken for two steps of ligation independent cloning by Gateway Technology involving two simple reactions. The first recombination reaction, is the BP reaction which according to the manufacturer instructions requires an overnight recombination reaction in the presence of BP Clonase enzyme mix at 25°C water bath (Pharmacia, USA) between the amplicons (150ng) with attB sites along with pDONR221 vector (containing attP sites and ccdB gene) (table 3.3). This step generates an entry clone containing attL sites and a ccdB fragment by-product containing attR sites. The recombination reaction was terminated by adding 1μl of proteinase K (2μg/μl) solution followed by an incubation at 37°C for 10min. This was followed by transformation into DH10β chemical competent cell line (3.1.10). After selection of appropriate entry clone with antibiotic (kanamycin 50 μg/ml) single colony was selected for plasmid extraction followed by sequencing and PCR with gene specific primer. The second recombination reaction, i.e. LR Reaction, takes place between the selected entry clone, pDONR221 (containing
the *cs* gene along with *attL* sites (Appendix 10) and a destination vector pHellSGATE8 (containing *attR* sites and *ccdB* gene, provided by CSIRO, Australia) (Appendix 11) in the presence of enzyme LR Clonase, at 25 °C overnight. This step generates an expression clone containing the gene insert with *attB* sites and a by-product containing the *ccdB* gene with *attP* sites. The reaction was terminated by adding 1 μl of proteinase K (2 μg/μl) solution and a similar incubation at 37 °C for 10 minutes as used in BP reaction. The recombined product thus generated was transformed into DH10β cell line and selected in spectinomycin (50 μg/ml) following which the selected clones were checked with restriction mapping with the appropriate restriction enzymes.

### Table 3.3- Reaction components used during BP and LR recombination reactions of Gateway Technology

<table>
<thead>
<tr>
<th>Components</th>
<th>Sample (in μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB-PCR product (80ng)</td>
<td>3</td>
</tr>
<tr>
<td>pDONR221 (150 ng/μl)</td>
<td>3</td>
</tr>
<tr>
<td>5X BP Clonase buffer</td>
<td>2</td>
</tr>
<tr>
<td>BP Clonase II enzyme mix</td>
<td>2</td>
</tr>
</tbody>
</table>

(1 μl of proteinase K is added and kept at 37°C for 10 min to terminate the reaction)
LR Recombination reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample (in µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry clone (50-150 ng/reaction)</td>
<td>3</td>
</tr>
<tr>
<td>Destination vector (150 ng/µl)</td>
<td>3</td>
</tr>
<tr>
<td>5X LR clonase buffer</td>
<td>2</td>
</tr>
<tr>
<td>LR clonase enzyme mix</td>
<td>2</td>
</tr>
</tbody>
</table>

(1 µl of proteinase K is added and kept at 37°C for 10 min to terminate the reaction)

Fig-3.1 Flow chart of steps followed for preparing the RNAi construct of Caffeine synthase.
3.1.10 Transformation of the recombination product

Preparation of chemically competent cells using INOUE method

The method of competent cell preparation as mentioned in Sambrook et al., (2000) was followed with slight modification. For a starter culture, single colony of DH10β was picked from plate and grown in a 150ml conical flask containing 30 ml LB broth at 37 °C incubation and 120 rpm overnight. About 50 µl from the starter culture was transferred into 150ml freshly prepared LB broth in a 250ml conical flask and cultured at 37 °C in a shaker incubator at 120 rpm. The OD$_{600}$ was determined periodically in a Spectrophotometer (BioPhotometer, Eppendorf, Germany) to monitor growth of the cell and as soon as the OD$_{600}$ reaches 0.5-0.6, the cultured cells were transferred aseptically into a 25ml centrifuge tube and kept in ice for 30 min. The cells were recovered from the media by centrifugation at 4000rpm for 10min at 4 °C. The media was discarded and the pellet thus formed was resuspended in 20ml of ice cold INOUE buffer (Appendix. 6) and stored in ice. This was followed by another round of centrifugation at 4000 rpm for 10min at 4 °C. The supernatant was discarded and the tube containing the cell pellet were kept in an inverted position for 30 sec to allow the last traces of buffer to drain away. The cell pellet was resuspended in 500µl of INOUE buffer and gently mixed with 0.75 µl DMSO. Aliquots of 30 µl cells were prepared in a 0.5ml sterile micro centrifuge tube, sealed with paraffin and snap chilled with liquid nitrogen for storage at -80 °C till further use.
Transformation of competent cells

The recombined product generated at each stage of BP and LR reactions are transformed with the competent cells prepared, for this the micro centrifuge tube containing 30 μl competent cells were taken out from -80 °C and kept in ice for 5 min. About 5 μl of recombinant product was added to the micro centrifuge tube, gently swirled and kept in ice for 30 min. Another aliquot of competent containing no recombinant product was taken as control. The tubes were transferred to a 42 °C water bath (Pharmacia, USA) to subject the cells for heat shock for 42 sec. The tubes were transferred to ice and allowed to chill for 1 min. This was followed by addition of 1 ml of SOC medium (Appendix 8) to each tube and cultured at 37 °C for 2 hr in a shaker incubator set at 225 rpm.

Selection of transformants

After each transformation event (i.e., BP and LR reaction) approximately 50 μl of transformation mix was selected in LB agar (Appendix 7) plates with two different antibiotics, kanamycin 50 μg/ml for BP reaction and spectinomycin 50 μg/ml for LR reaction and incubated at 37 °C for 16-18 hr. Hundreds of colonies were obtained from plates containing the recombined product while control plates showed no growth with antibiotic selection.

3.1.11 Isolation of plasmid DNA

The protocol for midi prep plasmid isolation mentioned in Sambrook et al (2000) was followed with slight modification. For this among hundreds of colonies obtained after each transformation event, single colony was picked and inoculated in 2 ml LB broth containing appropriate antibiotic for overnight
growth at 37 °C and 120rpm. The overnight culture was transferred into a 1.5ml micro centrifuge tube and centrifuged at 6000 rpm for 5 min. The supernatant was discarded and 100 μl of solution I was added, vortexed until no clumps were observed. The tube was kept in ice for 5min. Freshly prepared alkaline solution (solution II) of 200 μl was added into the tube and mixed gently by inverting the tube several times until the cell suspension becomes clear. The tube was kept in ice for 5min. This was followed by addition of 150 μl of ice cold solution III to the tube and inverted gently. The tube was centrifuged at 12,000rpm for 10min and the supernatant thus obtained was transferred into a fresh tube. Equal volume of phenol-chloroform was added to the tube and mixed by inverting the tube several times. The tube was then centrifuged at 12,000rpm for 10min after which two phases are obtained. The upper aqueous phase was carefully pipetted and transferred into a fresh tube into which 2 volumes of absolute ethanol was added. The tube was kept at -20 °C for 15min precipitation and centrifuged at 12,000rpm for 10min. The supernatant was discarded and a whitish pellet was obtained to which 500 μl of 70% ethanol was added, centrifuged for 10min. The supernatant was discarded and the tube was air dried. Pellet obtained was dissolved in 20 μl of nuclease free water and the purity of the sample was checked by 0.8% agarose gel electrophoresis.

