

Differential display-mediated identification of three drought-responsive expressed sequence tags in tea [*Camellia sinensis* (L.) O. Kuntze]

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There is no information on drought-modulated gene(s) in tea [*Camellia sinensis* (L.) O. Kuntze], a woody and perennial plant of commercial importance. Using differential display of mRNA, three drought-modulated expressed sequence tags (ESTs) were identified. Northern and BLAST analysis revealed that clone *dr1* (drought-responsive), induced only by drought but not by ABA, showed significant scores with PR-5 (pathogenesis related) family of PR-protein gene. Another clone *dr2*, repressed by drought but not by ABA, had nucleotide repeats for polyaspartate that are also present in chicken calsequestrin-like mRNA. Clone *dr3*, responded similarly to clone *dr2* but did not show significant homology with the reported genes, hence appears to be novel. Identification of these ESTs is an initial step to clone the full length genes and their promoters.

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1. Introduction

Drought is a major yield-limiting environmental factor identified across the globe. There is continuous search for new genes and characterization of their regulatory elements to design innovative strategies for better plant adaptation. Most of such genes have been cloned from model plants and annuals (Shinozaki and Yamaguchi-Shinozaki 1996; Grover *et al* 1999). These include *rd19*, *rd22*, *AtDi8*, *Rab18*, *AtDi21*, *Athb-7*, *Athb-12*, *Athb-8*, *HVA22*, *le16*, *Rab28*, *Rab17*, protease encoding genes and *HVA1* cloned from *Arabidopsis thaliana*, *Zea mays*, *Lycopersicon esculantum*, and *Hordeum vulgare* (Pla *et al* 1989; Plant *et al* 1991; Yamaguchi-Shinozaki and Shinozaki 1993; Straub *et al* 1994; Gosti *et al* 1995; Shen and Ho 1995; Shinozaki and Yamaguchi-Shinozaki 1997; Lee and Chun 1998).

As few reports on the molecular response to drought stress exist in perennial woody plants (Chang *et al* 1996; Chen *et al* 1996), the study was initiated in tea [*Camellia sinensis* (L.) O. Kuntze], which is a species of commercial importance wherein drought reduces yield as much as 40% (Barua 1989; Jain 1999) and where no work has yet been reported on the drought-mediated genes. Since, drought-responsive genes can be ABA-responsive or ABA-independent (Shinozaki and Yamaguchi-Shinozaki 1997), drought modulated genes in tea were also analysed in terms of ABA responsiveness. Tea has an advantage that plants are raised by vegetative cuttings from the same mother plant that ensures genetic homogeneity. Differential display of mRNA was employed to identify such genes owing to the ease and speed of comparing the transcripts in several treatments simultaneously (Liang *et al* 1994).

Keywords. *Camellia sinensis*; differential display; drought stress; genes; tea

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Abbreviations used: CO, Control; DS, drought stress; OLP, osmotin-like protein; PR, pathogenesis related; RC, recovery; TLP, thaumatin-like protein.

2. Materials and methods

Tea clone (T-78), a quality clone from Tukdah tea-garden in Darjeeling, was vegetatively propagated from the same mother plants in the well-maintained experimental farm of the Institute. Plants were raised in plastic pots (14.5 cm height \times 15 cm top diameter \times 9 cm bottom diameter) containing one part of soil from tea growing area, two parts of well-rotten farm yard manure and one part of coarse river sand and placed in a green house (temperature, $25 \pm 2^\circ\text{C}$; relative humidity, $75 \pm 10\%$). Fully expanded leaves at 4th node position from the top (average length, 9.5 ± 0.19 cm; average width 3.65 ± 0.1 cm) of two-year old plants were used for all physiological and molecular experiments.

2.1 Drought stress, ABA and recovery treatments

Control (CO) plants were watered (100 ml daily) regularly whereas, drought was imposed by withholding water in the treatment pots. Aqueous solution of ABA ($5 \mu\text{M}$) was applied to leaves at 2-day intervals to both adaxial and abaxial surface with cotton. In these pots, 100 ml of ABA solution ($5 \mu\text{M}$) was also applied in the soil near the base of the stem at 2-day intervals (Furini *et al* 1996; Sauter *et al* 2001). When ABA was not applied to the soil, these were watered as control. For recovery (RC) experiments, drought was applied for 14 days and the plants were watered thereafter. Data recording for various parameters was performed on day 0, 7, 14 and 18 after giving the treatments. Leaf samples for differential display and Northern analysis were collected on day 14 [for CO, drought stress (DS) and ABA (AB)] and on day 18 (for RC experiment). On these days the effect of stress, ABA treatment and recovery was visible as evidenced by physiological data.

