SUMMARY

Tea (Camellia sinensis L.) is the world’s most important caffeine beverage. Its tender shoot i. e. two leaf and a bud are commercially utilized for manufacturing black, green, and oolong tea. Tea has various important compounds but it also contains a purine alkaloid, caffeine. High intake of tea leads to an increased level of caffeine in addition to its important antioxidant constituents. Increased level of caffeine causes several problems such as palpitations, headache, gastrointestinal disturbances, anxiety, tremor, increased blood pressure, sleep disturbances, problems in nutrients absorption, and birth defects at least in sensitive people. Although, the exact amount of caffeine necessary to produce an adverse effect varies from person to person depending on their weight and sensitivity to caffeine. The moderate caffeine consumption is considered safe. But tea can become a source of useful compounds if its caffeine level is either decreased or eliminated all together from the plant itself without affecting its quality. Caffeine level in tea can be reduced through genetic engineering which has not been reported so far.

Therefore, the present thesis was mainly focused on three activities. First, analyzing the caffeine metabolism in tea; second, silencing of caffeine synthase (CS) gene in tea to reduce its caffeine content; third, effect of caffeine exposure to the plants which are generally not producing caffeine in vivo. Under caffeine metabolism, caffeine biosynthesis and caffeine degradation were studied in different tissues during non-dormant and dormant 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. 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 3.5-4.0% of DW. However, this CS sequence information was used for the CS expression as well as for silencing studies in Camellia sinensis (L.). For caffeine synthase silencing, Kangra jat tea cultivar was selected. Earlier established transformation and regeneration via somatic embryogenesis of Kangra jat tea cultivar in our Institute was followed. Using a partial cDNA fragment of CS from this cultivar, an RNAi construct was designed to transform the same and subsequently reducing its caffeine content. Kangra jat is a very popular tea 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 the highly valued Kangra tea. In tea, regeneration requires minimum 8-12 months. Therefore, for the first time a novel, rapid, and quite economical system of Agrobacterium-mediated tea root transformation was also developed for tea (Camellia sinensis L.) cv. Kangra jat.
Tea caffeine produces adverse effects on human health but the information regarding effect of caffeine on plants is scanty. Keeping this fact in view, influence of caffeine was also studied on model plants like *Arabidopsis* and tobacco. Results have documented the inhibitory effect of caffeine on seedling growth and this could be due to its senescent and down regulatory effect on Rubisco. The complete Ph.D. research work is summarized as below:

A partial cDNA fragment of caffeine synthase (*CS*) gene from young shoots of Kangra jat tea cultivar was cloned. In tea, caffeine synthase is one of the regulatory enzymes of caffeine biosynthesis. The isolated 376 bp caffeine synthase cDNA fragment was submitted to Genbank (accession number: FJ554589). Based on BLAST analysis, it showed very close sequence homology with the already reported single tea caffeine synthase (*TCSI*) from *Camellia sinensis* by Japanese scientist.

In present study, this *CS* sequence information was used for *CS* expression as well as for silencing studies in *Camellia sinensis* (L.). To study caffeine biosynthesis, *CS* expression and caffeine contents were analyzed in tea cultivars such as Kangra jat (KJ) and UPASI-9 (U9) belonging to Chinary, Tocklai variety (TV) belonging to Assamica, and Tocklai germplasm (TG) belonging to Cambod tea varieties. The *CS* expression was analyzed through reverse transcription-PCR and caffeine content through HPLC in different tissues of these cultivars during dormant (D) and non-dormant (ND) growth phases. Caffeine content and *CS* expression showed direct relation in various tissues except in young stem of TG and TV, suggesting caffeine synthase as one of regulatory enzymes of caffeine biosynthesis. The study revealed that the caffeine biosynthesis was higher in tissues which are used for tea drink manufacturing. Highest caffeine content was observed in U9 (4.7%) and KJ (3.7%). Similarly, these cultivars also showed the highest *CS* expression. This has documented the higher caffeine biosynthesis in Chinary tea clones compared to Assamica and Cambod.

To check caffeine degradation, allantoin content was estimated in all tissues which were used for caffeine biosynthesis study. Decrease in *CS* expression and caffeine content and increase in allantoin content in apical bud (AB), first leaf (IL), second leaf (IIL), young stem (YS; stem portion up to third leaf), and old leaf tissues (OL; fourth leaf from top) of the tea cultivars during D growth phase suggested that dormancy might be activating caffeine degradation and inhibiting the caffeine biosynthesis.

Low *CS* expression and caffeine content, and high allantoin content in old leaf (OL) of the tea cultivars during ND growth phase showed the faster degradation of caffeine in it as
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compared to other tissues. But high level of allantoin along with higher levels of CS expression and caffeine content in fruit coats compared to cotyledons suggested that the caffeine biosynthesis as well as caffeine degradation might be simultaneously operative at a competitive pace in fruit coats of all tea cultivars.

