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Tea (*Camellia sinensis* L.) is a medicinally and economically important beverage crop. It is valued for its young leaves which are used in making tea drink. Kangra jat tea is a cultivar of Chinary type and is extensively propagated in the Kangra valley at the Himalayan foothills of Himachal Pradesh, India. This cultivar is utilized for making high valued ‘Kangra tea’. Although its quality is similar to the Darjeeling tea, Kangra tea has a special flavour that makes it highly popular in India. Moreover, this tea is highly adapted to this region and thus can tolerate the adverse conditions more than the other cultivars. The cultivars belonging to Chinary (including Kangra jat), Assamica, and Cambod tea varieties were used in the present investigation. These are maintained in the Tea Experimental Farm, IHBT (CSIR) Palampur, India.

The present thesis is mainly focused on studying the caffeine metabolism in tea and caffeine synthase (CS) silencing in Kangra jat tea cultivar. Under caffeine metabolism, caffeine biosynthesis and caffeine degradation were studied in different tissues during non-dormant (ND) and dormant (D) growth phases in Chinary, Assamica, and Cambod tea varieties. Understanding caffeine biosynthesis is very important with respect to the silencing of caffeine synthase in tea as it provides the data on the relation between caffeine levels and CS expression in different tissues. For studying caffeine biosynthesis, a cDNA fragment of *CS* was isolated from high caffeine containing tea cultivar Kangra jat that contains caffeine in the range of 3.5-4% DW. Therefore, intake of high caffeine-containing tea may leads to an increased level of caffeine in addition to its important antioxidant constituents. High level of caffeine can produce some adverse effects on human health such as palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure, sleep disturbances, problems in nutrients absorption, and birth defects at least in sensitive people. Caffeine level in tea can be reduced through genetic engineering which has not been reported so far. For silencing, the isolated cDNA fragment of *CS* was used for making an RNAi construct to transform the same tea cultivar and subsequently reducing its caffeine content. However, this *CS* sequence information was used for *CS* expression as well as for silencing studies in *Camellia sinensis* (L.). In tea, regeneration requires minimum 8-12 months. Therefore, the attempt was also made to develop a rapid, efficient, and quite economical *Agrobacterium*-mediated root transformation system for tea (*Camellia sinensis* L.) cv. Kangra jat. This system was also used for *CS* silencing in Kangra jat tea seedlings using the developed RNAi construct. In addition to the caffeine metabolism and *CS* silencing studies, biochemical and molecular analysis of caffeine
induced growth inhibition were also studied on plants which are generally not producing caffeine in vivo.

5.1 Isolation of a caffeine synthase cDNA fragment from tea
Several caffeine biosynthesis pathway gene sequences from tea and coffee have been isolated (Kato et al., 2000, Uefuji et al., 2003, Mizuno et al., 2003a, Mizuno et al., 2003b, Yoneyama et al., 2006). The amino acid sequence alignment of the proteins encoded by these N-methyltransferase genes showed four conserved motifs necessary for caffeine synthesis (Mizuno et al., 2003a; Mizuno et al., 2003b). In present study, primers were designed to amplify the portion of tea genome which includes these conserved motifs for caffeine synthesis. Using cDNA-PCR approach, a methyltransferase gene fragment of 376 bp was fished out from young leaves of Camellia sinensis (L.) O. Kuntze cv. Kangra jat. The isolated fragment showed a very close sequence similarity with the already reported tea caffeine synthase1 (TCSI) from Camellia sinensis by Japanese scientist (Kato et al., 2000).

It has been observed that 100-400 bp gene fragment is generally sufficient for silencing through RNA interference in plants (Smith et al., 2000). Therefore, in present study the isolated 376 bp cDNA fragment of CS was employed to design an RNAi construct and used in CS silencing study. However, the sequence information of CS cDNA fragment was also used to study the caffeine biosynthesis.

5.2 Caffeine metabolism in tea
To study caffeine biosynthesis, CS expression and caffeine contents were analyzed in tea cultivars such as Kangra jat (KJ), UPASI-9 (U9), Tocklai variety (TV), and Tocklai germplasm (TG) belonging to different tea races. For studying caffeine degradation, allantoin content was estimated in these tea cultivars. Tea is essentially an out-breeding crop and is found in three major races Chinary, Assamica, and Cambod. These three tea races are morphologically distinct from each other. Therefore, these were included for studying caffeine metabolism. These cultivars are highly interfertile and therefore, the present tea populations are highly heterogenous (Barua 1989). Kangra jat is a Chinary type of tea clone possessing small serrated leaves with multiple stems at bottom. U9 is a Chinary hybrid possessing more Assamica type of morphological and systematic characteristics. TV is an Assamica type of tea clone possessing single stem at bottom and broad leaves. However, TG is a Cambod type of tea clone.
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These four tea cultivars were used for developmental and seasonal regulation of caffeine metabolism study. Tea is a perennial and the cheapest non-alcoholic caffeine containing beverage crop grown in the world. Its young shoots including two leaves and a bud are commercially utilized for making tea drink. However, during winter (Oct to Mar), tea undergoes a dormancy period. During this, growth of apical bud almost ceases, severely reducing the commercial yield of tea (Vyas et al., 2007). Thus, the vegetative tissues collected in the present study during dormant (D) and non-dormant (ND) growth phases represent two seasons. But the reproductive tissues collected represent only for one season. Because flowering in the first year results in seed setting of the second year. Rate of caffeine biosynthesis depends upon the amount and activity of enzymes, availability of substrates such as xanthosine and S-adenosyl-L-methionine (SAM), stage of development, tissue specific, and environmental growth period. High expression level of gene encoding NMT (TCS1) has been reported to be responsible for the production of high caffeine content in tea young leaves (Kato et al., 2000; Li et al., 2008).

