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Transcriptome of Pigeonpea Roots under Water Deficit Analyzed by Suppression Subtractive Hybridization

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ABSTRACT

Pigeonpea (*Cajanus cajan* (L) Millsp.) is a drought tolerant legume widely grown in the arid and semi-arid tropics of the world which possesses a deep and extensive root system that succors a number of important physiological and metabolic functions to cope with drought. Application of available functional genomics approaches to improve productivity under water deficit requires a better understanding of the mechanisms involved during pigeonpea's response to water deficit stress. In order to identify the genes associated with water deficit in pigeonpea, Suppression Subtractive Hybridization cDNA library was constructed from polyethylene glycol-induced water deficit young root tissues from pigeonpea and 157 high quality ESTs were generated by sequencing of 300 random clones which resulted in 95 unigenes comprising 37 contigs and 58 singlets. The cluster analysis of ESTs revealed that the majority of the genes had significant similarity with known proteins available in the databases along with unique and hypothetical/uncharacterized proteins. These differential ESTs were characterized and genes relevant to the specific physiological processes were identified. Northern blot analysis revealed the up regulation of ornithine aminotransferase, cyclophilin, DREB and peroxidase. The differentially expressed sequences are conceived to serve as a potential source of stress inducible genes of the water deficit transcriptome and hence may provide useful information to understand the molecular mechanism of water deficit management in legumes.

Keywords: cDNA, drought, PEG, Polyethylene glycol.

INTRODUCTION

Crop growth and productivity is under constant threat by environmental challenges in the form of various abiotic stresses, preventing them from reaching their full genetic potential and limiting crop productivity worldwide. Among them, water deficit is one of the major abiotic stresses that adversely affect nearly all aspects of plant metabolism and productivity (Bray *et al.,* 2000). The molecular responses of the plant to abiotic stresses include stress perception, signal transduction, gene expression, and finally, metabolic changes which not only protect cells from stress by the production of important metabolic proteins but also regulate downstream genes for signal transduction (Lata and Prasad, 2011). Drought tolerance is a quantitative trait with genetic, epigenetic and environmental components modulated by a set of characterized and uncharacterized transcription factors. Some of these are regulators of the ABA response mechanism, while others are involved in ABA-independent pathways (Cortes *et al.,* 2012). Numerous molecular responses of

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plants to water deficit stress have been observed in different plant species like *Arabidopsis thaliana* (Seki *et al.,* 2002), barley (Guo *et al.,* 2009), foxtail millet (Lata *et al.,* 2010), rice (Rabello *et al.,* 2008), pigeonpea (Priyanka *et al.,* 2010b), tea (Das *et al.,* 2012) etc. including transitions in gene expressions at different stages of water deficit. Since gene function is a complex phenomenon, the identification of genes that impart a vital role in drought response and tolerance will reveal insights into molecular adaptations of plants and could be essential for genetic improvement of drought sensitive legumes and crop plants.

Genes involved in drought responses and tolerance in plants have been identified through numerous techniques, including differential screening and serial analysis of gene expression, cDNA-AFLP, microarray analysis, subtractive hybridization and suppression subtractive hybridization (Deokar *et al.,* 2011; Li *et al.,* 2010; Liu and Jiang, 2010; Zhang *et al.,* 2009; Zheng *et al.* 2004), which proved to be powerful techniques to identify differentially expressed genes under water deficit conditions in different crop species. Suppression Subtractive Hybridization (SSH) is widely used to identify the genes in tissues which differentially expresses under various biotic and abiotic stresses. In order to identify differentially expressed genes induced during the drought stress in *Cicer arietinum* (Deokar *et al.,* 2011), *Gossypium hirsutum* (Wang *et al*., 2010) *Phaseolus vulgaris* (Recchia *et al*., 2013), salinity stress response in *Medicago truncatula* L. seedlings (Kang *et al.,* 2010) and differentially expressed cDNAs in *Ceratoides lanata*, during cold-stressed (Zeng *et al.* 2010) by SSH have led to isolation of valuable antifreeze, heat-resistant, drought-tolerant, and alkali-salt-tolerant genes from various crop species.