2.2 Measurement of physiological parameters

Water potential (ψ) was measured using a psychrometer (dew point microvoltmeter; model HR 33T, Wescor, USA) as per the manufacturer's instructions. Photosynthesis rate (A) was measured using a portable photosynthesis system (Li-6400, Li-COR, Lincoln, NE, USA) as described earlier (Kumar *et al* 2004). Chlorophyll fluorescence induction kinetics parameters (Fv/Fm, ratio of variable to maximum fluorescence) were measured using a plant stress meter (PSM Mark II, Biomonitor, Sweden) as per the manufacturer's instructions.

2.3 Differential display

Total RNA was isolated from leaf tissue (1 g) using a guanidine-hydrochloride based procedure as described

previously (Lal *et al* 2001). Contaminating residual DNA from RNA ($10 \mu\text{g}$) was digested with 10 units of DNase I (MessageClean® Kit from GenHunter Corporation, USA; Lal *et al* 2001). Differential display of mRNA was performed using the procedure as described earlier (Liang *et al* 1994; Lal *et al* 2001). A total of 6 arbitrary primers in combination with 3 anchoring primers (HT₁₁A, HT₁₁C, HT₁₁G; H, *Hind*III site sequence at 5' end) for 4 RNA samples [CO, DS, RC and AB] were used in the present work. Sequence of arbitrary primers were used as follows: 5'-AAGCTTGATTGCC-3' (A1); 5'-AGCTTCAAGACC-3' (A2); 5'-AAGCTTTATTTAT-3' (A3); 5'-AAGCTTCGACTGT-3' (A4); 5'-AAGCTTGCCTTTA-3' (A5); and 5'-AAGCTTCTTTGGT-3' (A6). Separation of radiolabelled PCR products, re-amplification of the cDNAs of interest, and cloning of the PCR products was performed essentially as performed earlier (Joshi *et al* 1996).

Cloned products to be used for Northern hybridization were radiolabelled using α -[³²P]dATP (4000 Ci/mmol; 10 mCi/ml; Bhabha Atomic Research Centre, Mumbai) and Klenow DNA polymerase (GenHunter Corporation, USA). After removing un-incorporated radioactive dATP using QIAquick nucleotide removal kit (Qiagen, Germany) probe was heat-denatured before using in hybridization experiments. Manufacturer's instructions were followed wherever necessary. Blotting of RNA and Northern hybridization was performed essentially as described earlier (Joshi *et al* 1996). Sequenase quick-denature Plasmid Sequencing kit (Cat# 70140, USB, USA) was used to sequence positive clones using sequencing primers from GenHunter Corporation (USA). Homology search of sequences in databases were conducted using the BLAST algorithm (Altschul *et al* 1990).

3. Results and discussion

Parameters such as ψ , A and Fv/Fm remained constant in control leaves throughout the experimentation period (figure 1). However, ψ , A and Fv/Fm dropped by 87.2%, 62.9% and 52.5%, respectively at 14 days of drought stress. In the case of ABA treatment, ψ , A and Fv/Fm dropped by 52.5%, 57.7% and 44.2%, respectively on the above days. The drop in values in all the above cases was expressed in relation to day zero value (figure 1). In recovery experiments, the values of ψ , A and Fv/Fm at day 18 (i.e. 4 days after re-watering) were -1.75 ± 0.02 MPa, $7.6 \pm 0.06 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ and 0.71 ± 0.23 , respectively. The experiment showed ability of tea to revive to its normal function in terms of A , Fv/Fm and ψ characteristic in spite of suffering severe drought stress, wherein ψ was only 12.8% of its fully hydrated value at day zero. Physiological parameters showed that tea did respond to drought, ABA treatment and during recovery from drought. These data also quantified the level of stress at which the