Lower expression of CS in old leaf (OL; fourth leaf from top) of tea compared to other younger tissues such as bud or apical bud (AB), 1st leaf (IL), 2nd leaf (IIL), and young stem (YS; stem portion up to third leaf) documented developmental effect on its expression. Constitutive production of caffeine in relevant tissues has also been reported in coffee, where transcript accumulation for CS was detected predominantly in immature fruits. However, other N-methyltransferase genes of the caffeine biosynthesis pathway accumulated in leaves, floral buds, and immature fruits (Uefuji et al., 2003).

Higher CS expression and caffeine content during ND and lower during D in various tea tissues documented the seasonal effect on caffeine biosynthesis. Before this study, seasonal variation in capacity of caffeine biosynthesis in tea leaves has been studied by measuring the rates of incorporation of radiolabeled 8-14C-adenine in caffeine and has suggested the biosynthesis of caffeine occurs in tea young leaves during April to June month of the year (Fujimori et al., 1991). Dormancy had a down regulation effect on the expression of CS. During dormancy, the CS expression was decreased significantly in the tea manufacturing tissues i.e. AB, IL, and IIL. However, the expression of CS in YS was appeared to be cultivar specific. There was no effect of dormancy on CS expression in YS of TG and TV cultivars. In contrast, expression of CS was decreased in YS of KJ and U9. Further, caffeine content and CS expression showed direct relation in various tissues except in YS of TG and TV, suggesting caffeine synthase as one of regulatory enzymes of caffeine biosynthesis. From the data, effect of dormancy on caffeine content was appeared.
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quite prominent in KJ as compared to other tea cultivars. The decrease in caffeine content of YS of TG and TV cultivars during dormancy and had no effect on CS expression suggested that other caffeine biosynthetic pathway genes might be regulating the caffeine synthesis in YS during D growth phase. Secondly, this could also be due to posttranscriptional regulation of CS in YS. The expressed CS transcript might not be translated to active caffeine synthase protein or CS transcript degraded at faster rate. Both have resulted in lesser caffeine content during D growth phase in YS compared to ND growth phase. Differential regulation of CS in YS needs to be deciphered.

Tea fruits had lower levels of CS expression and caffeine contents compared to other tissues used in this study. Higher caffeine content in fruit coat compared to cotyledons was also in direct correlation with higher expression levels of CS in the same tissue.

In spite of caffeine biosynthesis, a degradation pathway has been documented in coffee which is a close relative of tea in terms of caffeine containing plants. In coffee, caffeine is catabolized to xanthine via theophylline and 3-methylxanthine and these are then degraded further by the conventional purine catabolism pathway to CO₂, NH₃, and urea via uric acid, allantoin, and allantoic acid (Ashihara et al., 1996). Caffeine degradation pathways have also been well studied in lower organisms such as bacteria (Blecher and Lingens, 1997), fungi (Asano et al., 1993; Brand et al., 2000), and yeast (Sauer, 1982). Demethylase(s) activity for caffeine degradation has been documented in smaller organisms but it has not been elucidated in tea. However, based on tracer experiments several catabolites of caffeine degradation have been identified in Coffea arabica leaves and tea (Kalberer, 1965). Caffeine is mainly produced in young leaves of tea plants and it continuously accumulates during its maturation. It has also been observed that caffeine is slowly catabolized as the tissue ages (Ashihara and Crozier, 2001). From this, it is expected that demethylase(s) might be present in tea plants and is responsible for the removal of methyl groups from caffeine to convert it into xanthine. Caffeine degradation measured in terms of allantoin content was higher in OL during ND growth phase. Hence, low CS expression and caffeine content and high allantoin content in OL during ND growth phase documented the faster degradation of caffeine in OL. In contrast, AB, IL, IIL, and YS had higher rate of caffeine biosynthesis than degradation during ND. The lowest allantoin content was observed in YS of TV cultivar as compared to all other tea cultivars during ND growth phase documented very low caffeine degradation in it.
Furthermore, the decrease in CS expression and caffeine content and increase in allantoin content in all tissues of all the four tea cultivars during D growth phase had suggested that dormancy might be activating caffeine degradation and inhibiting caffeine biosynthesis.

Interestingly, fruits showed different behaviour than other tissues. High level of allantoin along with CS expression and caffeine content in fruit coat compared to cotyledons suggested that caffeine biosynthesis as well as caffeine degradation might be simultaneously operative at a competitive pace in fruit coat. Earlier reports based upon physiological and biochemical studies have documented that the ratio of caffeine biosynthesis and degradation in fruit and leaves determines the caffeine content. Furthermore, degradation of caffeine in fruits has been reported faster as compared to leaves (Mazzafera, 1991; Mazzafera, 1993; Mazzafera et al., 1994a). Among fruits, fruit coat was found to be possessing higher rate of caffeine biosynthesis and degradation compared to cotyledons. However, a high rate of caffeine biosynthesis accompanied by a slow rate of caffeine degradation has been reported in endosperm of developing seed of Coffea arabica, while Coffea dewevrei, which is characterized by low caffeine content, has been reported to catabolize caffeine much more efficiently than C. arabica (Mazzafera et al., 1991; Mazzafera et al., 1994a). The mature fruit and old leaves of tea have minimum amount of caffeine compared to young leaves (Ashihara and Crozier, 1999). The similar observations were concluded in this present study and this could be due to differential regulation of the biosynthetic and degradation pathways of caffeine in different tissues of tea.