Pigeonpea (*Cajanus cajan* L.) Millsp (2n= 22) is a major grain legume of the arid and semi-arid regions of the world (Nene and Sheila, 1990). It is cultivated globally in an area of 4.6 million ha with total productivity of about 3.4 million tons. Since, pigeonpea is widely grown in rainfed areas, a deep and extensive root system attributes these plants to withstand in several types of water-limiting stresses. The combination of physiological relevance and structural simplicity has made roots obvious targets for functional genomics analysis. The first pigeonpea EST dataset provides a transcriptomic resource for gene discovery and development of functional markers associated with biotic stress resistance (Raju *et al.,* 2010). Priynaka *et al.* (2010b) reported the generation of ESTs obtained from the subtracted cDNA libraries of pigeonpea whole plant tissues subjected to PEG/water-deficit stress conditions. Furthermore, knowledge of the pigeonpea transcriptome from various tissues will enhance basic understanding of how plants respond to water deficit stress and serve as a source of candidate genes of value to agriculture. In this study, our prime objective was the cloning and analysis of stress inducible genes expressed under PEG-induced water deficit from pigeonpea root tissues. Also, we aimed to construct the differential cDNA library of pigeonpea and verify the functionality of differentially expressed ESTs through northern blotting.

MATERIALS AND METHODS

Stress Induction and RNA Isolation

Drought tolerant pigeonpea cultivar (GRG-295 genotype) was screened for water deficit stress tolerance induced by polyethylene glycol-6000, a high molecular weight and non toxic compound, with diverse osmotic potential at seedling stage. The change in osmotic potential by PEG leads to the reduction of water absorption by seeds, which seems to be the main factor responsible for decrease in germination percentage and rate of root and shoot growth. The seeds were surface sterilized with 0.1% $HgCl_2$ and residual chlorine was eliminated by thoroughly washing the seeds with distilled water. For the induction of water deficit condition, PEG-6000 was dissolved in distilled water to achieve the

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diverse osmotic potentials of the test solutions to -0.15, -0.30, -0.45, and -0.89 MPa as described by Michel and Kaufmann (1973), while the seeds irrigated with distilled water served as the control. The seeds were allowed to germinate on double layer filter paper in petridish for 7 days under controlled conditions at 24±2°C and 12 hours photoperiod. The experiment was carried out in triplicate and at the end of the 7th day, the physiological parameters like germination percentage and root and shoot length were measured. On the basis of the results, stress level of -0.45 MPa was found optimal for the construction of SSH library. The total RNA from stressed root tissues along with the non-stressed root tissues was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), and mRNA was further isolated by using the PolyATract mRNA Isolation System (Promega, Madison ,WI). The quantification of RNA was verified by an absorption ratio of $OD_{260/280}$ and by formaldehyde gel electrophoresis.

Construction of Suppression Subtractive Hybridization (SSH) Library

The suppression subtractive library was constructed by using the Clontech PCR-Select™ cDNA subtraction kit (Clontech, Palo Alto, CA), starting with 5 µg of mRNA from tester and driver sample, respectively. In the subtractive library, the cDNA from non-stressed root tissues served as driver while the mRNA of stress induced (-0.45 MPa) root tissues served as tester. The first and second strand of the cDNA was synthesized as per the user manual of Clontech PCR-Select™ cDNA subtraction kit. The subtraction was performed according to manufacturer's instructions to identify the transcripts enriched in one sample relative to the other. Subtracted cDNAs were purified by the MinElute PCR purification kit (Qiagen, Valencia, CA) and ligated into a pGEM-T easy vector (Promega). Ligated plasmid DNAs were transformed into competent *E. coli* DH5α strain. Positive clones were selected on an ampicillin/IPTG/X-Gal LB plate. Plasmid DNA from positive clones was isolated by using the REAL 96 plasmid isolation kit (Qiagen), and purified DNA was single-pass Sanger sequenced with M13F/R universal sequencing primers on ABI 3500 XL Genetic analyzer.