3.1.12 Analysis of transformants

The transformants obtained after BP reaction were analyzed after isolating plasmid DNA and sequencing the clones. Plasmid obtained from transformants after LR reaction were checked for the presence of the insert by restriction enzyme digestion with Not I enzyme and PCR with gene specific primers.
**Sequencing of the clones**

Sequencing of the extracted plasmids carrying the insert (positive clones) after BP reaction was carried out using M13F and M13R primers by dideoxy chain termination method (Sanger *et al.*, 1977) in GA3130xl Genetic Analyzer (Applied Biosystems, CA, USA) using BigDye Terminator V3.1 cycle sequencing Kit (Applied Biosystems) following the instructions given in the manual. After removing the vector sequences using Sequence Scanner software (Applied Biosystems) the selected sequences are considered for further analysis using the BLASTX programme at the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) at default parameters against non-redundant (nr) protein database.

**Restriction digestion of destination vector for insert release**

The construct was prepared as per the instructions from the manufacturer of Gateway Technology (Invitrogen Co, Carlsbad, CA, USA). 1 μl of the resulting destination vector pHELLSGATE8 –cs construct (~1204 ng/μl) was digested with 0.2 μl (10u/μl) of NotI enzyme (NEB, New England BioLabs, Beverly, Mass) at 37 °C for 3 hr in a 20 μl reaction volume containing 2 μl 10X NEB Buffer 3 and 0.2 μl BSA (100μg/ml) . The digested product was gel electrophoresed in a 1.2% agarose gel along with undigested plasmid of pHELLSGATE8 and DNA ladder. The digested plasmid containing the desired fragment of cs along with vector sequences was eluted from the gel using GenElute PCR Clean Up Kit (Sigma, USA).
3.1.13 Dephosphorylation of binary vector, pART27

Hundred nanogram (ng) of freshly extracted plasmid of pART27 (fig 3.2) transformed in DH10β chemical competent E.coli cells (selected on LB spectinomycin 50 μg/ml) was digested with 0.01μl (10u/μl) of Not I enzyme (NEB, New England BioLabs, Beverly, Mass) in a 30 μl reaction volume containing 3 μl 10X NEB Buffer 3 and 0.3 μl BSA (100μg/ml) at 37 °C for 3 hr. Dephosphorylation of Not I digested pART27 was carried out using 1.5 μl of Calf Intestinal Phosphatase (CIP) (Invitrogen, USA) at 37 °C for 30 min in a micro centrifuge tube. This was followed by deactivation of CIP and purification. For this, to the contents of the micro centrifuge tube 0.25 μl of EDTA (0.5M), 0.5% of SDS (2%), 100 μg/ml of proteinase K was added and incubated at 65 °C for 30 min in a water bath (Pharmacia, USA), after which the reaction was allowed to cool at room temperature. An equal volume of phenol: chloroform: Isoamylalcohol (PCI) was added to the reaction mixture and centrifuged at 14000rpm for 15 min. This step was repeated again with PCI taking the supernatant after the centrifuge 1/10 volume of sodium acetate and 2.5 volume of ethanol was added to the supernatant mixed properly for precipitation and kept at -20 °C for 30 min incubation. After the incubation is over the reaction mixture was centrifuged at 14000rpm for 20 min at room temperature. Pellet obtained thereafter was washed with 70% ethanol, air dried and dissolved in water.
3.1.14 Ligation and transformation

Twenty nanogram (ng) of the NotI digested insert released from plasmid of pHELLSGATE8 – cs was taken for ligation into approximately 50ng of NotI digested and dephosphorylated binary vector, pART27 (CSIRO, Australia) having nptII as plant selectable marker. Ligation reaction was done in a sterile micro centrifuge tube containing, 1X Ligase buffer and 1 μl of T4 DNA ligase (3U/ μl) in a 20 μl reaction volume at 16 °C for 12 hr. The enzyme was inactivated by heating the reaction at 60°C for 10min. The ligation mix was added in a 100 μl suspension of chemical competent cells of DH10β, E.coli cells mixed gently by swirling the micro centrifuge tube and kept in ice for 30 min (Sambrook et al., 2000). The tube was transferred to a 42°C water bath (Pharmacia, USA) for 45 sec to give heat shock to the cells and rapidly transferred to a ice to give chilling treatment to the cells for 1-2 min. The transformation mix was resuspended in 1ml SOC media and incubated at 37°C.
for 45 minutes in shaking incubator at 225 rpm. Only 100 µl of the transformation mix was selected on pre-warmed LB agar plates containing spectinomycin 50 µg/ml and incubated at 37°C for approximately 16 hr.

Analysis of the transformants

The transformation reaction yielded more than hundred number of colonies out of which single colony was selected from the plate for checking the presence of desired plasmid containing pART27-cs by isolating plasmid DNA by midi-prep plasmid extraction (Sambrook et al., 2000). Quality of the plasmid was checked by performing 1% agarose gel electrophoresis and analyzed by taking it as a template for PCR with gene specific primers of cs and restriction enzyme digestion, at EcoRV (at 37°C) sites to check insert incorporation prior Agrobacterium mediated transformation. The pART27-cs contains a gateway cassette in two orientations with a PDK intron as spacer in between, thereby forming an intron containing hpRNA construct. The pART27-cs construct also contained CaMV35S promoter, OCS terminator along with spectinomycin as bacterial selection and nptII as plant selectable marker. The hpRNA construct so formed was thereafter lodged into chemically competent Agrobacterium tumefaciens strains of LBA 4404 and selected in YMB plates (Appendix 9) with spectinomycin 50(µg /ml) and rifampicin (50 µg/ml). Spectinomycin will select the colonies having the plasmid pART27-cs in it and rifampicin will select for the agrobacterium colonies which are resistant to it.
3.1.15 Preparation of the inoculum, transforming & regeneration of tea somatic embryos with gene construct of Caffeine synthase

Transformed colonies of *Agrobacterium* strain, LBA 4404 were checked by colony PCR with gene specific primers of *cs*. The screened colony was grown in YMB media (LBA 4404) media containing spectinomycin 50µg/ml for 48 hr at 28°C shaking incubator at 120 rpm and taken for isolation of plasmid DNA and restriction analysis with EcoRV. After confirmation, single selected colony was grown in a conical flask containing 25 ml YMB media (Appendix 9) containing spectinomycin 50 µg/ml for 48 hr at 28°C shaking incubator at 120 rpm. The cell suspension were taken out after two days and acetosyringone (400µM) was added (Joseph Lopez *et al.*, 2004) for virulence induction and kept in 28°C shaking incubator for about 2hrs at 120rpm.On the other hand, the somatic embryo of SA.3/3 clone, were sliced into small sections in a petriplate and *Agrobacterium* cell suspension cultures were poured into the petriplates and kept for about 45min at RT. The infected somatic embryos were recovered from bacterial suspension and placed on sterile filter paper above which L-glutamine (0.5mg/ml) was added for co-cultivation and sealed properly. After 36hr of co-cultivation the infected somatic embryos was washed several times (about 10 times) with sterile water to check bacterial overgrowth and air dried for 15min under laminar air flow. The somatic embryos were then cultured on somatic embryogenesis media (SEM) supplemented with cefotaxime (400 µg/ml) and kanamycin 15mg/ml. After 25 days from the date of inoculation development of secondary embryos could be seen after which they were sub-cultured in SEM media with decreasing cefotaxime concentration (200µg/ml, 100µg/ml,
50\mu g/ml) and increasing kanamycin concentration (25\mu g/ml, 35\mu g/ml, 50\mu g/ml). The antibiotic selected somatic embryos were transferred to SEM media without containing any cefotaxime and maintained there for two months. Following which the somatic embryos were transferred onto regeneration media and maintained for about 8 months by repeated sub culturing till plantlet stage containing kanamycin 50\mu g/ml as plant selection. The regenerated putative transformants were transferred to multiplication media containing kanamycin (50\mu g/ml) for multiplication. After the development of the plantlets the leaves were collected for further downstream analysis.

