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Identification of Possible Mechanisms of Chickpea (*Cicer arietinum* L.) Drought Tolerance Using cDNA-AFLP

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ABSTRACT

Drought sensitivity is considered as a major concern for chickpea (*C. arietinum*) seed production. Determination of drought adaptation mechanisms is an essential constituent of this crop breeding programs. With this purpose, the present research was conducted to distinguish the molecular basis of chickpea drought tolerance using cDNA-AFLP approach. The expression profile was compared between drought tolerant (ICCV2 and FLIP9855C) and susceptible lines (ILC3279) of chickpea under three drought treatments including well-watered, intermediate, and severe stress; where soil water content was kept at 85–90%, 55–60%, and 25–30% of Field capacity, respectively. Totally, 295 transcript-derived fragments (TDFs) were visualized. Among the differentially expressed TDFs, 72 TDFs were sequenced. Sequenced cDNAs were categorized in different functional groups involved in macromolecules metabolism, cellular transport, signal transduction, transcriptional regulation, cell division and energy production. Based on the results, ribosomal protein S8, mitochondrial chaperone, proteases, hydrolases, UDP -glucuronic acid decarboylase, 2-hydroxyisoflavanone dehydratase, NADPH dehydrogenase, chloride channels, calmodulin, ABC transporter, histone deacetylase and factors involved in chloroplast division were among genes that were affected by drought stress. Similarity search in data base showed that cell wall elasticity, isoflavonoids, maintenance of structure and function of proteins through increase in expression of mitochondrial chaperones, programed cell death, and remobilization of storage material from leaves to seeds were among mechanisms that distinguished differences between drought tolerant and drought susceptible lines.

Keywords: Drought stress, cDNA- amplified fragments length polymorphism, Transcript derived fragments (TDFs), Gene expression.

INTRODUCTION

Identification of drought tolerance mechanism(s) is one of the most important subjects in plant science. Several physiological, biochemical, and molecular changes result from exposure of plants to biotic and abiotic stress. Hence, an improved understanding of adaptive and general protective mechanisms conferring enhanced tolerance to plants becomes an important issue in stress physiology and is necessary to ensure optimal growth and yield of crop (Jayaraman *et al*., 2008). Screening of the genes expression profile under stress condition is one of the most straightforward approaches to reveal the molecular basis of a biological system (Wang *et al*., 2009). Induction of these

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molecular responses necessitates up- and down-regulation of specific genes (Nimbalkar *et al*., 2006).

These specific genes products are classified into three major groups: (1) the products that directly protect plant cells against stresses such as chaperones, LEA proteins, osmoprotectants and detoxification enzymes, (2) factors involved in signaling cascades and in transcriptional regulation, such as protein kinases, phospholipases and transcriptional factors, (3) transporters involved in water and ion uptake and their transport (Rodrıguez *et al*., 2006).

Investigation of possible regulatory responses in an organism to environmental challenges through gene expression profile can define both tolerant and sensitive genotypes (Rodrıguez *et al*., 2006). There are several transcript profiling techniques that allow the examination of gene expression. cDNA amplified fragments length polymorphism (cDNA-AFLP) is a gel-based transcript profiling method to analyze mRNA populations (Vuylsteke *et al*., 2007). This technique was first recognized by Bachem *et al*. (1996) for the study of differential gene expressions during potato tuber formation. Since then, it has been used to study transcript profile in a huge range of organisms. The most advantageous of cDNA-AFLP is that it is an open system and does not require specific sequence information (Vuylsteke *et al*., 2007). In this method, differences in band intensity reflect fluctuations in transcript levels and allow the determination of the relative expression profile of the corresponding gene (Breyne *et al*., 2003).

The objective of this study was to identify candidate genes that may be differentially expressed or exhibit a modulated expression following drought stress treatments in drought tolerant (ICCV2 and FLIP9855C) and drought sensitive (ILC3279) lines of chickpea. To this end, cDNA-AFLP analysis was employed, several differentially expressed cDNA fragments were isolated, sequenced, and their possible functions were discussed. Finding drought-related candidate genes provides further insight into elucidating the underlying mechanisms of drought tolerance in this crop. Besides, it could be helpful to give a good picture of relationship between gene expression and extent of drought tolerance and to find correlation between phenotypic drought adaptations and gene expression.

MATERIALS AND METHODS

Plant Material and Drought Treatment

This study was carried out in a greenhouse using three breeding lines of cultivated chickpea including ICCV2 and FLIP9855C, as tolerant, and ILC3279, as susceptible, genotypes. Growth conditions and three drought treatments including well-watered (T1), intermediate (T2), and severe (T3) stress were applied based on what was recommended by Pouresmael *et al*. (2013). Leaf sampling was done three times (one, three, and five weeks after exposure to drought stress treatments), then, immediately frozen in liquid nitrogen and stored at -80 °C prior to extraction of total RNA.

RNA Extraction and cDNA Preparation

Total RNA was isolated from drought treated and the control plants using RNeasy Plant Mini kit (Qiagene, Cat. No. 74904) based on kit manual. RNA quantity was tested using the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). About 3 µg of RNA was treated with DNaseI (Fermentas, EN0521) to remove genomic DNA. After that, DNase-treated total RNA and 1 µL of oligo-dT primer (0.5 µg μL^{-1}) was used to synthesize first strand cDNA by RevertAid™ first strand cDNA synthesis kit (Fermentas, K1622) as instructed by the manufacturer. Double-stranded cDNA was synthesized immediately using 20 µL first strand cDNA, DNA polymerase I (10 units μL^{-1}) and RNaseH (5 units/µL) (Fermentas).

cDNA-AFLP Analysis and Sequencing

The cDNA-AFLP protocol was applied as recommended by Bachem *et al*. (1998). The cDNA was digested with *ECORI* (ER0271)

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and *Mse*I (ER0981) restriction enzymes. T4DNA ligase (Fermentase, EL0014) and adapters (*ECORI*-Forw: 5'-CTC GTA GAC TGC GTA CC-3'; *ECORI* _Rev:5'-AAT TGG TAC GCA GTC TAC-3'; *MseI*-Forw: 5'-GACGAT GAG TCC TGA G-3'; *MseI*-Rev: 5'-TAC TCA GGA CTCAT-3') for ligation. Pre-amplification was performed with *Mse*I and *ECORI* primers carrying no additional nucleotide at the 3' end (MseI0: 5'-GAT GAG TCC TGA GTA A-3'; ECORI0: 5'-GAC TGC GTA CCA AT TC-3'). Pre-amplification PCR conditions were as follows: 4 min initial denaturation at 94 °C and then 15 cycles 30 s denaturation (94 °C), 60 s annealing (56 °C), 60 s extension (72 °C), followed by 10 min final extension at 72 °C.