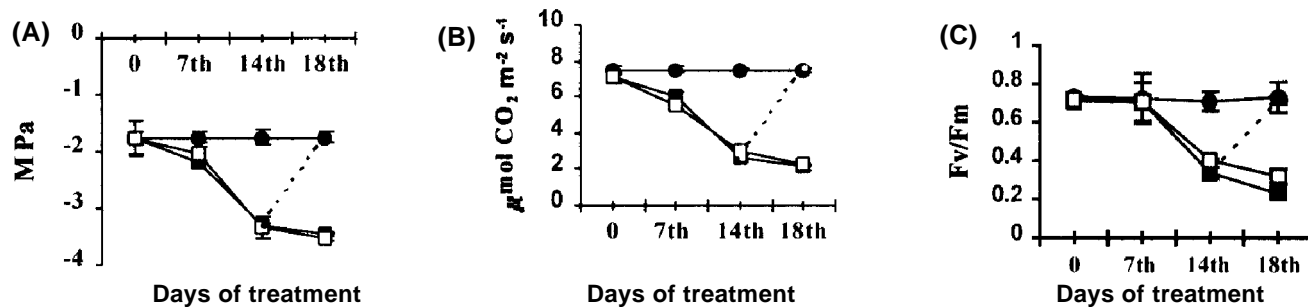


Figure 1. Water potential (ψ) (A); photosynthesis rate (B); and Fv/Fm ratio (C) of 4th leaf of 2-year old seedlings of tea plant subjected to drought stress by withholding water (■), ABA treatment (□) and subsequently re-watered (---) on day 14. Control values are shown by (●). Results are means \pm SE of four different measurements.

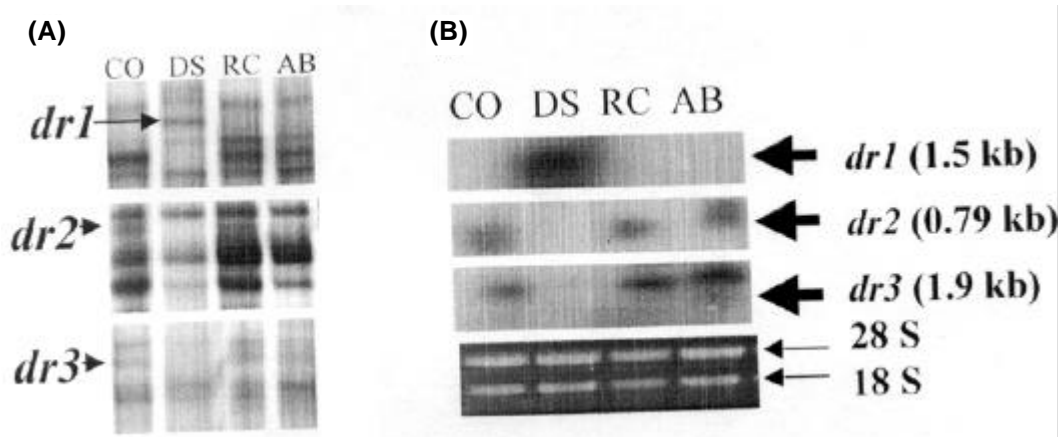


Figure 2. (A) Autoradiogram of representative differential display gels depicting the amplified products obtained using the template cDNAs derived from RNA extracted from control (CO), drought stressed (DS), recovery (RC) and ABA treated (AB) 4th leaf of tea. Primer combinations used in the reaction were: *dr1*, HT₁₁G+A2; *dr2*, HT₁₁A+A1; *dr3*, HT₁₁A+A2. Sequences of these primers are detailed in §2. (B) Northern hybridization results with probe *dr1*, *dr2* and *dr3*. Values in parentheses depict the transcript size. 28 S and 18 S ribosomal RNA bands are shown as loading controls.

observed alteration in gene expression took place. A decrease in ψ , A , and Fv/Fm is a general feature of plants experiencing drought stress (Hsiao 1973; Kumar and Gupta 1986; Angelopoulos *et al* 1996). Tea also showed the similar trend.