3.1.16 Experimental design

About 300-400 somatic embryos were inoculated with A. tumefaciens carrying the cs-RNAi construct for regeneration and subsequent antibiotic (kanamycin 50\mu g/ml) selection. The putative transgenic secondary somatic embryos were selected with increasing concentration of kanamycin (30, 40, 50 \mu g/ml) till plantlet stage. Throughout our experiment untransformed somatic embryos (without the RNAi construct) cultured in embryogenesis and regeneration media were taken as negative control. The protocol for transformation and regeneration already standardized at Biotechnology dept., TRA, in tea was followed.

3.1.17 PCR Detection of putative transformants

For molecular analysis of the putative transformants PCR was performed with nptII gene specific and vector T-DNA specific primers (P1P2 and P3P4) taking the isolated genomic DNA from control and 7 transgenic plants using CTAB method as described by Doyle and Doyle (1987) method. The primer pairs used
to check introgression were specifically designed encompassing both the sense and antisense fragment of vector T-DNA. The arrangement of T-DNA along with primer position is shown in the (fig 4.8). The primers were standardized in Gradient Mastercycler (Eppendorf, Manheim, Germany) and the combinations are mentioned in the table. (Appendix 12) PCR reaction mixture (20μl) included 10 pmol forward primer, 10 pmol reverse primer, 50 ng plant genomic DNA as template, 2 μl 10X Taq DNA polymerase buffer, 0.25 mmol/l dNTPs, 1 U Taq DNA polymerase (Bioline, London, UK). After amplification the PCR products were separated in a ethidium bromide stained 1% agarose gel and compared with a DNA ladder (New England BioLabs, Beverly, Mass).

3.1.18 Quantitative real time PCR analysis

Total RNA extraction

Total RNA was isolated from about 20-25mg of regenerated leaf samples and ground in liquid nitrogen with sterile mortar and pestle. Grounded frozen powder was transferred to micro centrifuge tube and total RNA was extracted using RNAqueous Kit (Ambion, USA, Cat. No. AM1912) following manufacturer’s protocol. The extracted total RNA was gel electrophoresed in 1% agarose gel and checked for integrity, purity and quantity. The concentration was also estimated by Spectrophotometer (Eppendorf, Germany).

mRNA isolation and first strand cDNA synthesis

The first strand synthesis for the samples were carried out for seven samples using transcriptor first strand cDNA synthesis kit (Roche, Germany) following the manufacturers protocol. For this, 3μl of total RNA (100ng/μl) was taken as
the starting material and gently mixed with 0.25μl of anchored-oligo (dT)18 primer in a 0.2 ml nuclease free PCR tube and incubated at 65 °C for 10 min in a thermal cycler (ABI 2720, Applied Biosystems, CA, USA) to denature RNA secondary structure. The tube was immediately chilled on ice and subsequently mixture of 1 μl 5X reverse transcriptase reaction buffer, 0.12 μl protector RNase inhibitor (40 U/ μl), 0.5 μl deoxynucleotide mix (10 mM each) and 0.12 μl transcriptor reverse transcriptase (20 U/ μl) were added and incubated at 50 °C for 1 hr in a thermal cycler (ABI 2720, Applied Biosystems, CA, USA). The Moloney Murine Leukaemia virus (MMLV) reverse transcriptase having RNaseH activity used for cDNA synthesis was deactivated by heating the tube at 85°C for 5 min. The first strand was stored at -20°C for downstream application.

**Primer designing and standardization**

Primer designing for cs was done using freely available primer3 software (http://frodo.wi.mit.edu/primer3/) using the sequence output of pDONR221 containing cs fragment (after BP reaction) in GA3130x1 Genetic Analyzer (Applied Biosystems, CA, USA). The primers used in real time, RTCSF and RTCSR (BioServe, India) (Appendix. 12) were specific and primarily designed from the ORF obtained after cloning of the cs fragment in pDONR221 using ORF finder. The primers were standardized in Gradient Mastercycler (Eppendorf, Germany) at 94 °C for 2 min followed by 35 cycles of 3 temperature holds 1st at 94 °C for 30 sec, 2nd at 60 °C for 30 sec (annealing temp.) and 3rd at 72 °C for 30 sec, the three holds were followed by a final extension at 72 °C.
for 7 min and the standardized primer specific PCR profiles was used in qRT-PCR analysis.

**Internal control genes**

Four housekeeping genes, *Camellia* tubulin, 18S rRNA, 26s rRNA and Ribulose 1,5 bis phosphate carboxylase/oxygenase were selected from public databases NCBI and DNA Data Bank of Japan (DDBJ) and the sequences were retrieved for primers designing using primer3 (http://frodo.wi.mit.edu/primer3/) software. All the primers were standardized before qRT-PCR analysis in a Gradient Mastercycler (Eppendorf, Germany). The PCR products when runned on a 1% agarose gel gave single amplicon of desired size for all the four genes at specific annealing condition. We have tested the expression levels of the four genes in experimental and control samples with the standardized PCR profiles for qRT-PCR analysis. The detailed information related to gene sequences, accession number and primer designing can be found in Appendix (13)

**Quantitative real time PCR for expression of Caffeine synthase gene**

The gene transcription level was determined by LightCycler 480 II real time machine (Roche, Germany) using LightCycler 480 SYBR green I Master kit (Cat. No. 04 707516 001, Roche Diagnostics, Germany). The PCR reaction was prepared in ice taking 0.4mM of RTCS forward and 0.4mM RTCS reverse primers and 4μl ROCHE Light Cycler 480 SYBR green I Master mix. After making the final volume to 4.7 μl with water 0.5 μl first strand cDNA template, was added to the multi-well plate and sealed with LightCycler 96 multiwell sealing foil. For each reaction two technical replicates were used to reduce the
error rate. Reactions were pre-incubated at 95 °C for 5 min, following which amplification occurred in three steps: 10 sec of denaturation at 95 °C, 10 sec of annealing at 60 °C, 10 sec of extension at 72 °C with a total of 45 cycles. This was followed by one cycle of melting curve analysis to check specificity of amplified product. The temperature for melting curve analysis was 95 °C for 5 sec, 65 °C for 1 min and 97 °C for continuous acquisition followed by one cycle of final cooling at 4 °C.

Real time data acquisition

The expression levels of each sample in an amplification reaction were obtained in the form of crossing point (Cp) at which visible fluorescence is obtained in an optimum number of PCR cycles to reach a threshold transcript. At this point as the transcripts start to accumulate in a particular PCR cycle (Ct) fluorescence starts to emit which is optimum enough for the camera to capture. The data for all the samples analyzed was obtained using 2nd derivative maximum method as computed by the software of Light Cycler (Roche Diagnostics) Carousel-based system.

Normalization factor of reference genes

For calculating normalization factor, at first the raw Cp values obtained from qRT-PCR of all the housekeeping genes are converted into relative quantities using delta Ct method. After which, the values obtained were computationally analyzed using geNorm software to find out the most stable genes among the set of housekeeping genes to calculate a gene expression normalization factor based on a geometric mean of user defined reference genes analyzed. As a result
unstable genes are eliminated from the group of housekeeping genes and at the end only two most stable reference genes remain.