After preamplification, the mixture was diluted 50 fold and 3 µL was used for selective amplification with six primer combinations. Selective amplification was done with primers which carried two selective additional nucleotides at their 3' end (MseI: AT, CC, GG; ECORI: AC, CT). Touch-down PCR conditions for selective amplifications were as follows: 5 min initial denaturation at 94 °C, followed by 30 s denaturation at 94 °C, 30 s annealing at 65 °C, 60 s extension at 72 °C (13 cycles, scale down of 0.7 °C per cycle in annealing step); 30 s denaturation at 94 °C, 30 s annealing at 56 °C, 60 s extension at 72 °C (23 cycles) and 10 min at 72 °C.

Selective amplification products were separated on a 6% denaturing polyacrylamide gel (acrylamide to bisacrylamide ratio 29: 1) containing 7 M urea in a Sequi-Gen (Bio-Rad) running for 2.5 h at 100 W and 50±2 °C. After silver staining, the expression profile visually was compared between tolerant and susceptible genotypes based on presence or absence of the band or expression pattern intensity. In fold intensity change criterion, expression level in control treatment of each line was used as threshold for differential expression. The bands of interest were cut using a surgical blade and eluted in 50 µL of distilled water and kept at 4° C overnight. An aliquot of 5 µL was used as a template for reamplification using primers which were used for selective amplification. PCR products were purified using QIAquicke Gel Extraction Kit (QIAGENE, Q28704) after running on 0.7% agarose gel and sequenced. In addition, the images of gels were quantitatively analyzed using *image J* 1.46 software based on band intensities for quantitative measurements of expression profiles.

Fragments Characterization

A total of 72 TDFs were recovered from gels, reamplified and sequenced in two way read by FAZA Biotec Co. (Tehran, Iran). After sequencing, sequences were identified based on their similarity with protein sequences in the database (http.//www.ncbi.nlm.nih.gov/BLAST) using the BLASTX algorithms and were classified into different functional groups. The biological activity of these TDFs and their role in drought tolerance mechanisms were predicted from their sequence homology to known proteins. E-value of 1e-5 was used for acceptance of similar functions. All the TDF sequences were submitted to NCBI as collection of ESTs. The alignments of TDF sequences with their homologues were generated using the Clustal W algorithm (http://www.genome.jp/tools/clustalw).

RESULTS AND DISCUSSION

cDNA-AFLP Analysis and Detection of Differentially Expressed Transcripts

Generally using six primer combinations, a total of 295 TDFs were amplified and visualized as band. The number of TDFs ranged from 41 to 56 per primer pair and their sizes were verified from 100 to 700 bp. Figure 1 shows an example of the expression patterns in ECORI+AC/ MseI+GG primer pair. Totally, 46 fragments showed constitutively differential expression between tolerant and susceptible lines. Among these TDFs, 22 TDFs were present only in tolerant lines. One TDF was present only in susceptible line. Twenty two TDFs had more expression levels in tolerant lines and one TDF had more expression level in the susceptible line.

A total of 158 TDFs showed quantitative variants, which means that their expression

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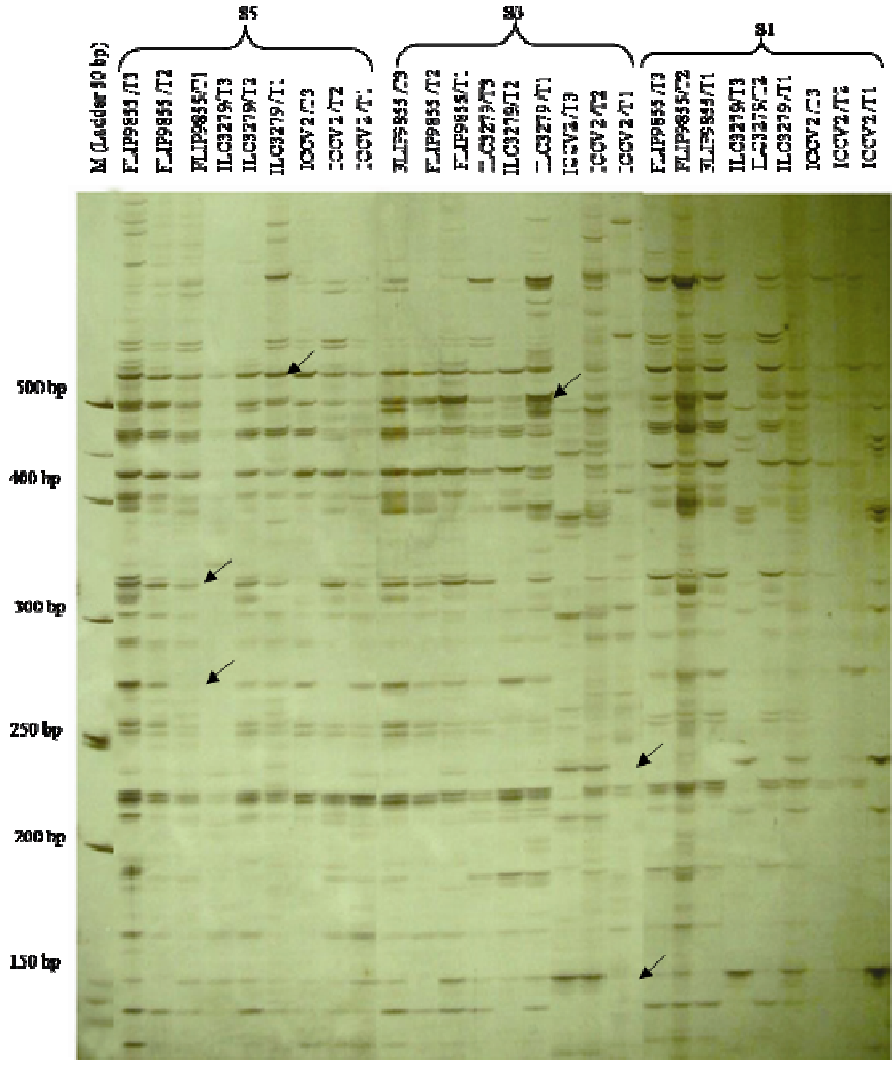