Caffeine and allantoin content of four tea cultivars during ND and D phases were negatively correlated. Strong correlations were observed between caffeine and allantoin content of KJ during D phase, U-9 during ND and D phases, TG during ND phase, and TV during D phase. Good correlations were observed between caffeine and allantoin content of KJ during ND phase and TV during D phase. However, a poor correlation was observed between caffeine and allantoin content of TG during D phase which could be due to the following reasons; first, TG belongs to Cambod tea variety which is distinct in morphological and systematic characteristics than Chinary and Assamica tea varieties; second, based upon the caffeine synthase transcript expression and caffeine content analysis, other caffeine biosynthetic pathway or genes might be regulating the caffeine synthesis during D phase. Further, the exact caffeine degradation pathway has not been
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elucidated in tea. Thus, a different caffeine biosynthesis and caffeine degradation pattern might be possible in TG tea cultivar.

In conclusion, data shown in the present study provided evidence for regulation of caffeine in different tissues of tea by caffeine synthase. Further results also documented that caffeine biosynthesis and degradation in tea was appeared to be cultivar-specific, tissue-specific, and season-dependent.

5.3 Tea regeneration system
An efficient regeneration system is required for successful transformation of tea with the RNAi construct to reduce its caffeine content. Tea regeneration via somatic embryogenesis has been well optimized and established in Kangra jat tea cultivar in our Institute. Thus, a similar regeneration protocol was followed in the present study.

However, there are a number of reports on tea regeneration from different explants such as embryogenic tissues (Jeyaramraja and Meenakshi, 2005), somatic embryos (Mondal et al., 2001a; Bhattacharya et al., 2006a), and in vitro leaves (Sandal et al., 2007). But somatic embryogenesis has been considered as one of the most worked out regeneration system in tea (Jain and Newton, 1990). Development of somatic embryos from tea cotyledons is their inherent characteristic. However, the efficacy of such a system for plant production depends upon the efficiency of multiplication and conversion rate of somatic embryos. The advantage of somatic embryogenesis is the development of adventitious embryos from explant tissue without an intervening callus phase which helps in maintaining genetic fidelity (Bano et al., 1991). Thus, it is regarded as an efficient means for multiplication of elite clones (Mondal et al., 2001b), artificial seed production (Janeiro et al., 1997; Mondal et al., 2000), and cryopreservation for germplasm storage of elite clones (Thorpe, 1988), embryo rescue of inter-specific crosses (Yamaguchi et al., 1987), and transgenic plant production (Mondal et al., 1999). Also, advantage of this tea regeneration system over conventional micropropagation is tap roots of somatic seedlings, which helps them better to combat drought. Following the usefulness of the somatic embryogenesis, it was adopted in the present study.

5.3.1 Production of tea somatic embryos
Induction of somatic embryogenesis depends upon several factors such as physiological stage and type of explant and media formulation including plant growth regulators. A judicious selection of all these factors may lead to a successful embryogenesis of tea. In
present study somatic embryos were derived from de-embryonated tea cotyleden based upon the earlier reports of Akula et al. (2000) and Mondal et al. (2001a). The secondary somatic embryos were formed without callus formation from the epidermal tissues of the initial primary somatic embryos on embryo induction medium containing high concentration of betaine along with ABA. Betaine at high concentration acts as an osmolyte while at low concentration it serves as nitrogen source in culture (Naidu et al., 1987). The osmotic stress produced by betaine might cause a disruption of plasmodesmal interconnections between the pre-embryogenic cells. Thus the cells become physiologically isolated to differentiate into somatic embryos. Various factors affecting the induction, growth, multiplication, maturation and germination of somatic embryos have been reviewed by Mondal (1999). For the first time, Kato (1982) reported the successful induction of somatic embryos on mature cotyledons. Later on, somatic embryos were also produced from immature cotyledons (Bano et al., 1991), de-cotyledenated embryos (Paratasilpin, 1990) and de-embryonated cotyledons (Rajkumar and Ayyappan, 1992; Ponsamuel et al., 1996) of C. sinensis.

5.3.2 Maintenance of tea somatic embryos

Somatic embryo-to-somatic embryo, commonly known as repetitive embryogenesis, growth pattern has been reported for the secondary embryogenesis in tea. This growth pattern has been more frequent as it can be exploited for mass propagation and obtaining true-to-type transgenic plants. In repetitive embryogenesis, an unlimited number of secondary embryos were generated in a cyclic manner from a single culture of primary embryos (Raemakers et al., 1995) and thus utilized for mass propagation. By controlling the synchronous and cyclic multiplication of recurrent somatic embryos offered a greater potential in development of transgenics (Levee et al., 1999). A temporary immersion system was also developed by controlling immersion cycles, synchronized multiplication and embryos development for repetitive embryogenesis of tea in Australia to obtain uniform globular somatic embryos. This system was utilized for bioreactor technology (Akula and Akula, 1999). But little attention has been paid towards the formation and long-term maintenance of secondary embryogenesis in tea. In present study, it was observed that the irregular subculturing of somatic embryos resulted in the change of their growth pattern i.e. somatic embryos-to callus phase. In addition, a little higher agar percent (0.8%) and pH of media (5.7) were also resulted in the similar type of changes in
their growth pattern. This could be due the inherent characteristic of these tea somatic embryos maintained at their initial stage of development and establishment.

