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An *A20/AN1-zinc-finger domain containing protein* gene in tea is differentially expressed during winter dormancy and in response to abiotic stress and plant growth regulators

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ABSTRACT

The present manuscript describes cloning and expression characterization of *A20/AN1-zinc-finger domain containing protein* (*CsZfp*) gene in an evergreen tree tea [*Camellia sinensis* (L.) O. Kuntze] in response to winter dormancy (WD), abiotic stresses (polyethylene glycol, hydrogen peroxide, and sodium chloride) and plant growth regulators [abscisic acid (ABA), and gibberellic acid (GA_3)]. *CsZfp* encoded a putative protein of 173 amino acids with a calculated molecular weight of 18.44 kDa, an isoelectric point (pI) of 6.50 and grand average of hydropathicity (GRAVY) value of −0.334. The gene did not have an intron, and belonged to a multi-gene family. During the period of active growth (PAG), *CsZfp* showed maximum expression in root and fruit as compared to leaf, floral bud and stem. Interaction studies between temperature and plant growth regulators on the expression of *CsZfp* showed that ABA upregulated *CsZfp* expression at growth temperature (GT; 25 °C) but had no effect at low temperature (LT; 4 °C). In response to GA_3, upregulation was observed at LT but not at GT. Further, the expression was not modulated by LT either in the tissue harvested during PAG or during WD. It was interesting to record that the expression of *CsZfp* was upregulated by hydrogen peroxide and sodium chloride, whereas it was non-responsive to polyethylene glycol. The possible role of *CsZfp* in playing key but differential roles in tea to various abiotic stresses is discussed.

1. Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is a perennial evergreen tree grown in different agro-climatic zones across the world. Tender young shoots, consisting of an apical bud and associated two leaves, known as "two and a bud (TAB)", are harvested at intervals of one to three weeks for the production of commercial tea. Growth of TAB is relatively uniform throughout the year near the equator. However, growth of TAB is restricted during winters in the plants at latitudes beyond 16° north and south of equator and the plants are said to be dormant, a phenomenon known as winter dormancy (WD). Duration of WD varies from 2 to 6 months, placing the areas experiencing WD at a disadvantage for crop yield compared to those areas where it does not occur (Kumar et al., 2012).

There is an interest to understand the phenomenon of WD and the evergreen nature of tea during winters. A subtracted cDNA library-based approach (Paul and Kumar, 2011) and RNA-sequencing on next generation sequencing (NGS) platform (Paul et al., 2014) were used to decipher the phenomenon. Analyses of unigenes obtained showed the operation of mechanisms of winter tolerance, and downregulation of genes involved in growth, development, protein synthesis and cell division (Paul and Kumar, 2011; Paul et al., 2014). Also, the data explained the evergreen nature of the tea tree wherein inhibition of leaf abscission due to modulation of senescence related processes during WD in tea played a decisive role (Paul et al., 2014). Though subtracted cDNA library and RNA-sequencing on NGS platform produced a huge amount of biological information, limited information is available on the derived gene sequences needed for proper annotation and functional analysis because of the partial sequence data.

We previously identified an expressed sequence tag (EST; GH454325) that was upregulated in winter-dormant tissue of tea (Paul and Kumar, 2011). RNA-sequencing also identified expression of this gene (scaffold24632_86.0) in the transcriptome of winter-dormant tissue of tea (Paul et al., 2014). Preliminary analysis of the EST suggested the presence of A20 and AN1 zinc-finger domains. Realizing the importance of A20 and AN1 zinc-finger domain containing proteins in plant stresses response (Mukhopadhyay et al., 2004; Vij and Tyagi,

Abbreviations: ABA, abscisic acid; ANOVA, analysis of variance; *CsZfp*, *Camellia sinensis A20/AN1-zinc-finger domain containing protein*; cDNA, complementary DNA; DR, dormancy release; EST, expressed sequences tag; FB, flower bud; GA_3, gibberellic acid; GT, growth temperature; LT, low temperature; ML, mature leaf; PAG, period of active growth; PCR, polymerase chain reaction; RACE, Rapid amplification of cDNA ends; REST, Relative Expression Software Tool; ROS, reactive oxygen species; TAB, two and a bud; WD, winter dormancy.