Figure 1. Representative amplification pattern of three lines of chickpea, ICCV2 (drought tolerant), ILC3279 (drought sensitive) and FLIP9855C (drought tolerant), under different drought treatments at the1st (S1), 3rd (S3) and 5th (S5) weeks of drought stress displayed by cDNA-AFLP visualized on 6% polyacrylamide gel using silver staining. T1, T2, and T3 show the control, intermediate, and severe drought stress treatments, respectively. Arrows indicates the position of some bands that were affected by drought stress. Lane M represents the molecular weight marker.

levels was affected by drought treatments. Of these TDFs, 106 were up-regulated and 52 were down-regulated. Among these TDFs, 130 fragments showed up- or down-regulation in tolerant lines and 28 TDFs showed up- or down-regulation in the susceptible line (Figure 2). This gene expression flexibility in tolerant lines can be involved in the compatible process of these lines. In addition, there were a total of 13 TDFs with alteration between three lines, with 3 TDFs commonly up- regulated and 10 contra-regulated (a mixture of regulation polarities at least between two lines).

Functional Categories of Transcripts

The size of sequenced fragments ranged between 81-540 bp. The percentages of chickpea genes assigned to different functional categories have are in Figure 3. The results of

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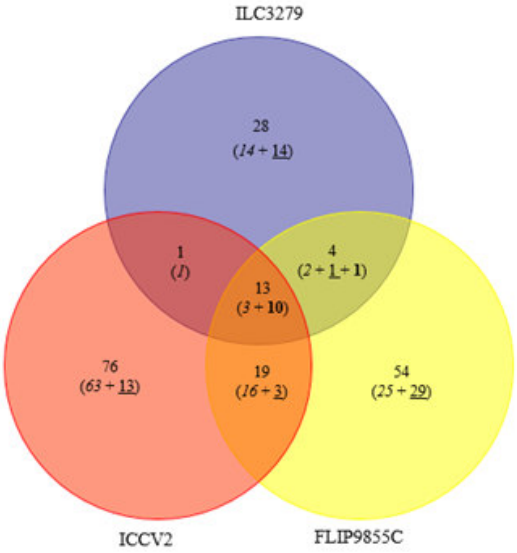

Figure 2. Venn diagram showing the number of TDF(s) affected by drought in three chickpea lines. *Italic*, under lined and normal text format shows up- down- or contra- regulated (a mixture of regulation polarities)TDFs in each line or intersection, respectively.

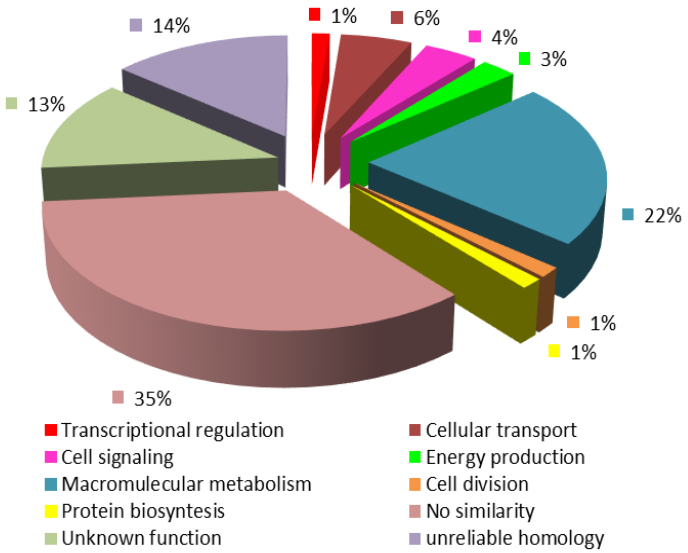

Figure 3. Functional category of the transcript derived fragments of chickpea leaves based on their homology. The pie chart is color-coded as per gene categories colors and represents the percentages of gene transcripts in each group.

BLAST searching database indicated that the majority of the sequenced TDFs were likely from *C. arietinum* (Table 1).

Comparison of the homologies of these sequences and those in the database revealed that about half of them belonged to either no hit (35%) or unknown proteins (13%), and the rest (52%) had homology with genes involved in putative functions. Unknown TDFs may represent new drought induced genes that have not been previously characterized and could contribute to future understanding of drought stress tolerance.

Among the sequences that were classified in later groups, different levels of homology with an E value ranging from 6e-51 to 13 were

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Table 1. The nucleotide-homology of the transcript-derived fragments (TDFs) with known gene sequences in the database using BLASTX algorithm.

