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Isolation and characterization of cDNA encoding cyclophilin gene from dormant bud of *Camellia sinensis* (L.) O. Kuntze

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Environmental stresses such as drought, salinity temperature *etc*. significantly limit crop productivity plants (Ingram and Bartels, 1996). In most of the studies, stress-associated proteins may confer stress tolerance by activation of a large set of cyclophilin genes, which leads to accumulation of specific cellular proteins. Cyclophilins are highly conserved, ubiquitous, stress-induced proteins present in all subcellular compartments (Galat, 1999) which exert cellular protection during stress (Chou and Gasser, 1997). These proteins also mimic their responses similar to the application of abscisic acid (Kullertz *et al*., 1999; Chaves *et al*., 2003). Cyclophilins are involved in a wide variety of processes such as protein trafficking and maturation (Shieh *et al*., 1989; Ferreira *et al*., 1996), receptor complex stabilization (Leverson and Ness, 1998), apoptosis (Lin and Lechleiter, 2002), receptor signaling (Brazin *et al*., 2002; Yurchenko *et al*., 2002), RNA processing (Krzywicka *et al*., 2001), and spliceosome assembly (Horowitz *et al*., 2002).

Cyclophilin possess peptidyl-prolyl *cis-trans* isomerase (PPlase) activity, catalyzing the rotation of X-pro peptide bonds from *cis* to *trans* conformation, a rate-limiting step in protein folding (Brandts *et al*., 1975). The expression of cyclophilin was also found to be induced by various biotic and abiotic factors such as fungal and viral infection, drought, salt stress, heat and cold shock, light, wounding, $HgCl_2$ (Marivet *et al*., 1992), ethephon (an ethylene releaser), salicylic acid, abscisic acid, and methyl jasmonate (Luan *et al*., 1994; Marivet *et al*., 1994; Chou and Gasser, 1997; Scholze *et al*., 1999; Godoy *et al*., 2000; Kong *et al*., 2001; Sharma and Singh, 2003).

Further characterization of cyclophilins in tea plant (*Camellia sinensis*) will improve our understanding on its diverse role in growth and development. Most of the important enzymes in tea are related to secondary metabolism, quality and stress tolerance, caffeine biosynthesis, floral aroma formation or disease and defense response (Chunlei and Liang, 2007). This study reports the cloning and characterization of full-length cyclophilin gene in tea and their expression pattern during the bud growth in relevance to dormancy.

UPASI-10, a prevalent Chinary tea cultivar (Balasaravanan *et al*., 2003), growing well in the tea garden of the UPASI- Tea Research Institute Valparai, India was selected in the present study. For cDNA library construction, bud tissues were collected from the shoots of dormant bud, stored immediately in liquid nitrogen and later transferred to -80 ºC till further use. For expression analysis by RT-PCR, bud tissues were collected from active and dormant shoots at 12, 24, 36, 48 and 60th days after shear harvesting (DAS). The samples were collected between 9 and 10 a.m., frozen

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immediately in liquid nitrogen and stored at -80 ºC till RNA isolation.

Total RNA was isolated from frozen bud tissue using RNeasy plant mini kit (QIAGEN) and quantified by measuring the ratio of absorption at 260 and 280 nm. RNA samples with an A260/A280 >2.0 were used for cDNA synthesis, using Creator SMART™ cDNA Library construction Kit (Clontech) and purified using QIAquick PCR purification kit (QIAGEN). The purified total cDNA from banji bud tissue were cloned into T/A cloning vector and transformed into *E. coli* DH5α strain using TransformAid™ bacterial Transformation kit (Fermentas). Glycerol stocks of recombinant clones were prepared and stored at -80 °C in duplicates. Plasmids were prepared from positive recombinant clones using QIAprep mini spin kit (QIAGEN) and sequenced on an ABI PRISM 3730 DNA Sequencer (Applied Biosystems). Sequences were searched for similarities to previously identified sequences in the public databases using the BLASTn and Delta BLAST algorithms for nucleotide and deduced amino acid sequences respectively (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Matches were considered significant when e-values and identity were <0.01 and >90 per cent, respectively. Sequencing of the clone revealed the presence of full length of the cDNA encoding *CsCyP* was 894 bp. Further analysis of the sequence revealed the presence of 624 bp open reading frame with coding capacity for a polypeptide of 208 amino acids length, flanked by 43 bp and 227 bp 5' and 3' untranslated sequences, respectively (Fig. 1). The sequence of the cDNA clone encoding *CsCyP* was submitted to NCBI database with accession number HM003242. The polypeptide predicted from the open reading frame was very similar to previously characterized cytosolic cyclophilins, with most similarities to cyclophilins from *Camellia oleifera* (FJ377540), *Nicotiana tabacum* (EF495223) and *Solanum tuberosum* (DQ284473) (Fig. 2). cDNA isolated from the present study represented the cyclophilin of 208 amino acids length, whereas the previously reported cyclophilin from *Camellia*