**Relative quantification analysis**

For relative quantification assays comparison of two different target sequences are done with a single sample. One of the target sequences refers to the gene of interest and the other a constitutively expressed gene or a reference gene. Expression of a target gene in a particular sample is expressed as the ratio of target-to-reference gene in the same sample. Here the mean Cp values of target gene considered in all the experimental and control samples were transformed into relative quantities using delta Ct method. The normalized expression levels of each experimental and control samples were calculated by dividing the quantities by their respective normalization factor. The normalized expression of a target gene thus obtained was compared within the experimental and control samples to know the relative expression level.

**3.1.19 Absolute quantification**

**Primer design and standardization**

The oligonucleotide primers were designed with freely available software primer3 (http://frodo.wi.mit.edu/primer3/) to perfectly match the sequences of nptII and pal. The nptII is a single copy gene of pART27 (Gleave, 1992) and pal is a single copy gene in tea (Kaundun et al., 2003). The detailed information related to primer sequences can be found in Appendix (12). Two separate PCR reaction tubes contained templates of 20ng extracted plasmid of pART27 (for nptII) and 50ng of chromosomal DNA of S.3A/3 (for pal) respectively. The PCR
reaction mixture for standardization of primers in each 20μl reaction included 10 pmol forward primer, 10 pmol reverse primer, 2μl 10X Taq DNA polymerase buffer, 0.25mmol/l dNTPs, 1 U Taq DNA polymerase (Bioline, London, UK). Standardization was carried out in a thermal cycler (ABI 2720, Applied Biosystems, CA, USA) at 94°C for 2 min followed by 35 cycles of 3 temperature holds 1st at 94°C for 30 sec, 2nd at 55°C for 30 sec (annealing temp.) and 3rd at 72°C for 30 sec, the three holds were followed by a final extension at 72°C for 7 minutes. The reaction product gave an amplification at 153bp (approx.) for nptII and 130bp (approx.) for pal and the standardized primer specific PCR profiles was used in qRT-PCR analysis.

Preparation of template DNA for real time PCR:

Approximately 50mg tissue was taken from 4 numbers of kanamycin selected regenerated T₀ plantlets for genomic DNA extraction using cetyl Trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1987). The selected number of regenerated plantlets were initially checked by PCR for presence of transgene followed by transcript determination by qRT-PCR. The template DNA extracted was normalized to 2ng/μl with nuclease free water for consistent qRT-PCR assay and analyzed to quantify nptII and pal in duplicates. Spectrophotometric readings of extracted genomic DNA was measured at 260 nm, while DNA purity was evaluated on the basis of the UV absorption ratio at 260/280 nm and analyzed by 1% agarose gel electrophoresis in 0.5X TAE with ethidium bromide staining.
Preparation of standard curve for absolute quantification

PCR generated fragment of _pal_ was used for cloning into vector pART27. Purified plasmid from the transformed cell lines of DH10β harboring pART27- _pal_ was obtained using alkaline lysis method. The resulting plasmid now contains two separate sequences specific for _nptII_ and _pal_ and can be detected easily using either of the primer sets _nptII_ or _pal_. The plasmid was denoted as pART27-NP. The purity of the extracted plasmid was checked after electrophoresing in an ethidium bromide stained 1% agarose gel and quantified with a spectrophotometer (Eppendorf). The extracted plasmid was serially diluted ranging from a concentration of 1 x 10^2, 1 x 10^4, 1 x 10^6, 1 x 10^8 and 1 x 10^{10} copies/μl and used to construct the standard curves for both _nptII_ and _pal_. Copy number of the extracted plasmid was calculated using the equation given below. (Whelan et al., 2003; Lee et al., 2005)

\[
\text{DNA (copy)} = 6.02 \times 10^{23} \text{(copy/mol)} \times \text{DNA amount (g)} \\
\text{DNA length (dp)} \times 660 \text{(g/mol/dp)}
\]  

Real time PCR with _nptII_ and _pal_ primer sets generated _C_p_ values in each dilution and the values were measured in duplicates to generate standard curves for _nptII_ and _pal_ respectively. The _C_p_ values were plotted against the logarithm of their initial template copy numbers. Standard curve of each was generated by a linear regression of the plotted points. From the slope of each standard curve, PCR amplification efficiency (E) was calculated according to the equation (Rasmussen, 2001):

\[
E = 10^{-1/slope} - 1
\]
Real time PCR conditions

The expression levels were obtained in the form of crossing point (Cp), the point where the sample’s fluorescence curve turns sharply upward. The data analysis were performed using the 2nd derivative maximum method as computed by the software of LightCycler Carousel-based system. RT-PCR was carried out in a LightCycler 480 II real time machine (Roche, Germany) for a 10μl reaction using LightCycler 480 SYBR green I Master kit (Cat. No. 04 707 516 001, Roche Diagnostics, Germany). The reaction mixture also contained 0.4mM nptII forward and 0.4mM nptII reverse primers with 20 ng template. The amplification condition consisted of 5 min at 95°C, followed by 45 cycles of denaturation (95°C, 10sec), annealing for 30sec at 55°C and extension at 72°C for 30sec. SYBR Green I dye typically binds to double-stranded DNA and emits fluorescence in a sequence-independent way. As a result both specific and non-specific PCR products are detected and therefore for accuracy in quantification non-specific PCR products are avoided. This was done with melting curve analysis of the double stranded DNA which causes a sharp reduction in the fluorescence signal around the melting temperature (Tm) of the PCR product, resulting in a clear peak in the negative derivative of the melting curve (−dF/dT). The cycling was followed by one cycle of melting curve analysis to check specificity of amplified product. The temperature for melting curve analysis was 95°C for 5 sec, 65°C for 1 min and 97°C for continuous acquisition. This was followed by one cycle of final cooling at 4°C. Each sample taken for analysis was estimated twice.
3.1.20 Biochemical analysis using HPLC

Biochemical estimation of the experimental and control plants were performed following the ISO method. This method specifies high performance liquid chromatographic method of separation determined on a phenyl bonded column using gradient elution with UV detection at 278 nm. In the present study, binary gradient elution system was used with a thermostatically controlled column compartment and a UV detector set at 278 nm. This system prefers to use phenyl bonded phases that gives additional selectivity over reverse phase materials and results in improved resolution. In the international standard procedure followed, the chromatographic condition and composition of the mobile phase specified are suitable for a phenomenex-LUNA 5 μm phenyl hexyl column of dimensions 250 mm x 4.6 mm fitted with 4 mm x 3.0 mm phenyl hexyl cartridge. External standards are used for quantification. External caffeine and catechins standards of defined purity and moisture are used (HPLC mobile phase composition, caffeine stock standard solution, catechin stock standard solution, preparation of test sample in Appendix. 16-18). Once the flow rate of mobile phase and temperature are stable, conditioning of the column was done with a blank gradient run. Mixed standard solutions A & B (Appendix. 22) of 10 μl each was injected into column followed by an equal volume of the diluted test solution. Repeated injection of the mixed working standard solution at regular intervals was performed. Data was collected for all the peaks in the mixed standards and test sample solutions. Quantification is performed by comparing the retention time from sample chromatograms with those obtained from the mixed standard solutions, under the same chromatographic conditions.
3.2 *Agrobacterium tumefaciens* mediated silencing of *n*-methyltransferase gene in *Camellia assamica*

3.2.1 Plant material

Seeds were collected from S3.A/3 clone, grown at Top replacement plot, Tocklai Camp's, TRA, Jorhat. S3.A/3, a standard Assam hybrid has a very good flavor and quality with average yield potential.