Fragment name	Query length(bp)	Gene Bank accession no.	Best match accession	Best match description	Organism	E value	Max identity
D 3	240	KC347021	XP_004511282.1	Presequence protease chloroplastic/mitochondrial-like	*Cicer arietinum*	2.00E-41	100%
D 4	198	KC347022	AAK72000.1	Calmodulin	*Elaeis oleifera*	9.00E-34	100%
D 7	202	KC347023	NP_001167662.1	Calmodulin	*Zea mays*	1.00E-33	98%
D 9	140	KC347024	ACN88213.1	NADH dehydrogenase subunit 4, partial (mitochondrion)	*Medicago prostrata*	1.00E-13	95%
D 16	289	KC306618	XP_004511282.1	Presequence protease chloroplastic/mitochondrial-like	*Cicer arietinum*	6.00E-51	99%
D 21	282	KC306619	XP_004511282.1	Presequence protease chloroplastic/mitochondrial-like	*Cicer arietinum*	1.00E-46	93%
D 37	188	KC306620	AHY20359.1	NADH dehydrogenase subunit 4, partial (mitochondrion)	*Brassica juncea*	3.00E-19	90%
A 54	161	KC347025	YP_005370425.1	Putative lipoprotein	*Corallococcus coralloides*	4.2	45%
A 56	121	KC347026	XP_004508862.1	CASP-like protein At2g36330-like	*Cicer arietinum*	1.00E-12	91%
A 63	92	KC347027	XP_004496323.1	Uncharacterized protein LOC101505985 isoform X1	*Cicer arietinum*	2.00E-07	100%
A 65	140	KC347028	AFB83619.1	NADH dehydrogenase subunit F, partial (chloroplast)	*Portulaca sp.*	8.2	50%
B 74	167	KC347029	ZP_08965458.1	Histone deacetylase superfamily	*Natrinema pellirubrum*	3.5	40%
B 90	227	KC347030	EJC79189.1	Nitrogen regulation protein	*Rhizobium leguminosarum*	13	65%
B 106	141	KC347031	XP_004507205.1	H/ACA ribonucleoprotein complex non-core subunit NAF1-like	*Cicer arietinum*	0.088	89%
B 111	126	KC306626	XP_004514810.1	30S ribosomal protein S8, chloroplastic-like	*Cicer arietinum*	1.00E-04	74%
B 114	284	KC306622	XP_004486919.1	Uncharacterized protein LOC101509652	*Cicer arietinum*	2.00E-44	100%
C 122	275	KC347032	XP_004496762.1	2-Hydroxyisoflavanone dehydratase	*Cicer arietinum*	1.00E-21	85%
C 124	540	KC184895	XP_004495956.1	UDP-glucuronic acid decarboxylase 2-like	*Cicer arietinum*	2.00E-45	99%
C 125	467	KC347033	XP_004495956.1	UDP-glucuronic acid decarboxylase 2-like	*Cicer arietinum*	2.00E-45	100%
C 128	111	KC347034	XP_003597539.1	Peroxisome biogenesis factor	*Medicago truncatula*	9.5	71%
C 129	102	KC306627	XP_004502706.1	Mitochondrial chaperone BCS1	*Cicer arietinum*	2.00E-08	96%
C 130	128	KC347035	XP_004502706.1	Mitochondrial chaperone BCS1	*Cicer arietinum*	5.00E-06	100%
C 132	499	KC347036	XP_004495956.1	UDP-glucuronic acid decarboxylase 2-like	*Cicer arietinum*	2.00E-44	97%
C 133	488	KC347037	XP_004495956.1	UDP-glucuronic acid decarboxylase 2-like	*Cicer arietinum*	2.00E-44	97%
C 134	91	KC306623	ZP_10191678.1	Sensor signal transduction histidine kinase	*Rhodanobacter*	0.83	80%
E 153	296	KC347038	XP_004490128.1	Cell division topological specificity factor homolog, chloroplastic-like	*Cicer arietinum*	8.00E-14	71%
E 166	342	KC347039	P55871.2	Eukaryotic translation initiation factor 2 subunit beta	*Malus domestica*	1.8	73%
F 224	128	KC306628	XP_003592991.1	Hypothetical protein	*Medicago truncatula*	2.00E-08	92%
F 247	215	KC306641	XP_004502291.1	Uncharacterized protein LOC101506977	*Cicer arietinum*	8.00E-39	100%
F 249	203	KC306629	XP_004511350.1	chloride channel protein CLC-d-like isoform X1	*Cicer arietinum*	2.00E-15	74%
F 251	268	KC306630	XP_004485995.1	ABC transporter C family member 2-like isoform X2	*Cicer arietinum*	2.00E-12	78%
F 252	186	KC306638	XP_004510120.1	UPF0481 protein At3g47200	*Cicer arietinum*	6.00E-28	98%
F 254	188	KC306631	XP_004510120.1	UPF0481 protein At3g47200	*Cicer arietinum*	1.00E-28	98%
F 257	166	KC306632	XP_004505145.1	uncharacterized hydrolase yutF like isoform X2	*Cicer arietinum*	4.00E-21	100%
F 264	112	KC306639	XP_004489351.1	Paired amphipathic helix protein Sin3-like 2-like	*Cicer arietinum*	0.007	66%
F 270	337	KC306633	XP_002329733.1	Predicted protein	*Populus trichocarpa*	0.12	63%
F 286	176	KC306640	ABB48578.1	Phospholipase	*Cryptococcus neoformans*	2.00E-07	100%
F 288	214	KC306634	CCF43572.1	Phospholipase/Carboxylesterase superfamily protein, partial	*Colletotrichum higginsianum*	4.9	34%
F 292	217	KC306624	XP_004505145.1	uncharacterized hydrolase yutF-like isoform X2	*Cicer arietinum*	2.00E-29	100%
F 293	186	KC306635	XP_004510120.1	putative UPF0481 protein At3g02645-like	*Cicer arietinum*	7.00E-16	70%
F 294	205	KC306636	XP_004511350.1	Chloride channel protein CLC-d -like isoform X1	*Cicer arietinum*	0.034	54%
F 295	246	KC306625	XP_004502291.1	Uncharacterized protein LOC101506977	*Cicer arietinum*	7.00E-49	99%
F 296	173	KC306637	XP_004485995.1	ABC transporter C family member 2-like isoform X2	*Cicer arietinum*	8.4	60%

observed. The homology of 38% of TDFs were reliable and the similarity of the remaining TDFs (14%) were unreliable, because the homology scores of these sequences were lower than 50 and their E value was more than 10^{-5}(Claveri and Notredame, 2007). Sequences with reliable similarity score had homology with genes involved in functions like protein biosynthesis, cellular transport, signal transduction, energy production, transcription regulation and cell division (Table 2). The majority of these TDFs (22%) were involved in macromolecular metabolisms including chaperones, protease, hydrolase, UDP glucuronic acid decarboxylase, and hydroxy isoflavanone dehydratase.