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2008) and that not much has been reported on this gene in its relationship with WD, the full-length cDNA of this gene was cloned through *R*apid *A*mplification of *c*DNA *E*nds (RACE) followed by detailed expression analysis. BLAST analysis of the full-length cDNA suggested this to be encoding for an A20/AN1-zinc-finger protein (A20/AN1-ZFP), therefore the gene was called *Camellia sinensis A20/AN1-zinc-finger domain containing protein* (*CsZfp*). Proteins of these families have an A20 zinc-finger domain present at the N-terminus and the AN1 zinc-finger domain present at the C-terminus (Mukhopadhyay et al., 2004; Vij and Tyagi, 2008). Evidence suggests that A20/AN1-ZFPs plays a crucial role in plant stress response. *A20/AN1-Zfps* are responsive to multiple environmental stresses like cold, salt, drought, submergence, wounding and heavy metals. Further, its overexpression confers tolerance to abiotic stresses in transgenic plants (Huang et al., 2008; Kang et al., 2011; Kanneganti and Gupta, 2008; Mukhopadhyay et al., 2004). A few reports also suggested their role in plant development (Li et al., 2011). The present work describes the characterization of *CsZfp* and discusses its role in relation to abiotic stresses response.

2. Materials and methods

2.1. Plant materials and stress treatments

TEENALI, an Assamica clone of tea was used in the present work (Paul and Kumar, 2011). The tea bushes were well maintained at the tea experimental farm of our Institute (32°6′N latitude; 76°8′E longitude; 1289 ± 15 m above sea level) and subjected to usual cultural practices. TAB was collected during the period of active growth (PAG, July; maximum temperature, 25 ± 2 °C; minimum temperature, 20 ± 2 °C), WD (December; maximum temperature, 15 ± 2 °C; minimum temperature, 4 ± 2 °C), and dormancy release (DR, April; maximum temperature, 26 ± 3 °C; minimum temperature, 15 ± 2 °C), frozen immediately in liquid nitrogen and stored at −80 °C until use. In addition to the above periods, TAB, mature leaf (ML), flower bud (FB), fruit, stem and root were also collected from a clonal bush from the field during the month of September, when both flower and fruit are available. Temperature data was collected from a weather observatory situated in the neighboring agriculture university, which is within 3 km of the periphery of the experimental tea farm.

In a separate experiment, shoot cuttings containing apical bud and associated five leaves were collected from clonal bushes of TEENALI from the field during PAG and WD and stabilized in deionized water overnight before the start of the experiment. Thereafter, shoot cuttings were transferred to deionized water (control), 100 μM 2-cis, 4-trans-abscisic acid (ABA; Sigma, USA) and 100 μM gibberellic acid (GA_3; Sigma, USA), separately as described by Paul and Kumar (2011). These were housed in a plant growth chamber set at 25 ± 3 °C (growth temperature, GT) and 4 ± 2 °C (low temperature, LT) (light intensity, 200 μE m^{-2} s^{-1}; RH, 70–80%; Saveer Biotech, India). For osmotic, oxidative and salinity stress, cuttings collected during PAG were transferred to 10% polyethylene glycol-8000 (Sigma, USA) and 0.25% hydrogen peroxide (Merck, Germany) and 100 mM sodium chloride (Sigma, USA) and housed in a plant growth chamber set at 25 ± 3 °C as described previously (Paul et al., 2012). Each experiment was carried out over a period of 48 h and the gene expression was analyzed in TAB as described in the relevant figures.