3.2.2 Explant preparation

The seeds were initially immersed in water for 12 hr. following which the seed coats were removed. Cotyledons obtained from the seeds were rinsed with sterile distilled water 4-6 times for 15 min. The washed cotyledons were treated with 0.3% blitox (Rallis, India), an antifungal agent and kept in shaking condition for 30 min at room temperature. This was followed by rinsing with sterile distilled water for 5-6 times. Washed cotyledons were again dipped in 0.1% HgCl₂ and kept in shaking condition for 10 min. The cotyledons were subsequently washed with sterile distilled water for another 5-6 times and dried on sterile filter paper for 1 hr. All the steps were performed under laminar air flow maintaining sterile conditions. The axenic cotyledons were taken as an explant for development of somatic embryogenesis.

3.2.3 Induction of somatic embryogenesis from S3.A/3 for transformation

Cotyledons obtained after sterilization were separated from the embryonal axis and placed in culture tubes containing half strength Murashige and Skoog's medium, as reported in other varieties of *Camellia* (Mondal et al., 2001). Various media compositions were used at different stages of culture with
following compositions: Induction media (IM): ½MS salts and vitamins, 0.5mg/l BA, 2.5mg/l NAA, sucrose 20g/l; pH 5.6; Secondary Embryogenesis media (SEM): ½MS; sucrose 20g/l, 0.5mg/l BA, 0.5mg/l NAA, 1mg/l L-glutamine; pH 5.6; Germination media (GM): ½ MS salts and vitamins, 30g/l sucrose, 250 mg/l ascorbic acid, 0.5mg/l IBA, 2mg/l GA₃, pH 5.8; Propagation & Maintenance media (PM): ½ MS and vitamins, 0.5mg/l IBA, 2mg/l BA, 30g/l sucrose, 1mg/l GA₃. For regular maintenance the cultures were kept for 16 hr photoperiod and a temperature of 25±2 °C. Somatic embryos thus obtained were sub cultured every 25 days and highly repetitive globular shaped embryos were taken for transformation work.

3.2.4 Protocol for cDNA isolation

RNA isolation- Fully developed expanded leaf samples of S3.A/3 clone were ground in liquid nitrogen with sterile mortar and pestle. Grounded frozen powder was transferred to micro centrifuge tube and total RNA was isolated following RNAqueous kit (Ambion, USA, cat no. AM1912) procedure. RNA integrity, purity and quantity were checked by running on 1.2% agarose gel and concentration was measured using BioPhotometer (Eppendorf, Germany).

mRNA isolation and first strand cDNA synthesis- From total RNA 3µl (100ng/µl) was taken as the starting material and gently mixed with 0.25µl of anchored-oligo (dT) 18 primer in a 0.2 ml nuclease free PCR tube and incubated at 65 °C for 10 min in a thermal cycler (ABI 2720, Applied Biosystems, CA, USA) to denature RNA secondary structure. The tube was immediately chilled on ice and subsequently mixture of 1 µl 5X reverse transcriptase reaction buffer, 0.12 µl protector RNase inhibitor (40 U/µl), 0.5 µl deoxynucleotide mix (10 mM each)
and 0.12 μl transcriptor reverse transcriptase (20 U/μl) were added and incubated at 50 °C for 1 hr in a thermal cycler (ABI 2720, Applied Biosystems, CA, USA). The Moloney Murine Leukaemia virus (MMLV) reverse transcriptase having RNase H activity used for cDNA synthesis was deactivated by heating the tube at 85°C for 5 min. The first strand was stored at -20°C for downstream application.

### 3.2.5 DNA gel electrophoresis

Gel electrophoresis of the extracted DNA sample was done in 1% (w/v) agarose gel and concentration was also determined spectrophotometrically (BioPhotometer, Eppendorf, Germany) before storing in –20 °C.

### 3.2.6 Designing of oligonucleotides

The primers were designed based on the available sequence information of n-methyltransferase gene of _Camellia sinensis_ from publicly available database (ACCESSION AX138776) using PRIMER 3 OUTPUT software. The nucleotide sequence of the primers were designed having attB sequence in the 5’ direction followed by the gene specific sequence for nmt in the 3’ direction as per the Gateway manual instructions is presented in table 3.4

**Table- 3.4 Primers used for cloning nmt gene following Gateway Technology.**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer(MTF)</th>
<th>Reverse primer(MTR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyltransferase</td>
<td>5’GGGGACAAGTTTGTACA</td>
<td>5’GGGGACCACTTTGTACAAG</td>
</tr>
<tr>
<td></td>
<td>AAAAACAGGCTNNGGC</td>
<td>AAAGCTGGGTNCAGCAATGG</td>
</tr>
<tr>
<td></td>
<td>CTGTCGTCTGAGTTATT</td>
<td>CCATAGCTAATA 3’</td>
</tr>
</tbody>
</table>

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3.2.7 PCR amplification of partial nmt gene fragment

The primers pairs used for amplification of nmt were initially standardized in a number of temperatures of which the best annealing was found at 65 °C giving an amplification of product size 430bp in size. The PCR reaction was carried out for 30μl reaction volume by briefly mixing the following reaction components in a micro centrifuge tube (Table 3.5).

Table- 3.5 Reaction components of PCR used during the study.

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>VOLUME(μl)</th>
<th>FINAL CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Reaction Buffer</td>
<td>3.0</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>2.1</td>
<td>1.75mM</td>
</tr>
<tr>
<td>Platinum Taq DNA polymerase(5U/μl)</td>
<td>0.3</td>
<td>0.05U/μl</td>
</tr>
<tr>
<td>Primer MTF(Forward)</td>
<td>1.2</td>
<td>0.2μM</td>
</tr>
<tr>
<td>Primer MTR(Reverse)</td>
<td>1.2</td>
<td>0.2μM</td>
</tr>
<tr>
<td>Template(~100ng)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>dNTP mix (10mM)</td>
<td>0.75</td>
<td>0.25mM</td>
</tr>
<tr>
<td>Nuclease- free water</td>
<td>19.45</td>
<td></td>
</tr>
</tbody>
</table>

Taking above reaction mixture in a PCR tube amplification of nmt was carried out in a Gradient Mastercycler (Eppendorf, Manheim, Germany) in a reaction condition of 94 °C for 2 min followed by 35 cycles of 3 temperature holds 1st at 94 °C for 30sec, 2nd at 65 °C for 30 sec (annealing temp.) and 3rd at 72 °C for
1 minute, the three holds were followed by a final extension at 72 °C for 10 minutes.

3.2.8 Purification of nmt amplicon

Gel electrophoresis of 30 μl PCR product was carried out in a 1.3% agarose gel and the size of the partial fragment of nmt was checked by comparing with a 1kb DNA ladder (NEB, New England BioLabs, Beverly, Mass). The amplicon was purified using GenElute PCR Clean Up Kit (Sigma, USA) following manufacturer’s instructions.