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1   GCTGCGAGCGAAACCAAAAGGGTGTGTCGAAACCCAGAGACAATGGCCACAAGAACTCGATCGTTCTCACTCGCACTGG
                                                M  A  T  R  T  R  S  F  S  L  A  L   12

81  TGTGGACGCTAGTCCTCTTCGCAACCCTAGCTCTCACTCAGGCGAAGAAATCGAAGGAGGATTTAAAGGAAGTGACTCAC
    V  W  T  L  V  L  F  A  T  L  A  L  T  Q  A  K  K  S  K  E  D  L  K  E  V  T  H 39

161 AAAGTTTACTTTGATGTTGAGATTGCCGGAAAACCTGCTGGTCGTATTGTCATGGGTCTCTTTGGGAAGGCAGTTCCTAA
    K  V  Y  F  D  V  E  I  A  G  K  P  A  G  R  I  V  M  G  L  F  G  K  A  V  P  K 66

241 AACAGCAGAGAATTTCCGAGCACTGTGCACAGGGGAGAAAGGTGTTGGAAAGAGTGGGAAACCTCTTCACTACAAGGGGA
     T  A  E  N  F  R  A  L  C  T  G  E  K  G  V  G  K  S  G  K  P  L  H  Y  K  G   92

321 GCAAATTCCATAGAATTATTCCCAGCTTTATGCTCCAGGGTGGTGATTTTACACTTGGTGATGGACGAGGTGGAGAATCA
    S  K  F  H  R  I  I  P  S  F  M  L  Q  G  G  D  F  T  L  G  D  G  R  G  G  E  S 119

401 ATTTATGGAGAGAAGTTTGCTGATGAGAATTTCAAGCTGAAGCACACTGGCCCAGGGTTTCTTTCAATGGCAAATGCTGG
    I  Y  G  E  K  F  A  D  E  N  F  K  L  K  H  T  G  P  G  F  L  S  M  A  N  A  G 146

481 CCCAGACACAAATGGCTCACAGTTCTTCATTACAACTGTCACAACTAGCTGGTTGGATGGCCGACATGTGGTGTTTGGAA
      P  D  T  N  G  S  Q  F  F  I  T  T  V  T  T  S  W  L  D  G  R  H  V  V  F  G  172

561 AGGTGCTATCGGGCATGGATGTGGTTTACAAGATTGAAGCTGAAGGCAATCAGAGTGGCACTCCCAAGAGCAATGTTCAA
    K  V  L  S  G  M  D  V  V  Y  K  I  E  A  E  G  N  Q  S  G  T  P  K  S  N  V  Q 199

641 ATTGCTGACAGCGGCGAACTTCCTTTGTGATGTTCTTGTTTGATTTATTTTCGATCAATGGCAGCTGTTTTGCTTTCCTT
    I  A  D  S  G  E  L  P  L  *                                                    208