Most of the known transcript categories were up-regulated under drought treatments in tolerant lines or had more expression in tolerant lines in comparison with the susceptible line. The only exception was the energy production category, where differentially-expressed TDF was down-regulated in ICCV2.

Predicted Function of AFLP-TDFs Homologues with Known Genes

In this study, we focused on TDFs which differentially expressed during drought treatments, had definite function corresponding to previously annotated protein encoding genes, their homology scores were more than 50, and their E value were lower than 10^{-5} (Table 1). These TDFs belonged to different functional group including:

Macromolecular Metabolisms

Enzymes like UDP glucuronic acid decarboxylase (C124, C125, C132 and C133 TDFs), protease (D16, D21 and A56 TDFs), hydroxy isoflavanone dehydratase (C122 TDF) and chaperones, were assigned to this functional group.

UDP glucuronic acid decarboxylase, which has been reported in a wide range of flowering plants, including mung bean, wheat, soybean, parsley, pea, tobacco, *Arabidopsis* and rice, is responsible for the synthesis of UDP xylose from UDP glucuronate (Zhang *et al*., 2005). Since the decarboxylation reaction catalyzed by this enzyme is essentially irreversible; its activity changes it and would be a target for regulatory control of cell wall composition through partitioning glycosyl residues between the synthesis of polysaccharides comprised of hexosyl residues and those containing pentosyl residues (Zhang *et al*., 2005). .

A mechanical property of the cell wall is known to be modulated in response to environmental stress (Hoson, 1998). High expression of arabinogalactan like proteins, which are abundant in the plant cell wall and plasma membrane, have been identified in the ICC 4958_drought_field library (Varshney *et al*., 2009). Zwiazek (1991) reported that preconditioning treatment by subjecting white spruce seedlings to three cycles of a mild and severe drought stress increased hemicellulose content of the cell walls. Increase in hemicelluloses and reduction of pectins was observed in the root apex of the drought tolerant durum wheat cultivar subjected to water stress (Leucci *et al*., 2008). Deokar *et al*. (2011) reported up-regulation of UDP galactose transporter in drought tolerant chickpea.

UDP-xylose is an important sugar donor in the synthesis of hemicelluloses (Suzuki *et al*., 2003). A fraction of cell wall that is important for controlling its strength and extensibility. Cell wall elasticity (CWE) and osmotic adjustment (OA) are important factors involved in turgor pressure maintenance under low water availability. More elastic cell walls would allow a reduction in cell volume and avoiding the plasma membrane to pull away from the wall inducing plasmolysis (Martinez *et al*., 2007). Jones and Corlett (1992) reported that plant metabolic processes are more sensitive to intermolecular distances. Hence, in comparison with absolute water potential, turgor and cell volume are critical factors in maintenance of metabolic activity. Martinez *et al*. (2007) indicated that CWE variations in common beans cultivars could be a genetic component of the water stress resistance. In contrast, Clifford *et al*. (1998) found that in *Ziziphus mauritiana*, maintenance of cell volume at low water potentials resulted from combination of solute accumulation and increased wall rigidity. These results suggest that CWE and OA are not present at the same time in a given plant. Taken together, the finding that UDP glucuronic acid

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decarboxylase gene has more expression in tolerant lines of chickpea in response to drought treatment suggests a possible role in protecting cells from water deficit by changing cell wall composition. Proteases are involved in all aspects of the plant life cycle ranging from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller, 2004). D16 and D21 TDFs which expressed in, respectively, drought tolerant genotype ICCV2 and drought sensitive line (ILC3279), showed homology to proteins similar to presequence protease. The expression of D16 TDF up-regulated under drought stress in ICCV2. The expression of D21 TDF increased up to two fold in ILC3279 under intermediate drought stress (T2) and then decreased to its expression level in the control under the severe drought stress treatment. Presequence protease or stromal processing peptidase (SPP) is an essential component of the chloroplast protein import machinery. The majority of chloroplast proteins are encoded in nucleus, translated in the cytosol as a protein precursor, then imported into the plastids. Import of the precursor proteins relies on post translational removing of transit peptide by SSP; the phenomenon that is necessary for chloroplast biogenesis and plant survival (Schaller, 2004).

Considering the frequency of chloroplast proteins, the importance of plastids for photosynthesis and the biosynthetic capacity of plants; removal of the transit peptide may be the most important posttranslational protein modification in the plant cell (Schaller, 2004). Up-regulation of this protease under drought stress probably helps plants to maintain their photosynthetic apparatus intact.

Up- and down-regulation of this TDF in the sensitive line indicated that this protein could have a positive role in maintaining photosynthetic apparatus of this line under mild drought stress, but not during the severe stress.

In plants, programmed cell death (PCD) is responsible for removal of redundant, misplaced, or damaged cells. This process is involved in various developmental events, such as differentiation of xylem, programmed abortion of floral organ in unisexual plants, and suspensor degeneration during embryonic development (Tian *et al*., 2000). Hyper sensitive response (HR) to pathogen attack is the most characterized kind of PCD in plants. However, recently it has been proved that abiotic stresses including salinity, cold stress, hypoxia, and waterlogging causes PCD, too (Shabala, 2009).