Sequence Processing and Annotation

All the ESTs were processed using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) to remove vector, cloning oligo sequences and various contaminants and to trim to a high quality region. All the generated ESTs were then compared with NCBI (http://www.ncbi.nlm.nih.gov) non-redundant protein database using BLASTx algorithm setting the default parameters. The similarity scores between the cDNA clones and known sequences were represented by BLASTx probability *E*-values. The sequences with the *E*-value≤ 1e-10^{-5} were considered for annotation. Further, the ESTs were classified into different functional categories based on the knowledge of biochemistry, plant physiology, and molecular biology (http://www.MetaCyc.org/), GO (http://www.ebi.ac.uk/GO/), COG (http://www.ncbi.nlm.nih.gov/COG/) tools and by searching related abstracts in PubMed.

Expression Validation by Northern Blotting

For Northern blotting total RNA (20 µg) from the control and stressed pigeonpea roots was separated by electrophoresis on a 1.5% formaldehyde agarose gel and transferred onto nylon membrane (Amersham) following the method of Sambrook and Russell (2001). The blots were air-dried and UV cross-linked at 150

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mJ using a UV cross linker (GS Gene linker, Bio-Rad). PCR-amplified individual cDNA fragments (amplified with M13 forward and reverse universal sequencing primers) were purified from the agarose gel and used as probes made by random primer α^{32}P-dATP labelling using DecaLabel™ DNA labeling kit (Fermentas Life Sciences). Amplified cDNA actin (EU529707) was the house-keeping gene control. Northern blots were scanned using a PharosFx Plus PhosphorImager (Biorad).

RESULTS AND DISCUSSION

To cope with major abiotic stresses imposed by water, extreme temperature, and high salt concentration, the sessile-terrestrial plants have evolved unique physiological and metabolic responses mediated by various stress-specific genes. An understanding of the mechanisms that regulate the expression of stress-responsive genes are of cardinal importance in basic plant biology, as they hold the key to genetic enhancement of plant productivity under adverse environmental conditions (Priyanka *et al.,* 2010a). Several methods, which range from water withdrawal to the use of chemicals such as polyethylene glycol, mannitol, etc., have been employed to create water stress in plants. It has been reasonably well established that polyethylene glycol induced water stress mimics that caused by withdrawal of water from plants (Kaydan *et al.,* 2008). Polyethylene glycol is commonly used to stimulate osmotic stress effects in petri dishes to control water potential in seed germination studies. The nontoxic PEG solution is favored for its high molecular weight, and its disability to pass plant cell walls (Kaydan *et al.,* 2008). Plants root systems were exposed to this solution, and no toxicities were observed at the plant level following the addition of PEG-6000 (Emmerich and Hardegree, 1990).

The germination response of pigeonpea cultivar at different levels of osmotic potential is depicted in Figure 1, which reveals that a decrease in osmotic potential of the medium resulted in gradual decrease in percentage of germination and root and shoot length. The germination was not observed beyond -0.89 MPa, indicating that the species resistance limit to water deficit was between -0.30 to -0.70 MPa. On the basis of the response to PEG induced water deficit and after determining the expression of RNA, the stress level of -0.45 MPa was chosen to isolate RNA and, subsequently, SSH libraries was constructed. Total RNA was isolated from stressed and non-stressed (control) pigeonpea root tissues at the end of 7 days of germination and mRNA was purified from total RNA using PolyATract mRNA Isolation System. To construct cDNA subtractive libraries, mRNA from the stress-induced root tissues served as tester and cDNA from healthy root tissues as driver. The unhybridized differential mRNA was further used for the construction of the subtractive cDNA library. The subsequent colony PCR analysis of clones revealed that the size of inserts ranged from 100 to 800 bp. Out of 475 bacterial colonies screened by colony PCR, the plasmid construct of 300 positive recombinant clones were sequenced in single pass sequencing reactions from 3' end using M13 forward/reverse primer. The leading sequences, tailing of the sequences, and poor quality sequences were excluded first, and 157 high-quality ESTs were retained from PEG induced water deficit library. In BLASTx analysis, it was observed that most sequences had significant homology with known proteins. Sequences that had no significant homology with entries in the protein database were compared to nucleotide BLAST using default parameters. Based on bioinformatics and differential screening, 157 differentially expressed genes were identified. The corresponding ESTs were clustered into 95 unigenes comprising 37 contigs and 58 singlets (Table 1). With the homology search against Genbank, the majority of the genes had significant homologs in other plants, of which many were related to the water-stress genes previously reported in