721 CGTTCCAACAACGATCTCATTAAGCTAGCTTAGTTTGTGTAATCAATAAACCCCATAGATTAAGGTGGTATTTTTCTGCA

801 TCAACCTTAATCATTAGGATATTCCTCTATATCAGACTTTTTCAAGTAATAAATCTTATTTACTGCTGGCAAAAAAAAAA

881 AAAAAAAAAAAAA
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of tea cyclophilin (*CsCyP*). The * symbol indicates the stop codon. The classical polyadenylation signal in the 3'-UTR is indicated in box. The conserved regions showing signature of peptidyl-prolyl cis-trans isomerase activity (Y-K-G-S-x-F-H-R-I-I-P-x-F-M-x-Q-G-G) are underlined. Sequence data was deposited in the GenBank (acc.no. HM003242)

sinensis (DQ904327) is of 164 amino acids length. Similar cDNA encoding stress activated cyclophilin of 172 amino acids was cloned from *Solanum commersonil* (Meza-Zepeda *et al*., 1998).

Full length cDNA sequence encoding *CsCyP* was used and the primary structural parameters of deduced protein, CsCyP (molecular weight, theoretical pI, amino acid composition, extinct coefficient, estimated half life, instability index, aliphatic index and grand average of hydropathicity) were determined using Protparam (http:// www.expasy.ch/ tools/protparam.html). Secondary structure conformational parameters of CsCyP were computed using SOPMA (Self-optimized prediction method; http://npsa-pbil.ibcp.fr/cgi-bin/ npsaautomat.pl?page=/NPSA/npsa_sopma.html). The theoretical molecular weight (MW) and isoelectric point (pI) of the deduced cyclophilin protein were predicted using Protparam to be 22.45 kDa and 9.36 respectively (www.expasy.org/tools/ pi_tool.html). Results of the present study was supported by cyclophilin A gene from *Venerupis philippinarum* and the cDNA encoded a polypeptide of 186 amino acids with the predicted molecular weight of 20.15 kDa and the theoretical isoelectric point of 9.39 (Chen *et al*., 2011). Cyclophilin of similar molecular weight was identified and characterized from *Triticum aestivum* and sugarcane under drought stress (Sharma and Kaur, 2009; Que *et al*., 2011). Multiple alignments of *CsCyP* sequence with other plant sources revealed signature sequences (Y-K-G-S-x-F-H-R-I-I-P-x-F-M-x-Q-G-G), which could be involved in PPIase activities. Chen *et al*. (2011) reported similar conserved regions with cyclophilin A (CsA) binding and PPIase activities from *Venerupis philippinarum*.

Analysis of amino acid composition showed 55.8 per cent hydrophobic amino acids (Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Cys), 43.2 per cent polar amino acids (Ser, Thr, Asp, Glu, Lys, Arg, Asn, Gln, His, Tyr) and 46 per cent charged amino acids (20% acidic and 26% basic amino acids). Further, SOPMA analysis revealed that *CsCyp* was predominantly α-helical protein, which mainly consisted of random coils (37.5%) and extended strand (28.37%), while α-helices (24.52%) and β-turn (9.62%) also contributed to the secondary structure conformations. Deduced amino acid sequence of *CsCyP* from other plant sources were retrieved from NCBI database and alignment was performed using ClustalW in BioEdit (Hall, 1999). Phylogenetic tree analysis of tea *CsCyP* protein with other related cyclophilin proteins were performed using neighbor-joining method in MEGA 5 program (Tamura *et al*., 2007). Amino acid sequence aligned by CLASTAL W revealed presence of conserved sequence between the sequences (Fig. 2A). The isolated gene (*CsCyP*) belongs to the subgroup II of dicot member in the constructed phylogenetic tree (Fig. 2B).