3.2.9 RNAi construct preparation

Cloning of n-methyltransferase gene fragment

The sequence selected for cloning was amplified with gene specific primers and purified to obtain RNAi constructs through Gateway® technology (http://www.invitrogen.com). It involved two basic steps; cloning attB-flanked PCR product into entry vector (pDONR221) by BP reaction, and shuttling cloned fragment from entry vector to destination vector (pWATERGATE) (Fig 3.3) by LR reaction. The reaction components used during the reaction is mentioned in table 3.6. For BP reaction, about 120 ng attB-PCR product, 140 ng of pDONR221 vector and 2 μl of 5X BP Clonase reaction buffer were added to a 0.5 ml centrifuge tube. 2 μl of BP Clonase II enzyme mix was added to the tube (containing PCR product, pDONR221 vector and 5X BP Clonase reaction buffer) and the contents were mixed well. After a short spin in centrifuge, the tubes were incubated at 25 °C water bath (Pharmacia, USA) for overnight. The reaction was terminated by adding 1 μl of proteinase K (2 μg/μl) solution
followed by incubation in a water bath at 37 °C for 10 minutes. The recombination product thus obtained was transformed into DH10β cell line. Clones selected after kanamycin (50 µg/ml) selection were also checked by sequencing and restriction digestion following which it was considered for entry clone in the next cloning step. The entry clone thus obtained contains attL sites and a ccdB fragment by-product containing attR sites. The second recombination reaction, i.e. LR Reaction, takes place between the selected entry clone, pDONR221 (containing the nmt gene along with attL sites) (Appendix 10) and a destination vector pWATERGATE (containing attR sites and ccdB gene, provided by CSIRO, Australia) in the presence of enzyme LR Clonase, at 25 °C water bath for overnight. This step generates an expression clone containing the gene insert with attB sites and a by-product containing the ccdB gene with attP sites. The recombined product thus generated was terminated by adding 1 µl of proteinase K (2 µg/µl) solution and a similar incubation at 37°C water bath for 10 minutes as used in BP reaction. This was followed by transformation into DH10β cell line and selection in spectinomycin (50 µg/ml) after which the selected clones were checked for restriction analysis with EcoRV (NEB, New England BioLabs, Beverly, Mass).
Table 3.6 Reaction components used during BP and LR recombination reactions of Gateway Technology.

**BP Recombination reaction**

<table>
<thead>
<tr>
<th>Components</th>
<th>Sample (in μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB-PCR product (80 ng)</td>
<td>3</td>
</tr>
<tr>
<td>pDONR221 (150 ng/μl)</td>
<td>3</td>
</tr>
<tr>
<td>5X BP clonase buffer</td>
<td>2</td>
</tr>
<tr>
<td>BP Clonase II enzyme mix</td>
<td>2</td>
</tr>
</tbody>
</table>

(1 μl of proteinase K is added and kept at 37 °C for 10 min to terminate the reaction)

**LR Recombination reaction**

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample (in μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry clone (50-150 ng/reaction)</td>
<td>3</td>
</tr>
<tr>
<td>Destination vector (150 ng/μl)</td>
<td>3</td>
</tr>
<tr>
<td>5X LR clonase buffer</td>
<td>2</td>
</tr>
<tr>
<td>LR clonase enzyme mix</td>
<td>2</td>
</tr>
</tbody>
</table>

(1 μl of proteinase K is added and kept at 37 °C for 10 min to terminate the reaction)
3.2.10 Transformation of the recombination product

Each of the recombined product generated after BP and LR reactions are transformed with the DH10β chemical competent cells. Competent cell was prepared following the INOUE method of cell preparation (Sambrook et al., 2000).

Selection of transformants

The transformants were selected in two different antibiotics after each transformation event (i.e., BP and LR reaction) about 50 μl of transformation mix was selected in LB agar (Appendix 7) plates with kanamycin 50 μg/ml for BP reaction and spectinomycin 50 μg/ml for LR reaction and incubated at 37°C.
for 16-18 hr. Hundreds of colonies were obtained from plates while control plates show no growth with antibiotic selection.

3.2.11 Isolation of plasmid DNA

Single colony was selected for midi prep plasmid extraction using alkaline lysis method. Purified plasmid obtained were used as a template for PCR reaction and restriction analysis.

Analysis of transformants

The transformation reaction yielded more than hundred number of colonies out of which single colony was selected from the plate for plasmid extraction obtained after BP reaction (positive clones) and analyzed by restriction analysis with NsiI followed by sequencing of the clones. Similarly, plasmid obtained from selected transformants after LR reaction were also checked for the presence of the insert by restriction enzyme digestion with EcoRV enzyme and PCR with gene specific primers.

Restriction analysis of entry clone after BP reaction

Plasmid extracted from selected clones after BP reaction were taken for restriction enzyme digestion with NsiI. For this 1 µl (1344ng/µl) of entry clone, pDONR221-nmt was taken for digestion with 0.2 µl (10u/µl) of NsiI enzyme (NEB, New England BioLabs, Beverly, Mass) at 37 °C for 3 hr in a 20 µl reaction volume containing 2 µl 10X NEB Buffer 3 and 0.2 µl BSA (100µg/ml). Gel electrophoresis of the digested product was done at 1.3% agarose gel along with undigested plasmid of pDONR221 for comparison.
Sequencing of the clones

Plasmids extracted from positive clones obtained after BP reaction were taken for sequencing using M13F and M13R primers in a GA3130xl Genetic Analyzer (Applied Biosystems, California, USA) using BigDye Terminator V3.1 cycle sequencing Kit (Applied Biosystems) following the instructions given in the manual. The vector sequences were removed using Sequence Scanner software (Applied Biosystems) and the selected sequences are considered for further analysis using the BLASTX programme at the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) at default parameters against non-redundant (nr) protein database.

Restriction digestion of destination vector for insert release

Selected plasmids obtained after LR reaction were considered for restriction enzyme analysis with EcoRV. For this about 1 µl of the resulting destination vector pWATERGATE-nmt construct (~ 1204 ng/ µl) was digested with 0.2 µl (10u/µl) of EcoRV enzyme (NEB) at 37 °C for 3 hr in a 20 µl reaction volume containing 2 µl 10X NEB Buffer 3 and 0.2 µl BSA (100µg/ml). The digested product containing the desired fragment of nmt along with vector sequences was gel electrophoresed in a 1.2% agarose gel along with undigested plasmid of pWATERGATE and DNA ladder. The pWATERGATE-nmt construct so formed contains a gateway cassette in two orientations with an intron in between. The intron containing hpRNA construct is driven by Arabidopsis rubisco promoter (ARbcS) and OCS terminator. It also has spectinomycin as bacterial selection and npt II as plant selection marker. The initial confirmation
of the plasmid analyzed was followed by *Agrobacterium* mediated transformation in tea somatic embryos.