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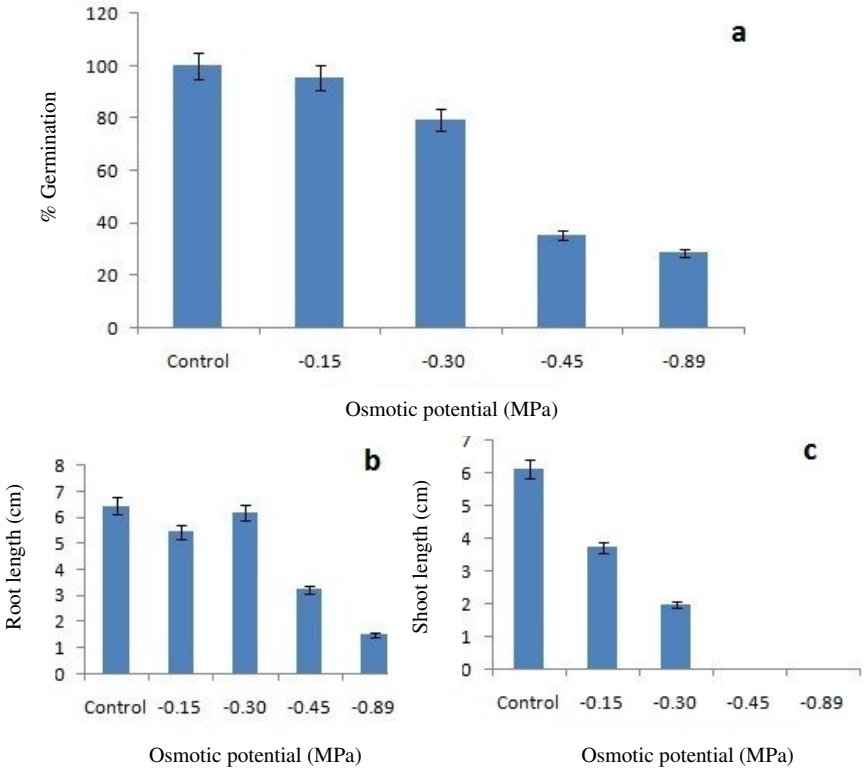

Figure 1. Germination parameters of the selected pigeonpea cultivar under diverse osmotic potentials: (a) Percentage of germination; (b) Root length, and (c) Shoot length.

Arabidopsis and *Medicago*. The ESTs were deposited in NCBI dbEST with the accession number JK973742 to JK973898 (Table 2).

The genes from pigeonpea generated by induction of water stress were classified into 7 diverse functional groups depicted in Figure 2. Out of 157 differentially subtractive clones, 13.37% were transporter genes, 24.84% genes encoded osmotic stress genes, 8.92% were signal transduction and transcriptional regulator genes, 31.21% biological (metabolism, structural organization, DNA and RNA processing) process genes, 7% hypothetical and uncharacterized genes, 10.83% of genes were involved in other functions, while 3.82% genes were not having significant match in database.

Table 1. Summary statistics of EST libraries.