CASP like proteins are special kind of proteases called caspases (cystein aspartate specific proteases) and involved in apoptosis-like phenomenon and programmed cell death (Segovia and Berges, 2005). Although the role of caspase orthologues in plants was controversial for decades, recently it was demonstrated that proteolytic activity of caspase like protease leads to PCD. Tian *et al*. (2000) found that heat shock-induced apoptosis in tobacco suspension cells occurred after activation of caspase-3-like protease. Wang *et al*. (2010 a) also showed that the PCD in halophyte *Thellungiella halophila* under salt stress occurred through a caspase 3-like dependent pathway.

Leaf senescence is another example of PCD, although PCD feature in leaf senescence differs from its feature in other processes (Lim *et al*., 2007). Liu *et al*. (2007) indicated that the difference in osmotic and salt induced cell death play an important role in drought tolerance difference between two rice ecotypes. *Arabidopsis* mutants with high level of ROS are more sensitive to drought stress and show accelerated leaf senescence and cell death (Lee and Park, 2012). Leaf senescence is a developmental process in plant life cycle. In addition to developmental age, this phenomenon is affected by various internal and external factors. This process, which is a coordinated action at the cellular, tissue, organ, and organism levels has been controlled by regulated genetic program (Lim *et al*., 2007).

In annual crops, leaf senescence happens along with maturity and grain filing. During senescence, changes in cell structure, metabolism, and gene expression occurr and catabolic activity increase. Increase in catabolic activity convert the leaf accumulated cellular material into the exportable nutrients needed for seed development or for growing other organs. Therefore, although leaf senescence is a destructing cellular process for leaves, it is an altruistic process for other organs that ensures optimal production of a plant under adverse condition (Lim *et al*., 2007). Hence, leaf senescence is the final stage of leaf development and is critical for relocation of nutrients from

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leaves to seeds. Leaf senescence has been controlled by presence or absence of reproductive organs in soybean and pea and removal of reproductive organs reversed senescing leaves fate to juvenile (Lim *et al*., 2007).

Taken together, more expression of CASP like protein TDF (A56) in tolerant line of chickpea in comparison with sensitive line provided new convincing evidence for the involvement of PCD and leaf senescence in relocation of nutrients from leaves to other organs, especially to seeds. In line with this result, Pouresmael *et al*. (2013) demonstrated that harvest index and partitioning of assimilates to developing seeds is one of possible mechanisms that influenced drought tolerance in chickpea genotypes. Expression of CASP like protein transcripts under drought stress have been demonstrated in rice by Fu *et al*. (2007), too.

C122 TDF with maximum similarity to hydroxyl isoflavanon dehydratase amino acid sequence up regulated in FLIP9855C under drought stress. The sequence of this TDF contains Abhydrolase conserved domain. 2-hydroxyisoflavanone dehydratase (HID) catalyzed dehydration of 2-hydroxyisoflavanones to produce isoflavones. Isoflavonoids, which are characteristic metabolites of the legumes, play significant roles in plant adaptation to different biological environments (Shimamura *et al*., 2007). In line with this result, application of low concentration of soybean isoflavones significantly reduced injury of rape seedlings growth under drought stress (Ye *et al*., 2008). Deokar *et al*. (2011) also reported up-regulation of flavonoid biosynthetic process in roots of drought tolerant chickpea.

C129 and C130 TDF showed high similarity to mitochondrial chaperone. The expression of C129 TDF in tolerant line (FLIP9855C) was about 1.5 fold more than that in sensitive line. The expression of C130 TDF decreased to less than half with increase in drought severity in sensitive line. Molecular chaperones are key components contributing to cellular homeostasis under both optimal and stress conditions (Wang *et al*., 2004). Chaperones are responsible for stabilizing proteins through regulation of their folding, assembly, translocation, and degradation. Hence, they can play a crucial role in protecting plants against stress by maintaining proteins in their functional conformations (Wang *et al*., 2004).

Cellular Transport

TDFs similar to ATP-binding cassette (ABC) transporter (F251) and Cl^- channel (F249) which their expression up- regulated under drought assigned to this functional group. ABC transporter are responsible for cellular detoxification processes and involved in sequestration of secondary metabolites and heavy metals into the vacuole (Klein *et al*., 2004). In addition to tonoplast, the localization of these transporters in plasmalma shows that they are implicated in regulation of ion channel activities (Klein *et al*., 2004). Also, Klein *et al*. (2004) demonstrated that ABC transporter gene (AtMRP4) was involved in regulation of stomatal aperture and concluded that coordinate action of several MRP-type ABC transporters was implicated in the stomatal opening regulation and its interaction with gaseous environment of plants.

Several literatures have pointed to the role of this class of transporters under adverse conditions. For example, induction of ABC transporter gene has been reported in *Aneurolepidium chinense* under heat stress (Shi *et al*., 2002). Over expression of ABC transporter transcripts under heat and drought stress have been demonstrated in *Arabidopsis* by Rizhsky *et al*. (2004). Keinanen *et al*. (2007) reported over expression of ABC transporter gene in *Betula pendula* tolerant to Cu treatment. Up-regulation of ABC transporter transcripts under drought stress have been demonstrated in chickpea roots by Molina *et al*. (2011). Selvam *et al*. (2009) also documented that the difference between drought tolerant and sensitive varieties of *Gossypium hirsutum* was based on their difference in ABC transporter expression.

The opening and closing of the stomatal pore are regulated by dynamic changes of guard cells osmotic pressure and ion channels are critical factors in this process. The cytosolic Ca^{+2} elevation and abscisic acid activate S-type anion channels in the plasma membrane of guard cells. This kind of anion channels, which are responsible for Cl^- efflux, cause depolarization of guard cells membrane and provide the driving

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force for K^+ efflux through outward K^+ channels, thereby triggering stomatal closure. Up-regulation of Cl^- channel TDF in tolerant line shows that modulating the stomatal conductance in response to drought is another possible mechanism for drought tolerance in chickpea. Stomatal closure balances rates of water loss and absorbance and maintains leaf water potential close to that of the control.