**Fig 3.4:** Flow chart of steps followed for preparing the RNAi construct of *n*-methyltransferase.
3.2.12 Preparation of the inoculum, transforming & regeneration of tea somatic embryos with gene construct of \textit{n-methyltransferase}

The desired construct obtained after LR reaction were lodged into chemically competent \textit{Agrobacterium tumefaciens} strains of LBA 4404 (prepared by INOUE method) and plated in YMB media (Appendix 9) having spectinomycin 50(\(\mu\)g/ml) and rifampicin (50 \(\mu\)g/ml) as selection. The colonies obtained after 48hrs of incubation at 28\(^{\circ}\)C were checked by colony PCR with gene specific primers of \textit{nmt}. The screened colony was taken for plasmid isolation and restriction analysis with \textit{EcoRV}, for which it was grown in YMB media containing spectinomycin 50 \(\mu\)g/ml for 48 hr at 28 \(^{\circ}\)C in a shaking incubator at 120 rpm. Single colony selected were taken after confirmatory results and grown in a conical flask containing 25 ml YMB media containing spectinomycin 50 \(\mu\)g/ml for 48 hr at 28 \(^{\circ}\)C shaking incubator at 120 rpm. Cell suspension obtained after two days were taken out and acetosyringone (400\(\mu\)M) was added (Joseph Lopez \textit{et al.}, 2004) for virulence induction and kept again in 28 \(^{\circ}\)C shaking incubator for about 2hrs at 120rpm. During this time, somatic embryo of S3.A/3 clone, were sliced into small sections in a petriplate and \textit{Agrobacterium} cell suspension cultures were poured into the petriplates and kept immersed for about 45mins at RT. The infected somatic embryos were recovered from bacterial suspension, placed on sterile filter paper under laminar hood above which L-glutamine (0.5mg/ml) was added, sealed properly and kept for co-cultivation of 48 hr. After which the infected somatic embryos was washed several times (about 10 times) with sterile water to check bacterial overgrowth and air dried for 15min under laminar air flow. The embryos were cultured on somatic
embryogenesis media (SEM) supplemented with cefotaxime (400μg/ml) and kanamycin 15μg/ml. Secondary embryos could be seen after 25 days from the date of inoculation after which they were sub-cultured in SEM media with decreasing cefotaxime concentration (200μg/ml, 100μg/ml, 50μg/ml) and increasing kanamycin concentration (25μg/ml, 35μg/ml, 50μg/ml). The selected somatic embryos were transferred to SEM media devoid of cefotaxime and maintained there for two months. After which embryos were transferred into regeneration media and maintained for about 8 months by repeated sub culturing till plantlet stage containing kanamycin 50μg/ml as plant selection. The putative transformants were then transferred to multiplication media containing kanamycin (50μg/ml) for multiplication and leaves were collected for further downstream analysis.

3.2.13 Experimental design

The protocol for transformation and regeneration already standardized at Biotechnology dept., TRA, in tea was followed. For this, RNAi construct of nmt lodged into A. tumefaciens was taken for infection of about 300-500 somatic embryos and subsequently regenerated with antibiotic (kanamycin 50μg/ml) selection. Putative transgenic secondary somatic embryos obtained about one month later were selected with increasing concentration of kanamycin (30, 40, 50 μg/ml) till plantlet stage. Throughout our experiment untransformed somatic embryos (without the RNAi construct) cultured in embryogenesis and regeneration media were taken as negative control.
Analysis of the putative transformants

To check the incorporation of the binary construct into the putative transformants, PCR was performed with nptII gene specific and vector T-DNA specific primers (T1T2 and T3T4) taking the isolated genomic DNA from control and transgenic plants using CTAB method as described by Doyle and Doyle (1987). The primer pairs used to check introgression were specifically designed encompassing both the sense and antisense fragment of vector T-DNA. The arrangement of T-DNA along with primer position is shown in fig 4.25. Standardization of the primers used to check the introgression were carried out in a Gradient Mastercycler (Eppendorf, Manheim, Germany) and the combinations are mentioned in Appendix (12). PCR reaction mixture (20μl) included 10 pmol forward primer, 10 pmol reverse primer, 50 ng plant genomic DNA as template, 2 μl 10XTaq DNA polymerase buffer, 0.25mmol/l dNTPs, 1U Taq DNA polymerase (Bioline, London, UK). After amplification the PCR products were separated in an ethidium bromide stained 1% agarose gel and compared with a 1kb DNA ladder (NEB, New England BioLabs, Beverly, Mass).

3.2.14 Quantitative real time PCR analysis

Total RNA extraction

About 20-25mg of regenerated leaf samples were taken out carefully under laminar hood. From this tissue total RNA was isolated by grounding in liquid nitrogen with sterile mortar and pestle. Grounded frozen powder was transferred to micro centrifuge tube and total RNA was extracted using RNAqueous Kit
(Ambion, USA, Cat. No. AM1912). The extracted total RNA was gel electrophoresed in 1% agarose gel and checked for integrity, purity and quantity. The concentration was also estimated by Spectrophotometer (Eppendorf, Germany).

**mRNA isolation and first strand cDNA synthesis**

Total RNA isolated from 5 samples were taken for first strand synthesis using transcriptor first strand cDNA synthesis kit (Roche, Germany) following the manufacturers protocol. For this, 3μl of total RNA (100ng/μl) was taken as the starting material and gently mixed with 0.25μl of anchored-oligo(dT)18 primer in a 0.2 ml nuclease free PCR tube and incubated at 65 °C for 10 min in a thermal cycler (ABI 2720, Applied Biosystems, CA, USA) to denature RNA secondary structure. The tube was immediately chilled on ice and subsequently mixture of 1 μl 5X reverse transcriptase reaction buffer, 0.12 μl protector RNase inhibitor (40 U/μl), 0.5 μl deoxynucleotide mix (10 mM each) and 0.12 μl transcriptor reverse transcriptase (20 U/μl) were added and incubated at 50 °C for 1 hr in a thermal cycler. This was followed by deactivation of reverse transcriptase used for cDNA synthesis (Moloney Murine Leukaemia virus, MMLV) by heating the tube at 85 °C for 5 min and stored at -20 °C for downstream application.

**Primer designing and standardization**

Primers used to detect the transcript levels of nmt gene in the transgenic plantlets were designed from the ORF obtained after sequencing using freely available primer3 software (http://frodo.wi.mit.edu/primer3/). The primers used in real time, RTMTF & RTMTR (Bio Serve, India) were specific and were
standardized in Gradient Mastercycler (Eppendorf, Germany) with the following parameters-94 °C for 2 min followed by 35 cycles of 3 temperature holds 1st at 94 °C for 30 sec, 2nd at 60 °C for 30 sec (annealing temp.) and 3rd at 72 °C for 30 sec, the three holds were followed by a final extension at 72 °C for 7 min and the standardized primer specific PCR profiles was used in qRT-PCR analysis.

**Internal control genes**

In the present study four housekeeping genes were considered namely, *Camellia* tubulin, 18S rRNA, 26S rRNA and Ribulose 1, 5 bis phosphate carboxylase/oxygenase. The respective gene sequences were retrieved from public databases (NCBI and DNA Data Bank of Japan (DDBJ) and taken for designing primers using primer3 (http://frodo.wi.mit.edu/primer3/) software. The primers were standardized in a gradient thermal cycler (Eppendorf, Manheim, Germany) before qRT-PCR analysis and the standardized PCR profiles were used to test the expression levels of the four genes in experimental and control samples. The detailed information related to gene sequences, accession number and primer designing can be found in Appendix (13).

