# 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 polyasparate 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 × 15 cm top diameter × 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 ± 2°C; relative humidity, 75 ± 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 μM) 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 μM) 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 ($\psi$) 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 μg) 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 ($HT_{11}A$, $HT_{11}C$, $HT_{11}G$; 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′-AAGCTTCG-ACTGT-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 $\alpha$-[$^{32}$P]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 $\psi$, *A* and Fv/Fm remained constant in control leaves throughout the experimentation period (figure 1). However, $\psi$, *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, $\psi$, *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 $\psi$, *A* and Fv/Fm at day 18 (i.e. 4 days after re-watering) were –1·75 ± 0·02 MPa, 7·6 ± 0·06 μmol $CO_2$ $m^{-2}$ $s^{-1}$ 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 $\psi$ characteristic in spite of suffering severe drought stress, wherein $\psi$ 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

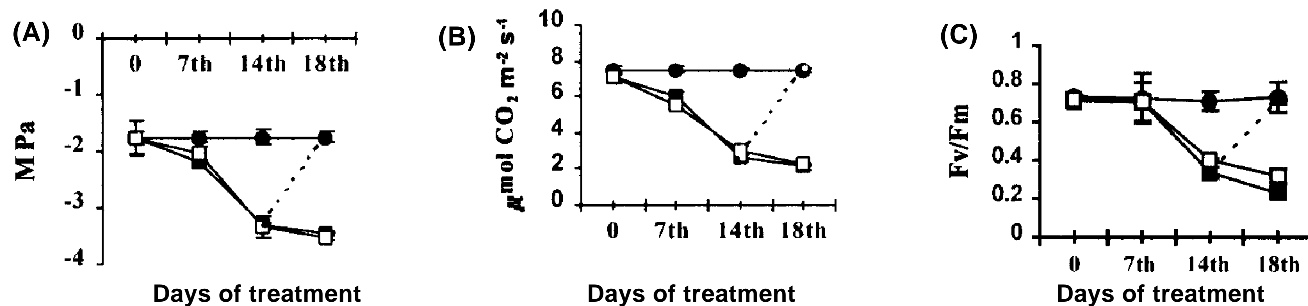


**Figure 1.** Water potential ($\psi$) (**A**); photosynthesis rate (**B**); and Fv/Fm ratio (**C**) of 4th leaf of 2-year old seedlings of tea plant subjected to drought stress by withholding water (■), ABA treatment (□) and subsequently re-watered (---) on day 14. Control values are shown by (•). Results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

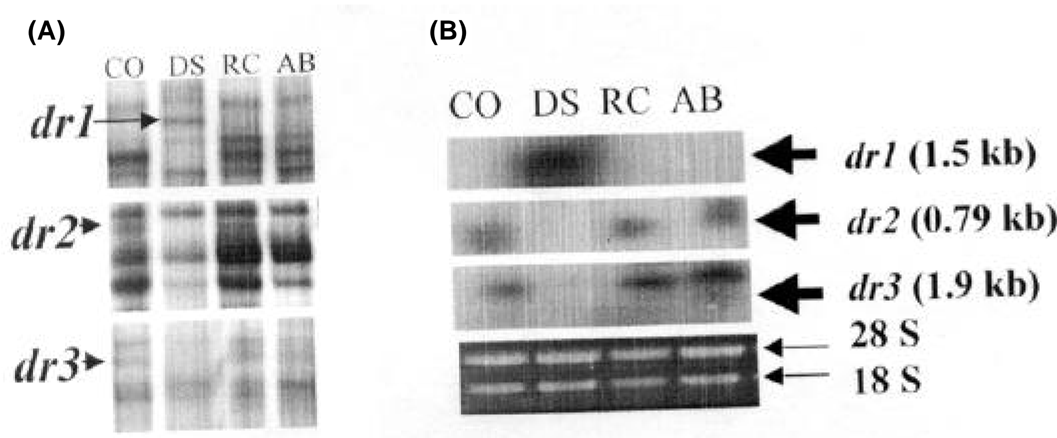


**Figure 2.** (**A**) Autoradiogram of representative differential display gels depicting the amplified products obtained using the template cDNAs derived from RNA extracted from control (CO), drought stressed (DS), recovery (RC) and ABA treated (AB) 4th leaf of tea. Primer combinations used in the reaction were: *dr1*, $HT_{11}G$+A2; *dr2*, $HT_{11}A$+A1; *dr3*, $HT_{11}A$+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 $\psi$, *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 $\psi$, *A* and Fv/Fm 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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## References

Altschul S F, Gish W, Miller W, Myers E W and Lipman D J 1990 Basic logical alignment search tool; *J. Mol. Biol.* **215** 403–415

Angelopoulos K, Dichio B and Xiloyannis C 1996 Inhibition of phoyosynthesis in olive trees (*Olea europaea* L.) during water stress and rewatering; *J. Exp. Bot.* **47** 1093–1100

Barua D N 1989 *Science and practice in tea culture* (Calcutta: Tea Research Association) pp 509

Casas A M, Nelson D E, Raghothama K G, D'Urzo M P, Singh N K, Bressan R A and Hasegawa P M 1992 Expression of osmotin-like genes in the halophyte *Atriplex nummularia* L.; *Plant Physiol.* **99** 329–337