Protein Synthesis

TDF B111, which expressed in FLIP9855C, is matched with ribosomal S8 proteins (Table 1) and contains special conserved domain of this kind of proteins. Ribosomal protein S8, a primary RNA binding protein of small ribosomal subunit, is a critical factor for correct folding of central domain of 16S RNA and stabilizing its tertiary structure (Davies *et al*., 1996). Hence, it causes translational regulation through participating in ribosome assembly. This protein is also one of the regulatory elements, responsible for controling ribosomal protein syntesis through translational feedback inhibition mechanism (Davies *et al*., 1996). It was proposed that this protein binds its own mRNAs in the same way that it binds to rRNA in the ribosome. Similar three dimensional structure between this protein mRNAs and rRNAs make this interaction possible. Competition between rRNAs and ribosomal protein mRNAs will guarantee the production of ribosomal proteins (Merianos *et al*., 2004).

Differential expression of this kind of proteins between chickpea lines shows that regulation of translation initiation is one of the critical mechanisms that differentiate tolerant and sensitive lines from each other. In line with this result, Molina *et al*. (2011) documented deployment of the protein machinery as prime response in the stressed roots of chickpea. Over expression of ribosomal protein transcripts under heat and drought stress have been demonstrated in *Arabidopsis* by Rizhsky *et al*. (2004). Keinanen *et al*. (2007) reported over expression of ribosomal protein gene in *B. pendula* tolerant to Cu treatment. Wang *et al*. (2010b) also documented the up-regulation of ribosomal protein in tolerant variety of *Arachis hypogaea* in response to *Aspergillus flavus* infection under drought stress.

Energy Production

The sequence of D9 TDF showed high similarity to NADH dehydrogenase proteins of *Medicago prostrata*. NADH dehydrogenase, also called mitochondrial complex I, is the first protein in the electron transport chain that participates in oxidative phosphorylation. Although oxidative phosphorylation is critical reaction for energy release in the cell, but inhibition of the full electron transport to molecular oxygen (a terminal electron acceptor in electron transport chain) produces reactive oxygen species (ROS). Hence, mitochondria represent a major source of ROS production and the consequent oxidative damage.

Reactive oxygen species are very harmful to cells, as they oxidize proteins and cause DNA denaturation. The plant cells use three different strategies for efficient defense against oxidative stress including the avoidance of ROS production, ROS detoxification, and the repair of ROS mediated damages. The first strategy, which is achieved by keeping the electron transport chain adequately oxidized, is more advantageous (Pastore *et al*., 2007).

Although ROS (s) are harmful for cell, they are also key factors for signal transduction under adverse condition. Hence, modulating ROS production may warrant quick control of cell homeostasis. It is well known that plant mitochondria play a critical role in cell adaptation to abiotic stresses through modulating cell redox homeostasis (Pastore *et al*., 2007). Rizhsky *et al*. (2004) demonstrated that, under drought and heat stress condition, increase in respiratory activity of plants is associated with expression of NADH dehydrogenase transcript. Keinanen *et al*. (2007) reported over expression of NADH dehydrogenase gene in *B. pendula* tolerant to Cu treatment. Liu *et al*. (2008) also documented over expression of NADH dehydrogenase protein in initial stages of rice flag leaf senescence.

In contrast to these examples, NADH dehydrogenase TDF (D9) down-regulated in ICCV2 and its expression under severe drought stress decreased to less than half of that under

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control treatment. Based on these results, the following hypothesis emerges: under control condition, constitutive level of this enzyme was high in drought tolerant line; therefore, the respiratory chain produced ROS at a basal level and activity of antioxidant enzymes was maintained at the low level (data not shown). Under severe drought stress treatment, a kind of feedback mechanism was activated that caused the expression of NADH dehydrogenase to decline. This reduction, in turn, increased NADH level, decreased the electron acceptor level in electron transport chain, and caused ROS production. In fact, severe drought treatment in ICCV2 changed cell homeostasis and ROS production works as a signal that elicits activation of stress responsive pathway and provides appropriate reaction against the stress. Increase in activity level of catalase in ICCV2 under severe drought stress treatment confirms this hypothesis (data not shown). But, further studies are necessary to check this possibility. In line with this hypothesis, Grabelnych *et al*. (2004) showed that the constitutive activity of alternative oxidase was higher in a pea under control condition, whereas the cold hardening decreased the activity of this enzyme.

Cell Signaling

Up-regulation of TDFs which contains EF hand calcium binding conserved domain (D4 and D7) in ICCV2 under drought stress shows that there is difference in tolerant and sensitive lines of chickpea in signaling cascades that are presumably contributing to the tolerance mechanism. Increased Ca^{2+} influx in response to biotic and abiotic stimuli can increase intracellular free calcium ion concentration (Liu *et al*., 2003).The Ca^{2+} elevations are sensed by Ca^{2+} sensors, which most often contain the EF-hand' motif(s) and a helix-loop-helix structure. The highly conserved Ca^{2+}-binding protein in plants is calmoduline (CaM), whose role in regulating calcium-dependent signaling pathways has been documented (Kim *et al*., 2010).

Identification of CaM-like proteins has been demonstrated by Fu *et al*. (2007) in study of drought tolerance candidate gene in rice. Molina *et al*. (2008) also reported up-regulation of CaM proteins and role of Ca^{2+} related signal transduction in chickpea under drought stress. Alignments of the amino acid sequence of D4 and D7 TDFs from ICCV2 and that of other species are shown in Figure 4.