During dormancy, maximum decrease of caffeine content was observed ranging from 0.1 to 0.25% in tissues of KJ. Thus, the effect of dormancy on caffeine content was appeared quite prominent in KJ as compared to other tea cultivars. Interestingly, the decrease in caffeine content of young stem of TG and TV cultivars during dormancy but no effect on CS expression suggested that other caffeine biosynthetic pathway genes might be regulating the caffeine synthesis in YS during D growth phase. Based on caffeine metabolism study, caffeine synthesis and degradation in tea appeared to be cultivar specific and under developmental and seasonal regulation.

An intron-containing hairpin RNA (ihpRNA) producing construct (pFGC1008-CS) was prepared employing the isolated cDNA fragment of CS from Kangra jat tea cultivar. The construct was used for Agrobacterium and biolistic-mediated transformation of Kangra jat tea somatic embryos using the earlier established protocols in our Institute. After 7th and 9th day of Agrobacterium-mediated transformation, a reduction of 80% and maximum reduction of 90% CS transcript expression was observed in transformed samples, respectively as compared to control (11th day transformed with RNAi vector alone). A decrease of 40, 65, and 70% caffeine content was observed in 7th, 9th, and 11th day transformed samples, respectively. These putative transformants were selected on 40 mg/l hygromycin and were regenerated into tea microshoots. But the regeneration efficiency was low and only 5 hygromycin resistant transgenic microshoots were produced from the positive tea transformants. Therefore, biolistic-mediated transformation was also attempted simultaneously. Tea somatic embryos were bombarded with pFGC1008-CS construct and with pFGC1008 vector (as control) coated on gold particles. A total of 27 independent transgenic microshoots were recovered on hygromycin containing somatic embryogenesis 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, three representative microshoots in addition to one positive control and a wild type tea plant were selected to analyze for the primary effects of RNAi. The obtained transgenic microshoots have shown similar morphological characteristics as that of control tea microshoots. These transgenic microshoots showed 51-78% reduction in CS transcript expression and 44-60% reduction in caffeine content
and 46-67% reduction in theobromine content. The low-caffeine tea microshoots produced through *Agrobacterium* as well as biolistic methods were micrografted on to root stocks of the same tea cultivar for hardening and fast multiplication.

The development of transgenic tea is very difficult, laborious and time taking process. Therefore, a novel, rapid, and quite economical system of *Agrobacterium*-mediated tea root transformation was developed. The protocol was tested by using the developed RNAi construct (pFGC1008-CS) to silence caffeine synthesis in Kangra jat tea seedlings. After one month of tea root transformation, the transformed lines were characterized at molecular and biochemical levels. Genomic DNA-PCR analysis in roots of transformed lines (K6a, K25, K29a, and K31a) was conducted. Use of T-DNA specific primer amplifications have confirmed the integration of T-DNA of RNAi construct in transformed roots. Out of 50 independent tea plants, only 7 were found positive for T-DNA integration. Further, dot blot analysis has resulted in detection of clear hybridization signals in genomic DNA of transformed tea roots (K6a and K31a) as compared to control tea root samples. One of the transformed lines was also confirmed through southern blot analysis. Hybridization signal was detected in transformed tea root sample (K6a). The integration of RNAi cassette for CS in tea root was resulted in formation of small interfering RNA (siRNA) upon transcription and processing. Thus, the presence of siRNAs 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. The exact mechanism for systemic spread of siRNAs in RNAi is not fully understood in plants. But the result documented that RNAi signals in the form of siRNAs were transferred from roots to young leaves of tea plants. These siRNAs might have served as RNAi signals to specifically target the active mRNAs of endogenous CS using the existing RNAi machinery of the plant system.

Young shoots of transformed lines were analyzed for the reduction in caffeine and theobromine contents. All the seven transformed lines have shown a significant reduction of 70 to 81% in CS expression and reduction of 26 to 61% and 37% to 67% in their caffeine and theobromine contents, respectively. Among the seven transformed lines produced, K24b showed maximum reduction in caffeine (61% DW) and theobromine (67% DW) contents. The significant reduction in CS transcript expression as well as in caffeine and theobromine contents in transformed lines has indicated the feasibility of this root transformation protocol for silencing/expression studies.
A plenty of literature is available on adverse effects of caffeine on human health. But studies on effect of caffeine on plants are scarce. Tea and coffee processing industries are adding lot of remnant, containing appreciable amount of caffeine to the soil that might be affecting the growth of other plants. Therefore in the present study, an effect of 1 mM and 5 mM caffeine concentration was analyzed in Arabidopsis and tobacco plants. Morphological alterations such as reduction in plant height, yellowing of leaf, diminished branching and reduction in rooting were observed. Caffeine content was found in exposed seedlings and the phenotypic differences in their growth pattern could be due to this. A significant decrease in chlorophyll content was observed in Arabidopsis and tobacco upon caffeine exposure which revealed that caffeine induces early senescence in plants. Further, a clear cut reduction in Rubisco gene (encoding larger subunit) expression as well as activity was observed in both plant seedlings. Thus, it was evinced that caffeine had inhibitory effect on seedling growth and this could be due to its senescent and down regulatory effect on Rubisco.