Total number of clones sequenced	300
ESTs taken for analysis	157
Number of unigenes	95
Number of contigs	37
Number of singlets	58
Average length of unigenes	439 bp
Average length of ESTs	430 bp
% GC content of unigenes	51.1
% GC content of ESTs	51.9

In the SSH library, various transcripts with known function *viz.* glutathione transferase (Gen Bank IDs |JK973835|, |JK973878|), peroxidase (Gen Bank IDs |JK973768|, |JK973776|, |JK973799|, |JK973801|, |JK973807|, |JK973888|), aldehyde dehydrogenase (Gen Bank IDs |JK973771|, |JK973773|, |JK973778|, |JK973880|, |JK973881|), alcohol dehydrogenase (Gen Bank IDs | JK973814|, | JK973817|, | JK973824|), DREB (Gen Bank IDs |

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Table 2. Functional classification of contigs and singlet of the ESTs generated under water deficit stress along with their E value on the basis of homology search using BLAST algorithm.

Accession no.	Description/species	No. of reads	E-value
	Transporter		
|XP_003573221.1|	ABC transporter family member [*Brachypodium distachyon*]	6	1e-08
|NP_001236298.1|	Sucrose transporter [*Glycine max*]	2	1e-109
|XP_003629906.1|	Potassium transporter [*Medicago truncatula*]	5	5e-102
|ZP_09292875.1|	Glycine betaine transporter periplasmic subunit [*Mesorhizobium*]	1	3e-07
|AAB87674.1|	Neutral amino acid transport system II [*Arabidopsis thaliana*]	1	7e-21
|NP_001184926.1|	Nodulin mtn21 /eama-like transporter protein [*Arabidopsis thaliana*]	2	2e-44
|XP_002530408.1|	Probable sugar phosphate/Phosphate translocator [*Vitis vinifera*]	1	3e-27
|AAY82249.1|	Mitochondrial voltage-dependent anion-selective channel [*Phaseolus coccineus*]	1	3e-32
|XP_002312708.1|	Porin/voltage-dependent anion-selective channel protein [*Populus trichocarpai*]	1	6e-11
|XP_003552598.1|	Solute carrier family 25 member 44-like [*Glycine max*]	1	2e-131
	Osmotic Stress Responsive Protein		
|CCF23020.1|	Drought responsive element binding protein 1[*Glycine max*]	2	5e-37
|AAB41811.1|	Peroxidase, putative [*Medicago sativa*]	6	7e-09
|ADD11814.1|	Hybrid proline-rich protein [*Cajanus cajan*]	3	5e-17
|NP_001236813.1|	Aldehyde dehydrogenase family [*Glycine max*]	5	1e-44
|XP_003629864.1|	Aquaporin [*Medicago truncatula*]	7	2e-109
|XP_003594839.1|	ABA-responsive protein ABR17 [*Medicago truncatula*]	1	5e-33
|NP_001234324.1|	Cysteine protease [*Solanum tuberosum*]	1	4e-44
|ACQ99773.1|	Alcohol dehydrogenase [*Cajanus cajan*]	3	2e-124
|ADI43217.1|	Osmotin-like protein [*Oryza sativa* Indica Group]	2	3e-16
|NP_001105510.1|	Glutathione S-transferase [*Zea mays*]	2	8e-20
|AAG09278.1|	Ornithine aminotransferase [*Vitis vinifera*]	2	1e-39
|XP_002510543.1|	Cyclophilin [*Ricinus communis*]	4	2e-58
|ACB59070.1|	Early response to drought 3 [*Pinus elliottii*]	1	4e-14
	Signal Transduction and Transcriptional Factors		
|NP_196014.1|	Signal recognition particle subunit [*Arabidopsis thaliana*]	1	9e-80
|AAS78476.1|	CCAAT-box transcription factor complex WHAP1[*Triticum aestivum*]	1	5e-10
|XP_003545749.1|	Transcription factor GTE11-like [*Glycine max*]	1	2e-30
|NP_182191.1|	Homeobox-leucine zipper protein ATHB-7 [*Arabidopsis thaliana*]	1	3e-43
|XP_003526539.1|	ZF-HD homeobox protein At4g24660-like [*Glycine max*]	2	1e-68
|XP_003526572.1|	F-box protein At1g78280-like [*Glycine max*]	3	1e-97
|AAF71806.1|	RNA recognition motif-containing protein [*Arabidopsis thaliana*]	1	6e-12
|AAG51243.1|	Zinc-finger protein [*Arabidopsis thaliana*]	3	2e-38
|XP_002891614.1|	Basic helix-loop-helix family protein [*Arabidopsis lyrata*]	1	9e-89
	Biological Processes (Metabolism, Cell structural organization, DNA and RNA Processing)		
|XP_003631280.1|	Mitochondria Outer membrane protein porin of 36 kda isform 2[*Vitis vinifera*]	1	3e-14
|XP_003610252.1|	Transmembrane protein-like protein [*Medicago truncatula*]	1	6e-25
|AAR99079.1|	Xanthine dehydrogenase 2 [*Arabidopsis thaliana*]	2	3e-17
|XP_002271255.1|	RNA polymerase II subunit A C-terminal domain phosphates [*Vitis vinifera*]	1	2e-61
|NP_174109.2|	ATP-dependent DNA helicase Q-like [*Arabidopsis thaliana*]	2	6e-19
|XP_002267930.1|	Aspartic proteinase nepenthesin-1-like [*Vitis vinifera*]	4	3e-116
|XP_002517672.1|	Hydrolase, Putative [*Riccinus communis*]	3	1e-14
|NP_568843.1|	Protein kinase family protein [*Arabidopsis thaliana*]	5	1e-121
JK973754	Ribonucleoside-diphosphate reductase small chain-like [*Glycine max*] (XP_003547645.1)	1	2e-34