5.3.3 Regeneration of tea somatic embryos

Regeneration of somatic embryos depends upon several factors such as optimal use of plant growth regulators and ionic strength of the medium supplied. However, the ionic strength of the medium is basically contributed by macro-elements, particularly from nitrogen and potassium sources. Sulphate is also an important macro-nutrient of plants. Sulphate ions react with ATP to yield adenosine 5'-phosphosulfate (APS) by an enzyme ATP-sulfurylase. APS is further reduced to sulfite and then to sulphide, which is finally incorporated into the amino acid cysteine. Mondal (1999) reported the role of optimal concentration of sulphate and nitrate salts in basal MS medium for the induction, maturation, and development of somatic embryos, and conversion of the developed somatic embryos into tea plantlets. The tea somatic embryos are recalcitrant (Berjak et al., 1993), therefore lack the required storage reserves during the embryo maturation phase (Bhattacharya et al., 2002). Thus, a lower concentration of nitrates was reported beneficial for the induction of normal, globular secondary embryo as well as synchronous development and maturation into heart-shaped and torpedo-shaped stages. It was due to an increase in accumulation of starch and other nonstructural carbohydrates by lowering the nitrate concentration in medium (Stitt and Krapp, 1999) which appeared to be important for normal maturation of embryos (Rock and Quatrano, 1995). Desiccation improved the germination of embryos in woody plants (Wetzstein et al., 1990; Deng and Cornu, 1992). But any desiccation treatment on tea somatic embryos was reported detrimental as it evoked desiccation damage in recalcitrant tea embryos. No effect of desiccation, chilling or culture treatment with GA3/ABA on improvement of maturation and germination of tea somatic embryos have been reported earlier by Mondal (1999). However, maltose has been reported as the dual purpose of acting as a carbon source and also as an inducer of desiccation tolerance during maturation and germination of somatic embryos (Lecouteux et al., 1993). Although maltose alone promotes the regeneration of somatic embryos, but based on the studies of Mondal (1999) a combination of maltose and trans-cinnamic acid was used in the present study. Maltose is usually broken down more slowly and provides a readily metabolizable carbon source over a long period of culture. Therefore, tea somatic embryos kept on maltose and trans-cinnamic acid containing medium during their maturation was followed and was found effective. There are different stages of somatic
embryogenesis *i.e.*, globular, heart-shaped, torpedo-shaped, and bipolar stage. The torpedo-shaped green somatic embryos with distinct poles are considered to be mature and easy to germinate. While globular somatic embryos are having high potential for repetitive embryogenesis and conversion. Therefore, the globular somatic embryos were chosen for transformation and regeneration in this study.

5.4 Caffeine reduction through gene silencing in tea

Several strategies for caffeine reduction have been reviewed recently by Mohanpuria et al. (2010). Due to several constraints in tea like woody, perennial crop plant conventional breeding approach seems difficult for its genetic improvement in a limited time period. Using genetic engineering to overexpress caffeine degradative (Demethylases) genes or to suppress the expression of caffeine synthase gene appears suitable approach for the purpose of reducing caffeine content in tea plants itself. But the caffeine degradation pathway and its enzymatic steps in tea have not been elucidated completely so far. Only the caffeine-deficient fully flavored coffee plants have been produced using the RNAi construct designed from 3'-untranslated region of theobromine synthase mRNA. After *Agrobacterium* mediated transformation, the resulted transgenic lines have shown a marked reduction of 30-80% and 50-70% in their theobromine and caffeine contents, respectively (Ogita *et al.*, 2003). In addition, the blocking of inosine monophosphate dehydrogenase, which catalyzes the formation of a caffeine precursor has been suggested as an alternative route for production of low-caffeine transgenic tea (Keyaa *et al.*, 2003). However, no success has been reported so far.

In the present work, a CS cDNA fragment specifically responsible for caffeine synthesis in KJ tea cultivar was used to design an RNAi construct to transform the same for reducing its caffeine content. CSIRO's hairpinRNA (hpRNA) construct transformation strategy has been shown to be highly efficient and effective in silencing plant genes. CSIRO in 1995 was the first to recognize the role of double-stranded RNA as the trigger for gene silencing *i.e.* popularly known as "RNA interference" or "RNAi". A construct with linked sense and antisense or inverted repeat sequences was invented by CSIRO. Waterhouse *et al.* (1998) reported that when this construct is transcribed in host system, a self complementary RNA is produced that forms a 'hairpin' structure by folding back on itself. Further, Smith *et al.* (2000) reported that the preferred version of hairpinRNA construct could be improved by using an intron or a spacer to separate the inverted repeat sequences. This makes the construct more stable in bacteria and increased silencing
efficiency in plants. It has been reported that hpRNA construct after transcription in host tissues resulted in the formation of functional small interfering RNA (siRNA). The formation of siRNA shows the functionality of RNAi mechanism in the transformants.

After the year 2000, Agrobacterium and biolistic-mediated approaches have gained a significant contribution in tea improvement by several researchers. Despite the fact that tea has difficult transformation and regeneration systems, the Agrobacterium as well as biolistic-mediated approaches have been attempted successfully (Mondal et al., 2001a; Sandal et al., 2002; Bhattacharya et al., 2006a; Sandal et al., 2007; Saini et al., 2010). Based on these earlier reports of genetic improvement of tea in present study, C's silencing was attempted using Agrobacterium as well as biolistic-mediated transformation of tea somatic embryos. After transformation, tea transformants were characterized at molecular level using reverse transcription-PCR and at biochemical level using HPLC. In addition, a novel Agrobacterium-mediated tea root transformation system was also developed. The feasibility of this protocol was also documented through silencing caffeine biosynthesis using the same developed RNAi construct. The transformed lines were analyzed at molecular level by genomic DNA-PCR with various sets of primers, dot blot analysis, southern hybridization, and reverse transcription-PCR. Further, these transformed lines were also analyzed at biochemical level using HPLC.