Differential display of mRNA showed that a number of genes were up- and down-regulated in response to drought as well as during recovery. With a total of six arbitrary primers in combination with three anchoring primers, 44 gene fragments were found to be induced whereas 84 were repressed in response to drought as compared to the control. Under ABA treatment 30 gene fragments were seen to be induced whereas 54 fragments were observed to be repressed compared to control. Thus under drought conditions, out of 44 apparently induced fragments, 14

were non-responsive to ABA treatment and out of 84 apparently repressed fragments, 30 were non-responsive to exogenous ABA. Out of all these gene fragments, 15 were such whose size ranged between 250–500 bp. Initially these were selected for further analysis due to ease of re-amplification, cloning and northern hybridization (Liang *et al* 1994 and the references mentioned therein). Since differential display of mRNA usually leads to the generation of false positives (Lievens *et al* 2001), these 15 apparently induced and repressed fragments were subjected to reverse Northern analysis, wherein 1 gene fragment was found to be induced and 2 gene fragments were found to be repressed under drought stress. The fragments were further subjected to Northern analysis. The gene fragment designated as *dr1* (figure 2A; 303 bp,

GenBank accession No. BQ825883) hybridized with the RNA isolated from the DS leaves (figure 2B). No signal was detected in RNA from CO, AB or RC leaves, which suggested *dr1* to represent a drought-induced, ABA non-responsive gene. We believe that these leaves responded to ABA – as was evident from altered y , A and F_v/F_m values (figure 1). Also, the differential display pattern from this tissue was altered in response to ABA (figure 2A), which suggested that leaves were sensitive to exogenous application of ABA. BLAST analysis revealed that *dr1* showed very significant score with the 3'-end of the genes for: (i) thaumatin-like proteins (TLP) from *Vitis vinifera* (Genbank accession No. gi|8980664|gb|AF227324.1|AF227324; E value, 6e-21) and *Nicotiana tabacum* (Genbank accession No. gi|19856|emb|X15223.1|NTE2TLP; E value, 9e-17); (ii) pathogenesis-related (PR) protein R major form with *N. tabacum* mRNA (Genbank accession No. gi|19979|emb|X12739.1|NTPRRMAJ; E value, 9e-17); and (iii) partial *olp2* gene for osmotin-like protein (OLP) from *Fagus sylvatica* (Genbank accession No. gi|12274935|emb|AJ298303.1|FSY298303; E value, 2e-17) and *Solanum commersonii* (Genbank accession No. gi|296775|emb|X72926.1|SCA81OLP; E value, 3e-04). TLP, PR protein with R major form, and OLP, all belong to the PR-5 family of PR proteins, which are known to be induced in response to osmotic stress (Singh *et al* 1987). Interestingly, one of the isogenes of OLP in *Atriplex nummularia* was found to be non-responsive to ABA, but responded to NaCl stress (Casas *et al* 1992). Also, LaRosa *et al* (1992) found osmotin not to be induced by ABA treatment. Further, experiments are needed to confirm if PR-5 family genes play an important role in conferring tolerance against drought.

Two gene fragments, designated as *dr2* and *dr3* (figure 2A) showed positive signals with RNA isolated from CO, AB and RC but did not show a hybridization signal against RNA isolated from DS leaf samples (figure 2B). BLAST analysis showed significant score of *dr2* (251 bp, GenBank accession No. BQ825884) sequence with the 3' ends of chicken (*Gallus domesticus*) calsequestrin mRNA (GenBank accession Nos gi|211496|gb|M58048.1|CHKCGS, gi|63561|emb|Y00789.1|GGLBPA; E value, 2e-04). Sequence 'GAT GAC GAT GAT GAC GAT GAT GAT' in *dr2* encodes polyaspartate, which is a characteristic feature of C terminus of calsequestrin (Katti *et al* 2000). However, due to availability of only a few nucleotides, it will be difficult to authentically identify *dr2* as being a calsequestrin and comment on its possible role. Nucleotide sequence of the clone *dr3* (361 bp; GenBank accession No. BQ825885) did not exhibit similarity with the reported sequences, which tentatively suggested that the gene could be novel.

The present communication for the first time identified 3 drought-modulated genes from tea. Availability of these

sequences provides an opportunity to clone full-length genes and their promoters.

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