Further to characterize the role of cyclophilin in dormancy, the change in expression pattern of transcript encoding *CsCyP* using RT-PCR was studied (Fig. 3). Single strand cDNAs were synthesized from total RNA (1µg) isolated from the bud tissues using cDNA synthesis kit (Clontech). cDNA samples (50 ng) were subjected to PCR amplification using *CsCyP* gene specific primers (FP: 5'-ATGTTGAGATTGCCGGAAAACC TGCTG - 3' and RP: 5'-TCACAAAGGAA GTTCGCCGCTGTCA- 3'). PCR conditions for each primer set was optimized and amplification was carried for 23 cycles with denaturation at 94 ºC for 30 s, standardized annealing temperature at 66 ºC for 45 s, and extension at 72 ºC for 1 min. PCR products were analyzed on 1 per cent agarose/ EtBr gels. A minimum of two sets of RT-PCR reactions were conducted independently and analyzed. Reverse (5' – GATATCTCGGCTCTCGC ATC - 3') and forward (5'- GCCCTCAACCTAATG GCTTC – 3') primers of *Cs5. 8SrRNA* were used as internal control. The intensity of bands was quantified using ImageJ software (http://rsb.info.nih.gov/ij/) and normalized against *Cs5.8S rRNA* band intensity. The average expression ratio was calculated between the normalized relative intensity of *CsCyP* gene amplification band in banji bud and the normal bud from two independent RT-PCR reactions. The expression pattern of *CsCyP* in active and dormant (banhji) bud tissues indicates its increased expression in dormant bud than the active bud (Fig. 3A, B). During the dormancy period, the expression level was high (3.64 fold) in dormant bud during the dormancy period (upto 24th DAS, Fig. 3A, B) and then showed progressive decrease during the conversion of dormancy to active growth phase (36th, 48th, and 60th DAS; Fig. 3A, B). It emphasizes that the distinct dormancy induced