5.4.1 Agrobacterium-mediated transformation of somatic embryos

Based on earlier established protocol of tea transformation, somatic embryos of Kangra jat tea cultivar were transformed with Agrobacterium harboring RNAi construct (pFGC1008-CS) and pFGC1008 vector (as control). For Agrobacterium-mediated tea somatic embryos transformation, several parameters such as type of explant, its physiological state, plant genotype, pre-culture, bacterial growth and cell density, bacterial strain, wounding method, co-cultivation period and pH of co-cultivation medium have been standardized earlier (Mondal et al., 2001a). The use of phenolic inducer, acetylsyringone for treating the explants or in co-cultivation medium has been reported in many woody crops including tea. It might produce promotional effects (Matsumoto and Fukui, 1998; Lopez et al., 2004) or suppression effects (De Kathen and Jacobsen, 1990) or no effects (Mannie et al., 1991; Confalonieri et al., 1997; Mondal et al., 2001a). Therefore, the acetylsyringone was not used in the transformation of tea somatic embryos. Further, the co-cultivation periods in tea transformation have been documented in the range of 2-7 days under dark at 28°C (Matsumoto and Fukui, 1998; Mondal et al., 2001a). Five days of co-cultivation period
was found sufficient for infection as well as transfer of the T-DNA in tea somatic embryos.

The RNAi construct used for tea transformation contained hygromycin phosphotransferase as plant selection marker. The hygromycin has been found to be a better selective agent as compared to kanamycin for a number of crops (Mathews et al., 1995). Earlier, phytotoxic levels of hygromycin were studied in the range of 20-75 mg/l for screening tea calli after transformation (Luo and Liang, 2000; Lopez et al., 2004). However, very few reports are there for determining its phytotoxic effects on growth of tea somatic embryos. Thus, different concentrations (20, 30, 40, 50, and 60 mg/l) of hygromycin were selected for analyzing its lethal effect on tea somatic embryos. Finally the 40 mg/l of hygromycin was used for selection of the tea transformants.

Of three bactericidal antibiotics tested by Mondal (1999), sporidex (400 mg/l) has shown a negligible effect on the growth and organogenic response of the somatic embryos. It was found to be most effective (followed by carbenicillin and cefotaxime) in controlling overgrowth of Agrobacterium. In the present study, a combination of sporidex and/or carbenicillin was used to check the Agrobacterium growth after somatic embryos transformation.

The transiently transformed somatic embryos were checked for CS transcript expression and caffeine content. Caffeine synthase transcript level was down-regulated by 80% just after 7th day of transformation. Later on, approximately equal and maximum of 90% down-regulation in CS transcript was obtained after 9th and 11th day of Agrobacterium inoculation. Earlier, Mohanpuria et al. (2008) also reported the down-regulation in transcript level of glutathione synthetase transcript after 11th day of transformation of tea somatic embryos and almost complete absence after 15th day of tea transformation. A maximum of 70% decrease in caffeine content was observed in the transformed tea somatic embryos. The decrease in caffeine content was much lower compared to the decrease in relative levels of CS transcript in these tea transformants. It was probably due to differences in regulations of the gene at post-transcriptional level. Results also suggested a longer lag between CS suppression and reduction in caffeine content. The minor pathways were also reported to operate in tea along with the major caffeine biosynthesis pathway (Kato et al., 1996). This could be the reason for the presence of some caffeine even after complete suppression of CS expression in tea somatic embryos.
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Tea microshoots were regenerated from the transformants. However, the regeneration of somatic embryos transformed with pFGC1008-CS RNAi construct was delayed by two months compared to somatic embryos transformed with pFGC1008 construct. The regeneration efficiency of such transformants was also low. Similarly, a very low frequency (0.5%) of transgenic plant recovery was earlier reported in Agrobacterium-mediated tea transformation (Bhattacharya et al., 2004). Slow rate of regeneration could be due to following reasons. The regeneration of somatic embryos depends upon their inherent growth potential. Also, repeated washing of tea transformants with the antibacterial agents might degrade their regeneration potential. The presence of high levels of polyphenols might be responsible for the low rate of transformation and regeneration competence of tea (Biao et al., 1998; Mondal et al., 2004). The delay in regeneration of microshoots from transformed tea somatic embryos as compared to control somatic embryos was obviously due to the introduction of foreign DNA sequences into these tea somatic embryos.

5.4.2 Biolistic-mediated transformation of somatic embryos

A low efficiency of Agrobacterium-mediated tea transformation was resulted in the optimization of biolistic-mediated transformation for the development of tea plants expressing stress tolerance gene (Bhattacharya et al., 2006a; Saini et al., 2010). The biolistic-mediated tea transformation was resulted in stable transformation with higher frequency of transgenic plant recovery (0.75-1.0%) as compared to Agrobacterium-mediated transformation (Bhattacharya et al., 2004). It is devoid of laborious works involving repeated washing of explants with the phytotoxic antibacterial agents to kill the bacterial overgrowth after transformation. Thus, based on earlier established protocol, tea somatic embryos were bombarded with the microcarriers coated with the developed RNAi construct (pFGC1008-CS) for silencing CS. The transformants were selected on 40 mg/l hygromycin. A total of 27 independent transgenic microshoots were recovered on selection medium. But after analysis through reverse transcription-PCR using endogenous CS specific primers, only 8 transgenic microshoots showed the reduced CS transcript expression. Out of these, 3 representative microshoots (K5b, K7b, and K8a) in addition to one positive control (C-I) and a wild type tea plant (C-II) were selected to analyze for the primary effects of RNAi.