Chang S, Puryear J D, Dilip M A, Dias D L, Funkhouser E A, Newton R J and Cairney J 1996 Gene expression under water deficit in loblolly pine (*Pinus taeda*): Isolation and characterization of cDNA clones; *Physiol. Plant.* **97** 139–148

Chen J, Reinisch A J, Adair L S, Newton R J, Funkhouser E A and Cairney J 1996 Isolation, sequence and expression analysis of two water stress deficit related cDNA clone from the halophyte *Atirplex canescens* (saltbush); *Physiol. Plant.* **96** 401–410

Furini A, Parcy F, Salamini F and Bartels D 1996 Differential regulation of two ABA-inducible genes from *Craterostigma plantagineum* in transgenic *Arabidopsis* plants; *Plant Mol. Biol.* **30** 343–349

Gosti F, Bertauche N, Vartanian N and Giraudat J 1995 Abscisic acid-dependent and independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*; *Mol. Gen. Gen.* **246** 10–18

Grover A, Sahi C, Sanan N and Grover A 1999 Taming abiotic stresses in plants through genetic engineering: current strategies and perspective; *Plant Sci.* **143** 101–111

Hsiao T C 1973 Plant responses to water stress; *Annu. Rev. Plant Physiol.* **24** 519–570

Jain N K 1999 *Global advances in tea science* (New Delhi: Aravali Books International) pp 882

Joshi C P, Kumar S and Nguyen H T 1996 Application of modified differential display technique for the cloning and sequencing of the 3′ regions from three putative members of wheat two members of HSP70 gene family; *Plant Mol. Biol.* **30** 641–646

Katti M V, Sami-Subbu R, Ranjekar P K and Gupta V S 2000 Amino acid repeat patterns in protein sequences: Their diversity and structural-functional implications; *Protein Sci.* **9** 1203–1209

Kumar S and Gupta R K 1986 Influence of different leaf water potentials on photosynthetic carbon metabolism in sorghum; *Photosynthetica* **20** 391–396

Kumar N, Kumar S and Ahuja P S 2004 Differences in the activation state of ribulose-1,5-bisphosphate carboxylase/oxyge-

nase in barley, pea and wheat at two altitudes; *Photosynthetica* **42** 303–305

Lal L, Sahoo R, Gupta R K, Sharma P and Kumar S 2001 RNA isolation from tea leaves, a highly polyphenolic containing tissue and extension of the procedure to diverse plant species; *Plant Mol. Biol. Rep.* **19** 181a–f

LaRosa P C, Chen Z, Nelson D E, Singh N K, Hasegawa P M and Bressan R A 1992 Osmotin gene expression is posttranscriptionally regulated; *Plant Physiol.* **100** 409–415

Lee Y H and Chun J Y 1998 A new homeodomain-leucine zipper gene from *Arabidopsis thaliana* induced by water stress and abscisic acid treatment; *Plant Mol. Biol.* **37** 377–384

Liang P, Zhu W, Zhang X, Guo Z, O'Connell R P, Averboukh L, Wang F and Pardee A B 1994 Differential display using one-base anchored oligo-dT primers; *Nucleic Acids Res.* **22** 5763–5764

Lievens S, Goormachtig S and Holsters M 2001 A critical evaluation of differential display as a tool to identify genes involved in legume nodulation: looking back and looking forward; *Nucleic Acids Res.* **29** 3459–3468

Pla M, Goday A, Vilaradell J, Gómez J and Pages M 1989 Regulation of the abscisic acid-responsive gene rab28 in maize viviparous mutants; *Mol. Gen. Gen.* **230** 394–400

Plant A L, Cohen A, Moses M S and Bray E A 1991 Nucleotide sequence and spatial expression pattern of a drought- and abscisic acid-induced gene of tomato; *Plant Physiol.* **97** 900–906

Sauter A, Davies W J and Hartung W 2001 The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot; *J. Exp. Bot.* **52** 1991–1997

Shen Q and Ho T-H D 1995 Functional dissection of an abscisic acid ABA-inducible gene reveals two independent ABA-responsive complexes each containing a G-Box and a novel cis-Acting element; *Plant Cell* **7** 295–307

Shinozaki K and Yamaguchi-Shinozaki K 1996 Molecular responses to drought and cold stress; *Curr. Opin. Biotechnol.* **7** 161–167

Shinozaki K and Yamaguchi-Shinozaki K 1997 Gene expression and signal transduction in water-stress response; *Plant Physiol.* **115** 327–334

Singh N K, Bracker C A, Hasegawa P M, Handa A K, Buckel S, Hermodon M A, Pfankoch E, Regnier F E and Bressan R A 1987 Charaterization of osmotin. A thaumatin-like protein associated with osmotic adaptation to plant cells; *Plant Physiol.* **85** 529–536

Straub P F, Shen Q and Ho T-H D 1994 Structure and promoter analysis of an ABA- and stress-regulated barley gene, HVA1; *Plant Mol. Biol.* **26** 617–630

Yamaguchi-Shinozaki K and Shinozaki K 1993 The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of rd22, a gene responsive to dehydration stress in *Arabidopsis thaliana*; *Mol. Gen. Gen.* **238** 17–25

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