Expression analysis of drought stress specific genes in Peanut (*Arachis hypogaea*, L.)

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Abstract Improving drought tolerance through gene manipulation has been of importance for modern agriculture, which requires identification and validation of candidate genes. Prospecting candidate genes from drought adapted crop species is of immense significance. To identify candidate stress responsive genes from adapted crop, we carried out expression analysis of a few drought responsive ESTs from *Arachis hypogaea* L. (peanut). The expression patterns of nine AhDR (*Arachis hypogaea* drought responsive) clones were analysed under drought. Quantitative reverse transcription PCR analysis revealed stress responsive nature of the selected genes. The clones AhDR 118 (putative cyclin T-like), AhDR185 (aldehyde reductase-like), AhDR193 (cholin kinase-like) and AhDR 76 (proline amino peptidase-like) showed more than five fold increase in expression. Highly upregulated genes analysed for expression pattern against salinity at seedling level indicated that these genes provide cross protection. This paper is the first report indicating the association of peanut genes cyclin T, proline amino peptidase and choline kinase to drought tolerance, and the possible roles of these genes are discussed.

Keywords *Arachis* · Drought genes · Gene expression

Introduction

Drought response of plant is very complex and degree of tolerance varies among different species and genotypes. The strategies adapted by crop species to tolerate desiccation stress could be different in spite of a few common stress responses.

Crop plants growing in drought prone areas may have several efficient adaptive mechanisms that allow them to survive and complete their life cycle under stressful conditions. Peanut (*Arachis hypogaea*, L.) a drought-adapted legume maintains better cellular metabolisms under stress by osmotic adjustment leading to maintenance of turgor. At whole plant level, better survivability, leaf area retention, and increased water use efficiency has been reported in peanut under stress (Nautiyal et al. 2002; Govind et al. 2009). All these traits make peanut an excellent crop system for mining genes associated with drought tolerance. The complete genome sequence of peanut has not been deciphered so far. Expressed Sequence Tags (ESTs) resources gain importance in the absence of whole genome, as they are efficient and cost effective solution for gene discovery. A large number of ESTs have been generated in peanut by different researchers using various types of tissues subjected to both abiotic and biotic stress and submitted to NCBI Gene bank (www.ncbi.nlm.nih.gov/genbank). NCBI dbEST has nearly 2,50,000 peanut ESTs of which 25914 are drought responsive. Guo et al. (2008) have conducted a large-scale project to generate ESTs (21,777) of peanut challenged with *Aspergillus parasiticus* and drought stress. Similarly, Govind et al. (2009) have created EST library to identify, isolate and characterize the genes expressed during gradual drought stress in peanut. Although a larger number of drought specific ESTs have been developed, functional validation has been carried out for only a few genes. For example, the transcript levels of flavonol 3-O-glucosyl-transferase (F3OGT) gene from peanut showed marked increase in response to stress (Gopalakrishna 2001). Govind et al. (2009) has studied expression pattern of regulatory (25) and functional (25) genes under drought. With the aim to enrich the candidate drought responsive genes in peanut, we identified nine genes that have not been characterised for their drought stress responsive nature, so far and studied their expression pattern in the present study. The selected genes represented

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various pathways and a few of them showed cross protection to salinity stress.

Materials and methods

Imposition of drought stress

a. Whole plant level:

Peanut plants (*Arachis hypogaeae*; cv., TMV2) were raised in pots by following plant growth and protection methods in greenhouse. Different levels of drought were imposed at whole plant level by controlled irrigation to 25 days old plants by gravimetric approach (Karaba et al. 2007). Control plants were maintained at 100 % field capacity (FC), while stressed plants were maintained at 60–70 (mild stress) and 20–30 % FC (severe stress) for 2 weeks. The plants were then subjected for biochemical and physiological assays to assess the extent of stress effects.

Fig. 1 Expression analysis of a few AhDR genes in the leaf tissues of peanut subjected to drought at whole plant.