Similar to the characterization of transformants obtained through Agro-transformation, these transformed somatic embryos were also checked for CS transcript
expression through reverse transcription-PCR and caffeine and theobromine contents through HPLC measurement. In one of the three transgenic microshoots (K7b), maximum of 78% down-regulation in CS transcript expression and 60% reduction in caffeine content and 67% reduction in theobromine content was obtained. The degree of caffeine reduction in coffee plants was 70% (Ogita et al., 2003; Ogita et al., 2004). This has indicated the similar degree of caffeine reduction in tea and coffee by RNAi technology. In transgenic microshoots, caffeine was not reduced completely. This has suggested the existence of other minor or bypass pathway(s) of caffeine biosynthesis and their activation due to blockage of the major pathway. The production of caffeine from 7-methylxanthine via paraxanthine as one of the important minor pathway has been suggested. However, the gene encoding N-methyltransferase catalyzing this minor pathway is not known (Kato et al., 1998). Also, based on the gene expression data it was expected that other caffeine biosynthetic pathway genes might be regulating the caffeine synthesis in young leaves and stem portion. The constitutive production of caffeine could be possible in tea through other minor pathway(s) or by several other N-methyltransferases just like in coffee (Mohanpuria et al., 2009).

The primary target of RNAi in the present study was caffeine synthase which is a bifunctional enzyme that catalyzes the second and third methylation steps of caffeine biosynthesis. The reduction of the final product, caffeine, was subsequently not due to suppression of a single gene but might be due to the disturbance of whole caffeine biosynthesis pathway in tea. Interestingly, only a single gene encoding N-methyltransferase of caffeine biosynthesis pathway has been reported in tea (Kato et al., 1999). Since various N-methyltransferases have high protein sequence homology (>80% identity) (Kato and Mizuno, 2004), it is possible that the siRNAs generated from the double-stranded RNA of present tea CS could have targeted the transcripts of other members of this pathway in tea like NMTs using the existing plant RNAi mechanism. It is firmly ascertained that the suppression of CS transcript and reduction in caffeine and theobromine contents was due to the small functional gene sequence of CS from C. sinensis, which was used for RNAi construct preparation and tea transformation. It is possible that the xanthosine might be accumulated by blocking the upstream steps of caffeine biosynthesis pathway. But there is a possibility of active system of degrading xanthosine to CO₂ and ammonia by purine catabolic pathway similar to coffee (Ashihara and Crozier, 1999). Thus, the xanthosine if accumulated in the transgenic tea plants is removed.
To stabilize and harden the low caffeine transgenic microshoots produced through Agrobacterium as well as biolistic methods, these were micrografted on tea seedlings of the same tea cultivar following the procedure as reported earlier (Prakash et al., 1999). In tea like important plantation crop, there is growing demand for producing superior planting materials for varietal improvement and their rapid propagation in field. Micrografting improves the tea quality, increases vigour, and simultaneous hardens the microshoots for field transfer (Prakash et al., 1999). The main advantage of micrografting is time saving as tea plants with grafted microshoots can be transferred to field within 10 months. This method instead of reducing the gestation time from in vitro to field transplantation also facilitates the adaptation of tailored teas like low-caffeine tea to new environment. In future, this employed technique and methodology could be utilized with more efficiency for the commercial utilization of low-caffeine tea production. However, the degree of caffeine reduction and its effects on the tea production in terms of quality and yield need to be considered critically. The stability and heritability pattern of this low-caffeine trait also need to be studied.

5.4.3 Development of a tea root transformation protocol and use in caffeine biosynthesis silencing

The development of transgenic tea is very difficult, laborious and time taking process. In tea, regeneration requires minimum 8-12 months. Therefore, the attempt was made to develop a rapid, efficient, and quite economical Agrobacterium-mediated root transformation system for tea (Camellia sinensis L.). The feasibility of the developed protocol was documented through silencing caffeine biosynthesis using the developed RNAi construct (pFGC1008-CS). Agrobacterium infiltration based gene expression/silencing has been a relatively noninvasive and cost effective technique favored in model plant species for gene functional analysis (Yang et al., 2000; Goodin et al., 2002). But such infiltration was not possible in tea (Camellia sinensis L.) like woody and perennial crops. Furthermore, there was no report of any silencing through root transformation for gene functional analysis in tea. Only the silencing system using tea somatic embryos has been documented recently (Mohanpuria et al., 2008).

The age of plant and type of explants used for transformation were reported to be affecting the Agrobacterium-mediated transformation efficiency in addition to various other factors (Opabode, 2006; Mannan et al., 2009). Earlier reports in tea have suggested no expression for endogenous CS in roots. Similarly, low transcript expression has also
been reported for most of secondary metabolites biosynthetic pathway genes such as catechins and flavonoids in roots and cotyledons compared to other parts of tea (Ashihara et al., 2010). Even in this study, no endogenous expression of CS was found in tea roots. The *Agrobacterium* infiltration of leaves generally yields low and highly transient expression in several species (Wroblewski et al., 2005). Thus, the roots have been reported to serve as the best substrate for *Agrobacterium*-mediated gene expression in case of *Arabidopsis* (Levy et al., 2005; Gelvin, 2006). The main root elongation zone and lateral root primordial regions in plants like *Arabidopsis* have been reported to be the most susceptible to *Agrobacterium* transformation. Because these regions contain cells that are most highly expressing the histone H2A-1 (RAT5) gene (Yi et al., 2002) and CSLA9 gene (Zhu et al., 2003) which contained conserved motifs necessary for T-DNA integration into the plant genome. The exposure of these root segments to wounding or phytohormones has been reported to increase the H2A-1 expression as well as susceptibility to *Agrobacterium* transformation. Recently, the overexpression of several *Arabidopsis* histone genes especially H2A-1 gene has been shown to increase the *Agrobacterium*-mediated transformation and transgene expression in plants. It has been observed that the certain histones enhance the transgene expression and efficiency of *Agrobacterium*-mediated transformation by protecting the incoming transgene DNA during the initial stages of transformation (Tenea et al., 2009). Therefore, in the present tea transformation protocol, specifically the root elongation zone of tea seedlings was selected for fresh wounding. Further, incubation of these wounded roots in *Agrobacterium* suspension containing RNAi construct was resulted in bacterial infection.