2.2. Rapid amplification of cDNA ends (RACE) and sequence analysis

As a part of our ongoing program on understanding the molecular basis of WD of tea, we deposited ESTs at Genbank at NCBI (accession numbers FF682697 to FF682833, GH454303 to GH454326, FE942774 to FE943102, and JG017532 to JG017536). For the present work, EST (accession number GH454325) was used to design primers for RACE (SMART RACE cDNA amplification Kit, BD Biosciences, USA) to clone full-length cDNA. Since the 3′ end of the gene was complete as cloned through screening of a subtracted cDNA library, 5′-RACE (Supplementary Fig. 1) was performed as per manufacturer's instructions using gene specific primers (GSPs): GSP1: 5′-TCCATCAAACCAGCACAAACAAACAA GT-3′ and GSP2: 5′-CCACTGAAATATCCATCAAACCAGCACA-3′. Total RNA isolated from winter dormant tissues as described by Muoki et al. (2011) was used for RACE-ready cDNA synthesis. The amplified fragment (Supplementary Fig. 1) was cloned in pGEM-T easy vector (Promega, USA). The nucleotide sequence was determined using BigDye terminator v 3.1 cycle sequencing mix (Applied Biosystems, USA) on an automated DNA sequencer (ABI 3130*xl* Genetic Analyzer, Applied Biosystems, USA) following the manufacturer's instructions. A search for homologous nucleic acid and protein sequences was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/). A multiple sequence alignment was generated by ClustalW multiple sequence alignment programs (http://www.ebi.ac.uk/clustalw/). The deduced amino acid sequence was analyzed by ProtParam Programs at ExPASy proteomics server (http://ca.expasy.org/) to calculate pI/Mw. The phylogenetic tree was constructed using MEGA 6.06 software (Biodesign Institute, A240, Arizona State University, Tempe, AZ).

2.3. Intron and southern blot analysis

Genomic DNA was isolated from TAB using plant DNAzol (Invitrogen, USA). To check for the presence of intron within the coding region, gene-specific forward (5′-ATG GAGCAAAATGAGACAGGATGCC-3′; start codon underlined) and reverse (5′-CTAGAGCTTATCAAGCTTTTCAGC-3′; stop codon underlined) primers designed from the start and stop codon were used for polymerase chain reaction (PCR) with 3 μl of RACE-ready cDNA or 10 ng of genomic DNA. The PCR amplification involved an initial denaturation step at 94 °C for 1 min, followed by 28 cycles of denaturation at 94 °C for 10 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. Cloning and sequencing were performed as described in the previous section.

Southern blotting was performed essentially as described by Sambrook et al. (1989). Genomic DNA (10 μg) was digested to completion with *Dra*I, *Eco*RI, *Eco*RV and *Spe*I (no cut site within the probe) in separate reactions. Digested genomic DNA was separated by electrophoresis on a 0.7% agarose gel, denatured, and blotted onto a nylon membrane (Amersham Pharmacia, UK). The membrane was hybridized with full-length of *CsZfp* cDNA probe labeled with [α-^{32}P] dATP. Hybridization was performed overnight at 55 °C in ExpressHyb™ solution (Clontech, USA). After hybridization, the blot was washed twice with 2× SSC and 0.01% SDS for 20 min each at room temperature and twice with 0.1× SSC and 0.1% SDS for 10 min each at 55 °C. The blot was then exposed to X-ray film and stored at −80 °C until developed as described by Bhardwaj et al. (2010).

2.4. Expression analysis

Gene expression was performed as described by Paul and Kumar (2011). Total RNA was treated with DNase I, Ampl Grade (Invitrogen, USA) to remove contaminating genomic DNA. First-strand cDNA was Synthesized using 2 μg of DNA free RNA, SuperScript III First-strand synthesis Kit (Invitrogen, USA) and an oligo $(dT)_{12–18}$ primer (Invitrogen, USA). Gene expression was performed using forward: 5′-CAACAACAGACTGA ACTTGC-3′ and reverse: 5′-GCAGTCATGTTTGTCAGAGT-3′ gene-specific primers on a Stratagene Mx3000P system (Agilent Technologies, Germany) using 2× Brilliant III SYBR® Green qPCR Master Mix (Agilent Technologies, Germany). All qPCRs were run in triplicate with a no-template control to check for contamination. PCR was conducted under the following conditions: 10 min at 95 °C (enzyme activation), 40 cycles each of 30 s at 95 °C, 30 s at 55 °C and 72 °C for 30 s and a final melting curve analysis was performed (55° to 95 °C) to verify the specificity of amplicons. The raw threshold cycle (Ct) values were normalized against a housekeeping gene *actin* (forward: 5′-GCCATATTTGATTGGAATGG-3′