**Quantitative real time PCR for expression of n-methyltransferase**

Transcript level of *nmt* gene was determined by LightCycler 480 II real time machine (Roche, Germany) using Light Cycler 480 SYBR green I Master kit (Cat. No. 04 707516 001, Roche Diagnostics, Germany). The PCR reaction was prepared in ice taking 0.4mM of RTMT forward and 0.4mM RTMT reverse primers (Appendix 12) and 4μl ROCHE Light Cycler 480 SYBR green I Master
mix. The final volume was made to 4.7 μl with water after which 0.5 μl first strand cDNA template, was added to the multi-well plate and sealed with LightCycler 96 multiwell sealing foil. To reduce the error rate three technical replicates were used for each reaction. The reactions were pre-incubated at 95°C for 5 min, following which amplification occurred in three steps: 10 sec of denaturation at 95°C, 10 sec of annealing at 60°C, 10 sec of extension at 72°C with a total of 45 cycles. This was followed by one cycle of melting curve analysis to check specificity of amplified product. The temperature for melting curve analysis was 95°C for 5 sec, 65°C for 1 min and 97°C for continuous acquisition followed by one cycle of final cooling at 4°C.

**Real time data acquisition**

Expression levels of individual samples considered in the study were obtained in the form of crossing point (Cp). At this point visible fluorescence is obtained in an optimum number of PCR cycles to reach a threshold transcript which is optimum enough for the camera to capture. The data for all the samples analyzed was obtained using 2nd derivative maximum method as computed by the software of LightCycler (Roche Diagnostics, Germany) Carousel-based system.

**Normalization factor of reference genes**

Normalization factor for the samples taken during the study were calculated using the raw Cp values obtained from qRT-PCR for all the housekeeping genes. The values obtained were converted into relative quantities using delta Ct method and computationally analyzed using geNorm software to find out the most stable genes among the set of housekeeping genes.
Relative quantification analysis

Relative quantification assays of individuals samples are performed by comparing two different target sequences. One of the sequence refers to the gene of interest and the other a constitutively expressed gene. The expression of the target gene is expressed as ratio of target-to-reference gene in the same sample. Cp values of target gene considered in all the experimental and control samples were transformed into relative quantities using delta Ct method. The normalized expression levels of each experimental and control samples were calculated by dividing the quantities by their respective normalization factor. The normalized expression of a target gene thus obtained was compared within the experimental and control samples to know the relative expression level.

3.2.15 Absolute quantification for copy number study

Primer design and standardization

Freely available software primer3 (http://frodo.wi.mit.edu/primer3/) were used to design oligonucleotide primers for nptII sequence present in the binary vector of pWATERGATE–nmt used for transformation in tea. Information related to primer sequences can be found in Appendix (12). The primers were standardized in a thermal cycler (ABI 2720, Applied Biosystems, CA, USA) having the parameters at 94 °C for 2 min followed by 35 cycles of 3 temperature holds 1st at 94 °C for 30 sec, 2nd at 55 °C for 30 sec (annealing temp.) and 3rd at 72 °C for 30 sec, the three holds were followed by a final extension at 72 °C for 7 minutes. The reaction mixture for a 20μl reaction included 10 pmol forward primer, 10 pmol reverse primer, 20 ng extracted plasmid as template, 2 μl
10XTaq DNA polymerase buffer, 0.25 mmol/l dNTPs, 1 U Taq DNA polymerase (Bioline, London, UK). The reaction product gave an amplification at 153bp and the standardized primer specific PCR profiles was used in qRT-PCR analysis.

**Preparation of template DNA for real time PCR**

Genomic DNA was extracted from five numbers of kanamycin resistant regenerated T₀ plantlets. For this, approximately 50mg tissue was taken and DNA was isolated using CTAB method as described by Doyle and Doyle (1987). Presence of the transgene in the regenerated plantlets was initially checked by PCR and the transcript level of *nmt* were determined by Real time PCR. The extracted genomic DNA was estimated spectrophotometrically, at 260 nm, while DNA purity was evaluated on the basis of the UV absorption ratio at 260/280 nm and analyzed by 1% agarose gel electrophoresis in 0.5X TAE with ethidium bromide staining.

**Preparation of standard curve for absolute quantification**

Plasmid was isolated from single colony of DH10β transformed with binary construct of pWATERGATE-*nmt* in LB broth with kanamycin selection using plasmid extraction kit (Hi-media) following manufacture’s protocol. Plasmid purity was checked after electrophoresing in an ethidium bromide stained 1% agarose gel and quantified with a spectrophotometer (Eppendorf). Serial dilution of extracted plasmid to a range of $1 \times 10^2$, $1 \times 10^4$, $1 \times 10^6$, $1 \times 10^8$ and $1 \times 10^{10}$ copies /µl were used to construct the standard curves for *nptII*. The standard curve prepared for *pal* (3.1.19) is taken as reference. Copy number of the
extracted plasmid was calculated using the equation given below. (Whelan et al., 2003)

\[ \text{DNA (copy)} = 6.02 \times 10^{23} \text{(copy/mol)} \times \text{DNA amount (g)} \]

\[ \text{DNA length (dp)} \times 660 \text{ (g/mol/dp)} \]  \hspace{1cm} (1)

Real time PCR with nptII and pal primer sets generated \( C_p \) values in each dilution and the values were measured in duplicates to generate standard curves for nptII and pal respectively. The \( C_p \) values were plotted against the logarithm of their initial template copy numbers. Standard curve of each was generated by a linear regression of the plotted points. From the slope of each standard curve, PCR amplification efficiency (E) was calculated according to the equation (Rasmussen, 2001):

\[ E = 10^{-1\text{slope}} - 1 \]  \hspace{1cm} (2)

**Real time PCR conditions**

Absolute quantification of the samples taken in the study was carried out in a Light Cycler 480 II real time machine (Roche, Germany). For each sample a 10\( \mu \)l reaction was made using Light Cycler 480 SYBR green I Master kit (Cat. No. 04 707 516 001, Roche Diagnostics, Germany). The reaction mixture also contained 0.4mM nptII forward and 0.4mM nptII reverse primers (Appendix 12) having 20 ng template. The PCR parameters consisted of 5 min at 95 °C, followed by 45 cycles of denaturation (95 °C, 10 sec), annealing for 30 sec at 55 °C and extension at 72 °C for 30 sec. The cycling was followed by one cycle of melting curve analysis to check specificity of amplified product. The temperature for melting curve analysis was 95 °C for 5 sec, 65 °C for 1 min and
97 °C for continuous acquisition. A final cooling of another one cycle at 4 °C was followed and each sample taken for analysis was estimated twice.

3.2.16 Biochemical analysis using HPLC

For biochemical quantification of caffeine and theobromine, an ISO method using high performance liquid chromatographic (HPLC) was performed. In this method separation is determined on a phenyl bonded column using gradient elution with UV detection at 278 nm. In the international standard procedure followed, the chromatographic condition and composition of the mobile phase specified are suitable for a phenomenex-LUNA 5μm phenyl hexyl column of dimensions 250nm x 4.6mm fitted with 4mm x 3.0 mm phenyl hexyl cartridge. Quantification of caffeine and catechins was carried out using the relative response factor concept of ISO 14 505-2 (Anonymous, 2005). External standards are used for quantification. External caffeine and catechins standards of defined purity and moisture are used (HPLC mobile phase composition, caffeine stock standard solution, catechin stock standard solution, preparation of test sample in Appendix 16-18). Once the flow rate of mobile phase and temperature are stable, conditioning of the column was done with a blank gradient run. Mixed standard solutions A & B (Appendix 22) of 10μl each was injected into column followed by an equal volume of the diluted test solution. Repeated injection of the mixed working standard solution at regular intervals was performed. The chromatographic peaks were identified and estimated by an external standard method from response factors (concentration of standard/peak area of standard) determined from different catechin standards (Sigma Aldrich).
The solvents used for extraction and analysis were of HPLC grade (E. Mark, Mumbai, India).