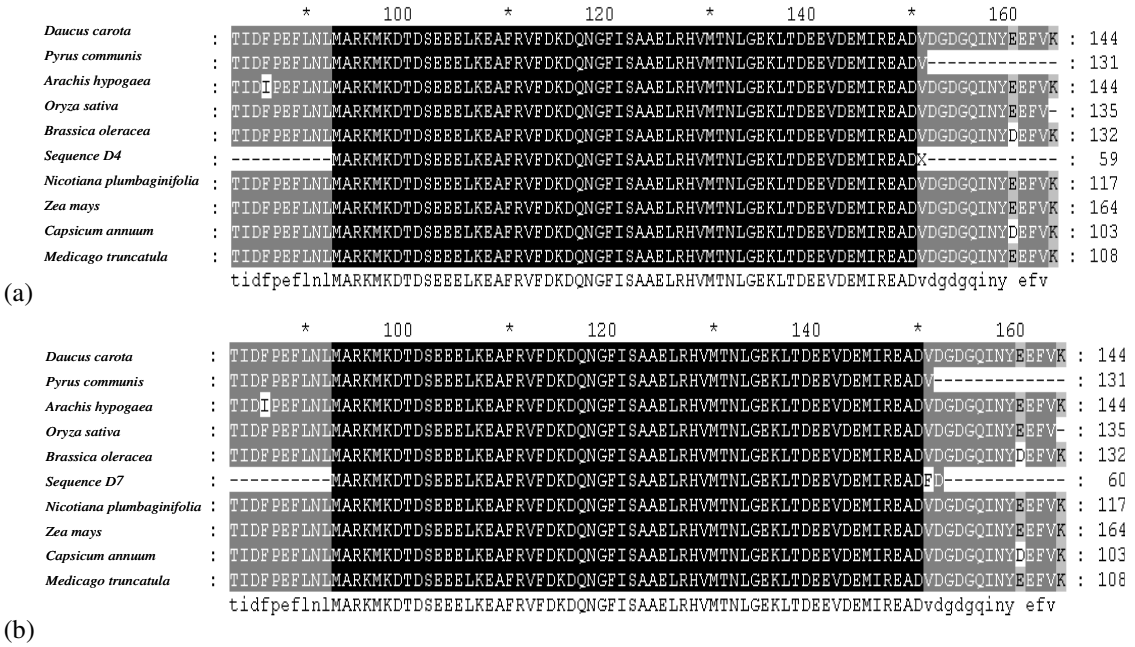

Figure 4. The multiple sequence alignment between D4 (a) and D7 (b) TDFs sequence from ICCV2 line of chickpea and their homologous CaM protein from other species.

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CONCLUSIONS

The present study demonstrated that cDNA-AFLP is a powerful technique to study candidate genes involved in drought tolerance of chickpea. About 38% of sequenced TDFs had reliable homology to protein with known function. These genes that were categorized in macromolecules metabolism, cellular transport, signal transduction, transcriptional regulation, cell division, and energy production functional groups provide insight in understanding the chickpea drought tolerant mechanism for future functional studies and can be targeted for increasing drought acclimation of this crop. On the other hand, about half of the sequenced TDFs encode proteins whose roles cannot be predicted from their amino acid sequences similarity and their biological activity remains to be determined. Working on these unknown proteins or ESTs with no hit in the data bank may be an important priority in future for further investigation and for exploring better understanding of new and unknown drought tolerance mechanisms.

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شناسایی مکانیسم های احتمالی تحمل خشکی در نخود زراعی با استفاده از تکنیک cDNA-AFLP

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چکیده

تنش خشکی یکی از چالش های اصلی تولید نخود بوده و تعیین مکانیسم های سازش به خشکی یکی از اجزاء اصلی برنامه های اصلاحی این گیاه به شمار می رود. این پژوهش به منظور درک اساس مولکولی تحمل خشکی در ژنوتیپ های حساس (ILC3279)و متحمل (ICCV2وFLIP9855C) گیاه نخود تیپ کابلی با استفاده از روش cDNA-AFLP به اجرا در آمد. مقایسه بیان ژن های واکنش به تنش در سه تیمار کنترل، خشکی متوسط و خشکی شدید که به ترتیب در آنها محتوای آب خاک در سطح ۹۰-۸۵، ۶۰-

۵۵ و ۳۰-۲۵ درصد ظرفیت زراعی حفظ شده بود انجام شد. در مجموع ۲۹۵ قطعه مشتق از رونوشت مشاهده شد که ۷۲ قطعه از انها تعیین توالی شد. قطعات تعیین توالی شده در گروه های عملکردی متفاوت نظیر ژن‌های دخیل در نقل و انتقال سلولی، متابولیسم ماکرومولکول ها، تنظیم رونویسی، ترارسانی، تقسیم سلولی و ژن‌های فراهم‌کننده انرژی، قرار گرفتند. بر اساس نتایج به دست آمده پروتئین ریبوزومیS8، چپرون های میتوکندریایی، پروتئازها، هیدرولازها، UDPگلوکورونیک اسید دکربوکسیلاز، ایزوفلاونوئید دهیدراتاز، NADHدهیدروژناز؛ ناقل‌های کلر و ناقل های ABC ، کالمودولین، هیستون داستیلاز و ژن‌های دخیل در تقسیم کلروپلاست به عنوان ژن‌های واکنش به تنش خشکی در گیاه نخود شناسایی شدند. بررسی میزان شباهت قطعات تعیین توالی شده با قطعات ثبت شده در پایگاه های اطلاعاتی نشان داد که الاستیسیته بالاتر دیواره سلولی، بیان بالاتر ایزوفلاون ها، حفظ ساختار و عملکرد پروتئین ها توسط بیان بیشتر چپرون های میتوکندریایی، مرگ سلولی برنامه ریزی شده و تحرک مجدد مواد غذایی از برگ ها به سمت دانه ها از جمله مکانیسم هایی است که اختلاف بین ژنوتیپ های متحمل و حساس را با یکدیگر مشخص می سازند.

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