The CSLA9 promoter activity in the root is restricted only to the root elongation zone and the lateral root primordial regions up to a limited period after seed germination (Yi et al., 2002). Further, McIntosh et al. (2004) reported the variable results in such types of transient assay system which were due to the use of plants with different ages. Thus, for developing root transformation protocol, one month old tea seedlings were used.

Fresh wound induces some phenolic compounds like acetosyringone, a chemoattractant for the bacterium which subsequently increases the transformation efficiency (Potrykus, 1990). Also, the cortex region of root stores sugars and starch for the frequent growth of new tea young shoots after pruning. These sugars and starch may also act as chemoattractant for the *Agrobacterium* (Smith and Townsend, 1907). Thus, tea roots wounded up to the cortex region has resulted in efficient root transformation. However, the wounding beyond the cortex has resulted in loss of many tea plants.
The co-cultivation for more than 3 days led to a decrease in transformation frequency and loss of most of the explants because of bacterial overgrowth as reported in previous experiments to produce transgenic garden pea (De Kathen and Jacobsen, 1990), flax (Dong and McHughen, 1993) and Datura (Ducrocq et al., 1994). Although, the differential requirement of co-cultivation period depends upon the Agrobacterium strain used, the medium for bacterial culture and the host plant. Earlier, the co-cultivation period of 3-5 days has been reported for the transformation of tea somatic embryos (Mondal et al., 2001a). However, only two days of co-cultivation was found to be sufficient for transferring T-DNA in the present root transformation protocol.

After one month of tea root transformation, the transformed lines were characterized at molecular and biochemical levels. For molecular characterization, genomic DNA-PCR analysis in roots of transformed lines (K6a, K25, K29a, and K31a) was done. Various T-DNA specific primer amplifications confirmed the integration of T-DNA of RNAi construct used. Out of 50 independent tea plants, only 7 were found positive for T-DNA integration. Further, dot blot analysis was resulted in detection of clear hybridization signals in genomic DNA of transformed tea roots (K6a and K31a) as compared to control tea root samples. The integration of RNAi cassette for CS in tea root was resulted in formation of siRNA upon transcription and processing. Thus, the presence of siRNAs specific to CS gene was determined in the root and leaf tissues by northern hybridization to check the site of their formation and spreading in other parts of the plant. Although in plants, the amplification of the silencing signal and cell-to-cell RNAi spreading has been observed earlier (Ahlquist, 2002; Hannon, 2002; Szweykowska-Kulinska et al., 2003; Stanislawksa and Olszewski, 2005; Voinnet, 2005). But the exact mechanism for systemic spread of siRNAs in RNAi is not fully understood in plants. In this study, the presence of siRNA specific to CS in roots documented their formation in roots of transformed tea lines. At the same time, detection of siRNA in leaves suggested their spreading in other parts of the plant. This result documented that RNAi signals in the form of siRNAs were transferred from roots to young leaves of tea plants. These might have served as RNAi signals to specifically target the active mRNAs of endogenous CS using the existing RNAi machinery of the plant system. Since the effect of RNAi is post-transcriptional, reduced expression of endogenous CS was analyzed through reverse transcription-PCR in young shoots of these transformed lines. A maximum reduction of 81% and 78% was observed in CS transcript expression of K23b and K24b transformed lines, respectively.
For biochemical characterization, young shoots of transformed lines were analyzed for the reduction in caffeine and theobromine contents using HPLC. All the seven transformed lines have shown 26-61% and 37-67% reduction in their caffeine and theobromine contents, respectively. However, the control lines showed nearly equal (39.1 mg g\(^{-1}\) DW) caffeine and (2.36 mg g\(^{-1}\) DW) theobromine contents but higher than transformed ones. Among the seven transformed lines produced, K24b showed maximum reduced caffeine (15.45 mg g\(^{-1}\) DW) and theobromine (0.79 mg g\(^{-1}\) DW) contents. Although, the maximum caffeine levels were reported to be 4 mg g\(^{-1}\) for the decaffeinated leaf teas and 10 mg g\(^{-1}\) for the decaffeinated instant teas (Ye et al., 2007). Thus, the reduction of caffeine content in present study is lesser than that to the required for decaffeination. But the significant reduction in CS transcript expression as well as in caffeine and theobromine contents of transformed tea lines documented the feasibility of the developed root transformation protocol for silencing/expression studies. The efficiency of tea root transformation through *Agrobacterium*-infiltration was also good. The *Agrobacterium* infiltration-based transformation protocol developed in tea would be quite useful for analyzing various constitutive as well as tissue specific promoters, gene regulatory elements and reporter genes, localization studies of proteins targeted to the different cell compartments, and protein overexpression. Further, this protocol could be utilized for various other genes functional analysis in tea where the transformation and regeneration system is difficult as compared to other plant species.

This is the first report of genetically modified low-caffeine transgenic tea plants produced through RNAi. The produced plantlets were morphologically similar and at maturity, these could be expected to produce essentially normal tea except for low-caffeine content. However, the degree of their reduction and their effects on the tea production in terms of quality and yield could be considered critically. The stability and heritability pattern of this low-caffeine trait could also be studied.