Phenotype of peanut plants exposed to drought by gravimetric approach (a), the relative water content (RWC) of drought stressed plants (b) and end point PCR showing the expression analysis of 9 AhDR clones under drought in leaf tissue (c). The names of ESTs representing the said IDs are given along with their *Arabidopsis* homolog IDs in parenthesis AhDR 38 - Glutathione S- transferase (At2g02390); AhDR 57 - GA regulated proteins (At1g75750); AhDR 193- Choline kinase (At4g34220); AhDR 78-Extensin precursor (At3g54590); AhDR 118- Cyclin T (At1g27630); AhDR 146 - Protein phosphatases (At3g50870); AhDR 171-ACC oxidase (At1g06620); AhDR 185-Aldehyde reductase (At1g10310); AhDR 76-Proline amino peptidase (At4g30920)

b. Seedling level:

Peanut seeds were germinated in petri dishes and uniform seedlings (48 h after germination) were exposed to polyethylene glycol (PEG) 6000-induced dehydration (-14 and -18 bars for mild and severe stress, respectively) and salinity stress (150 mM NaCl for 12 h and later transferred to plates containing either 250 mM or 500 mM NaCl for 24 h). At the end of the stress period, fresh weight of root and shoot of seedlings was taken to assess the stress effect. Simultaneously, a control set was maintained by growing seedlings in water for same time interval. Root and shoot samples were harvested for gene expression studies.

Relative Water Content (RWC)

The leaves collected from plants exposed to different drought treatments were quantified according to Barrs and Weatherly (1962). Fresh weight (FW) of the leaf discs was recorded and the discs were then floated in 5 ml of water for

Fig. 2 Quantitative expression analysis of a few AhDR genes for drought response. RNA was isolated from leaf tissues exposed to mild drought stress (60–70 % FC) and cDNA synthesized was subjected to quantitative real time expression analysis. The graph represents the fold change in transcript under stress compared to unstressed control condition

6 h to gain turgidity. After recording the turgid weight (TW), the samples were dried in oven at 80 °C to constant weight to record dry weight (DW). The RWC was estimated and expressed in percent using the following formula.

$$\text{RWC} = \left[\frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \right] \times 100$$

Assessing the expression pattern of a few known stress responsive genes

RNA was isolated from peanut leaves (Datta et al. 1989) and 5 µg of total RNA was reverse-transcribed using 200 U Molony Murine Leukaemia Virus reverse transcriptase (MMLV-RT) at 42 °C for 1 h. The reaction was primed with 40 picomoles oligodT primers in the presence of a mix of 10 mM dNTPs (Nethra et al. 2006).

- RT-PCR:** For end point reverse transcription PCR, a 20 µL reaction was set containing 1 unit of Taq polymerase (MBI, Fermentas) in 1X reaction buffer, 25 mM MgCl₂, 2 mM dNTP mix, 3 picomoles of primers and template from RT reaction. The amplified products were separated on agarose (1 %) gel and documented.
- Quantitative Real Time RT-PCR analysis:** The real-time PCR was performed with gene specific primers under standardized annealing temperatures. The reaction mixture without template DNA (cDNA) was treated as blank and the real-time fluorescence value was subtracted from the blank values for analysis (Nethra et al. 2006). The optimization of the real time PCR reaction was performed according to the manufacturer's instructions (MJ Research, USA & MJ Bioworks, Inc.). The PCR conditions were standardized for the genes selected using the DyNamo SYBR-Green qPCR master mix

containing *Tbr*-DNA polymerase, SYBR-green 1.5 mM MgCl₂ and optimized PCR buffer with dNTPs (DyNamo SYBR-Green qPCR Kit FINNZYMES, Finland, www.finnzymes.fi).

- Northern blot for gene expression studies:** Northern blot hybridizations were carried out according to Sambrook and Russell (2001). RNA (15 µg) from each sample was separated in formaldehyde denaturing gel and transferred to Hybond nylon membrane and fixed by UV (1,200 J for 60 s) in a UV cross-linker. The blots were probed with respective probes, prepared by labelling with [³²P] dCTP (3,000 Ci/mmol) during PCR. Pre-hybridization was carried out at 42 °C for 2 h and hybridization at 60 °C overnight with blocking solution (0.5 M sodium-phosphate buffer, pH 7.2, 1 mM EDTA and 7 % SDS). The blots were exposed to phosphorimager plate for 2 days; the intensity of band was quantified and normalized for variation in RNA loaded.