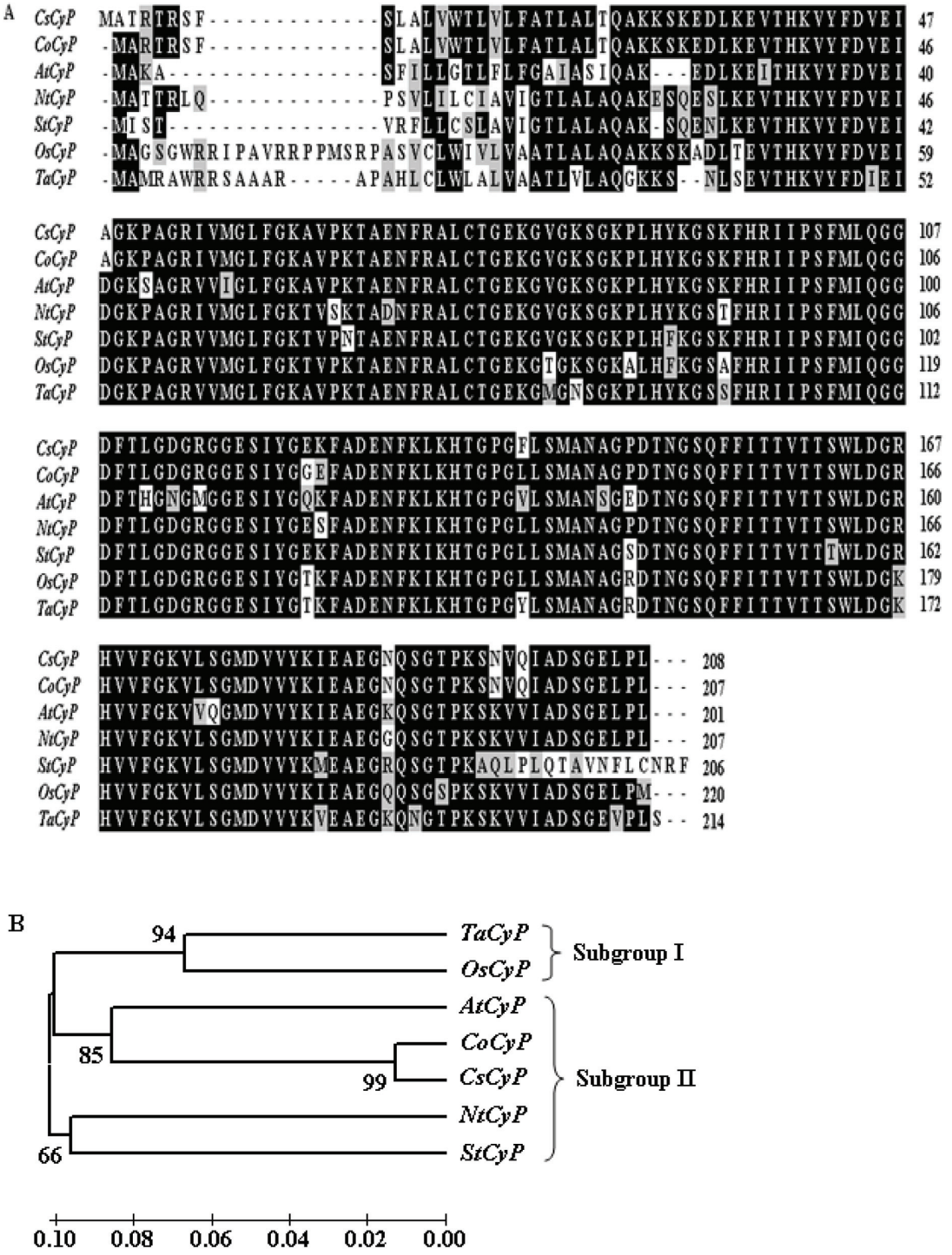

Fig. 2. Comparison of the deduced amino acid sequence of CsCyP with other cyclophilins from various plants (CoCyP - *Camellia oleifera* (FJ377540), NtCyP-*Nicotiana tabacum* (EF495223), StCyP - *Solanum tuberosum* (DQ284473), AtCyP – *Arabidopsis thaliana* (AAB71401), OsCyP – *Oryza sativa* (BAD53622), TaCyP – *Triticum aestivam* (ACF49500)). (B) Un-rooted phylogenetic tree derived from the amino acid sequences. The tree was generated from a consensus of 1000 bootstrap replicates using Neighbor-Joining method in *MEGA 5* program. The *scale bar* indicates the relative amount of change along branches

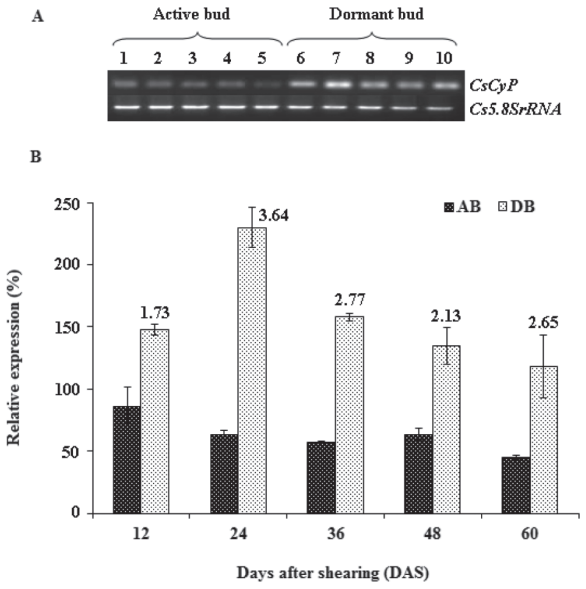

Fig. 3. Expression pattern analysis of *CsCyP*. Semi-quantitative RT-PCR analysis (A) and expression ratio (B) from active and dormant bud tissues collected at different DAS. Lanes 1 and 6: 12th DAS; Lanes 2 and 7: 24th DAS; Lanes 3 and 8: 36th DAS; Lanes 4 and 9: 48th DAS; Lanes 5 and 10: 60th DAS

expression pattern of *CsCyP* in bud tissues may lead to specific stress related protein accumulation in subcellular compartments during the stress (Chou and Gasser, 1997; Galat, 1999).

A full length cDNA encoding *CsCyP* was identified and characterized from dormant bud cDNA library of tea. The present study provides the first information of *CsCyP* expression details in bud growth. It provides valuable information for exploitation of cyclophilin genes and further identification of interacting proteins in responses to bud dormancy and stress condition in tea plant growth and development.

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