5.5 Effect of caffeine exposure to the plants

Tea and coffee processing is a huge business because of their demand in the market. Although, we are fulfilling the demands of people by providing good and improved quality of tea and coffee but we are doing injustice with other plants. While doing so industries are adding lot of remnant, containing appreciable amount of caffeine, left out after processing to the soil that might be affecting the growth of other plants. A plenty of literature is available on the adverse effects of caffeine on human health. However, a few
studies have been done on effect of caffeine exposure in plants which are generally not producing caffeine in vivo. Keeping the above fact in view, 1 mM and 5 mM caffeine treatment was given to Arabidopsis and tobacco and its effects were analyzed on seedling growth, caffeine absorption, total chlorophyll content, and Rubisco.

Caffeine treatment inhibited growth of roots to a greater extent than shoots in both the model plants. Similarly, the inhibition in shoot elongation by 50% and root elongation by 90% after 6-day of 2.5 mM caffeine treatment has been documented in rice seedlings (Smyth, 1992). Thus, apparently, shoots have a more effective mechanism than roots for maintaining growth in the presence of caffeine (Smyth, 1992). Morphological alterations upon caffeine exposure such as reduction in plant height, yellowing of leaf, diminished branching and reduction in rooting were some of the common features observed in this study and also reported in previous study (Smyth, 1992). Similar morphological changes in plants were also observed upon exposure to various other compounds. Heavy metals and salt stress exposure of plants resulted in a similar kind of symptoms (Yadav et al., 2005a, 2005b; Singla-Pareek et al., 2006). Further, the caffeine content was estimated in Arabidopsis and tobacco seedlings grown on MS medium containing no caffeine (control), 1 mM and 5 mM caffeine. Caffeine accumulation was similar in both the model plants. The results documented that both these seedlings absorbed caffeine to an appreciable level causing the phenotypic differences in their growth pattern.

The ability of plants to maintain chlorophyll under any kind of environmental stresses is taken as an index for determining the stress induced effect or injury (Singla-Pareek et al., 2006). Arabidopsis and tobacco exhibited significant decrease in their chlorophyll content upon caffeine exposure. The loss in chlorophyll content upon increasing the caffeine treatment concentration suggested that caffeine has promoted early senescence in plants. It has earlier been reported that the amount of chlorophyll decreased substantially during senescence of rice (Inada et al., 1999) and wheat leaves (Ono et al., 1995). During senescence, one of the most obvious enzymatic events of leaves has been identified as proteolysis (Yoshida, 2003; Donnison et al., 2007; Imai et al., 2008).

Since caffeine induces early senescence in plants and Rubisco is one of the major proteins (Makino et al., 2003), Rubisco gene (encoding larger subunit) expression as well as activity was measured in response to caffeine exposure in Arabidopsis and tobacco seedlings. Photosynthesis-related genes showed higher expression levels in young, expanding leaves in contrast to senescent leaves (Mullet, 1993), and hence promote photosynthesis (Nakano et al., 1997). When a leaf is senescent, photosynthesis-related
proteins are actively degraded and are hardly synthesized again (Yoshida, 2003; Krupinska and Humbeck, 2004). Thus, a senescent leaf appears not to have the ability to synthesize Rubisco protein. It is still unknown whether caffeine induces senescence that leads to down-regulation of Rubisco or if it is the caffeine down-regulated Rubisco that leads to senescence. In conclusion, for the first time this study evinced that caffeine had inhibitory effect on seedling growth and this could be due to its senescent and downregulatory effect on Rubisco.

In conclusion, the present thesis advances the understanding of caffeine metabolism in tea. The caffeine biosynthesis was documented to be higher in commercially utilized tissues of tea and minimum in mature fruit and old leaf tissues. This could be due to differential regulation of the biosynthetic and degradation pathways of caffeine in different tissues of tea. Further results also documented that caffeine biosynthesis and degradation in tea seems to be cultivar-specific, tissue-specific, and season-dependent. First time use of RNAi technology in tea opens up the way to engineer the tea for other metabolic pathways for useful purposes. Significant reduction in CS transcript expression as well as in caffeine and theobromine contents of transformed tea lines has documented the feasibility of developed root transformation protocol for silencing/expression studies. Further, this protocol could be utilized for various other genes functional analysis in tea where the transformation and regeneration system is difficult as compared to other plant species. This study also advances the knowledge on transferring the RNAi signals from roots to shoots in tea. This study evinced the inhibitory role of caffeine on seedling growth and this could be due to its senescent and downregulatory effect on Rubisco. But it is still unknown whether caffeine induces senescence that leads to down-regulation of Rubisco or if it is the caffeine down-regulated Rubisco that leads to senescence. Also, the inhibitory effects of caffeine on model plants suggest the safe disposal of tea and coffee waste.

After perusing the points of discussion it emerges that the following gaps in knowledge still prevail. The future studies should be focused on:

- Based upon the present investigation, other regulatory genes encoding N-methyltransferases and N-demethylases involved in caffeine metabolism could be isolated from tea.
- Several caffeine biosynthetic pathway metabolites such as 7-methylxanthine and paraxanthine which are present at low level need to be isolated. This would help in analyzing the other primary effects of RNAi in such type of study.
The developed *Agrobacterium*-mediated root transformation protocol in tea can be utilized for gene functional analysis in tea like perennial plants. But the mechanism of transferring the RNAi signals from tea roots to shoots is not yet explored. In addition, various important proteins like Dicer-like (DCL) element and Argonaute responsible for the RNAi mechanism can be explored in tea.

- The technique and methodology of producing low-caffeine tea through RNAi can be utilized with more efficiency for the commercial utilization of decaffeinated tea production.
- As the caffeine is mainly synthesized in tea manufacturing tissues, silencing of caffeine synthase can be employed using shoot or leaf specific promoter.
- The present study evinced that caffeine had inhibitory effect on seedling growth and this could be due to its senescent and downregulatory effect on Rubisco. The mechanism by which the caffeine downregulated the Rubisco need to be studied.