Fig. 3 Expression analysis of selected AhDR clones under varied levels of salinity stress. Phenotype of peanut seedling experiencing salinity stress (a) and northern blot analysis showing the expression pattern of AhDR genes (b)

Result and discussion

Nine genes namely glutathione s-transferase (AhDR38, EC365204), proline aminopeptidase (AhDR76, EC365242), choline kinase (AhDR193, EC365358), protein phosphatase (AhDR146, EC365311), GA regulated protein (AhDR57, EC365223), extensin precursor (AhDR78, EC365244), cyclin-T (AhDR 118, EC365284), aldehyde reductase (AhDR185, EC365350) and ACC oxidase (AhDR171, EC365336) were selected for characterization as they were significantly associated with drought stress response as per the e-northern analysis.

For gene expression analysis, peanut plants were drought stressed and leaf samples were collected. The stressed plants exhibited wilting symptoms (Fig. 1a) and the severity increased with decrease in soil field capacity. The RWC was significantly reduced under stress indicating effect on plant water relation (Fig. 1b). The end point reverse transcription PCR showed gradual increase in transcripts of cyclin-T and GA-regulated protein with increase in stress. The genes glutathione s-transferase, proline amino peptidase, aldehyde reductase and choline kinase showed increased expression under mild stress, whereas the clones ACC oxidase and protein phosphatase were down regulated under stress when compared to well irrigated condition. The clone extensin precursor did not show any change in the expression pattern. Drought inducible protein (DIP) gene was used as indicator of stress effect at cellular level, as this gene is highly stress responsive. The elongation factor -A (*elfa*) was used as an internal control (Fig. 1c).

Real time PCR analysis for the clones glutathione s-transferase proline amino peptidase, aldehyde reductase indicated five, three and eight fold increase in expression over control whereas only two fold increase was seen in GA-regulated protein. The genes like extensin precursor, protein phosphatase and ACC oxidase were down regulated under stressful conditions. Cyclin T and choline kinase were significantly up regulated to 18 and 20 fold, respectively when compared to control (Fig. 2). Four genes cyclin T, aldehyde reductase, proline aminopeptidase and choline kinase showed increased expression under salinity stress indicating its role in cross protection (Fig. 3).

Drought signal transduction pathway involves regulation of various pathways like osmo-regulation, cell cycle, ubiquitination and ion homeostasis. This study aimed at characterizing genes that were component of a few of these pathways for their drought responsive nature. Plant cyclinT proteins binds to cyclin dependant kinase (CDK9) to activate transcript elongation by phosphorylating the CTD of RNA polymerase II (Fulop et al. 2005). Choline kinase catalyses the first step of choline pathway for phosphatidylcholine biosynthesis (PC) wherein it converts choline to phosphorylcholine (Pcho). Although choline kinase has

been reported to be induced under many abiotic stresses (Seki et al. 2002; Abdeen et al. 2010; Krasensky and Jonak 2012), the characterization for drought response was not elusive. The two genes cyclinT and choline kinase has been shown to be up-regulated under drought and salinity for the first time in our study (Figs. 2 and 3).

Proline/propyl amino peptidase (APP) hydrolyzes the peptide bond between any amino acid and a penultimate proline residue at the N terminal of oligopeptide and protein substrates. Recently, *Triticale* APP (TsPAP1) was reported to be expressed under drought, saline, cadmium and aluminium stress (Szawłowska et al. 2012). Aldehyde reductase (ALR), member of aldo keto reductases superfamily of proteins detoxifies the degradation products of lipid peroxidation. The alfalfa aldose/aldehyde reductase (MsALR) over expression has shown reduced concentration of reactive aldehyde and increased tolerance against oxidative and drought stresses (Oberschall et al. 2000). Aldehyde reductases have also been shown to provide tolerance against UV-B radiation (Hideg et al. 2003), low temperature and cadmium stress (Hegedus et al. 2004). In this study, peanut APP and ALR have been shown to be upregulated under drought and salinity stress, indicating their relevance in abiotic stress tolerance.

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