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Continued of Table2.

Accession no.	Description/species	No. of reads	E-value
|ABX88962.1|	Cytochrome oxidase subunit II [*Cajanus cajan*]	1	1e-69
|BAA97361.1|	Ca^{2+}-transporting ATPase-like protein [*Arabidopsis thaliana*]	3	3e-46
|XP_003610980.1|	UDP-glucose 4-epimerase [*Medicago truncatula*]	2	2e-53
|ACG31465.1|	Hydroxyacylglutathione hydrolase [*Zea mays*]	1	3e-45
|XM_008683179.1|	Fiber protein, Predicted [*Zea mays*]	1	2e-41
|BAB86292.1|	Nucleoside diphosphate kinase 1 [*Brassica rapa*]	1	1e-51
|XP_003543661.1|	Bifunctional aspartate aminotransferase and glutamate/aspartate-prephenate aminotransferase-like [*Glycine max*]	1	4e-128
|XP_002298312.1|	Isomerase peptidyl-prolyl cis-trans isomerase [*Populus trichocarpa*]	3	9e-06
|NP_001078556.1|	Malate dehydrogenase [*Arabidopsis thaliana*]	1	2e-75
|NP_201190.1|	Amino acid permease 4 [*Arabidopsis thaliana*]	2	6e-149
|NP_001234432.1|	N-acetyl-glutamate synthase [*Solanum lycopersicum*]	1	1e-19
|ACG25973.1|	Farnesyl pyrophosphate synthetase [*Zea mays*]	1	5e-47
|ABD96608.1|	Xyloglucan endotransglucosylase XET2 [*Capsicum annuum*]	2	1e-07
|NP_187277.1|	Galacturonosyltransferase-like 4 [*Arabidopsis thaliana*]	1	6e-178
|XP_003517586.1|	Probable carboxylesterase 2-like [*Glycine max*]	1	2e-88
|XP_002509491.1|	Acyltransferase [*Ricinus communis*]	1	6e-24
|XP_002870710.1|	Galactinol-sucrose galactosyltransferase 5, Putative [*Arabidopsis thaliana*]	2	9e-07
|XP_003527110.1|	Glucan endo-1,3-beta-glucosidase-like protein [*Glycine max*]	1	1e-04
|NP_200041.1|	Myosin heavy chain-related protein [*Arabidopsis thaliana*]	3	6e-28
	Hypothetical and Uncharacterized Protein		
|XP_003601178.1|	Hypothetical protein MTR_3g076860 [*Medicago truncatula*]	1	7e-39
|XP_002442114.1|	Hypothetical protein SORBIDRAFT_08g012980 [*Sorghum bicolor*]	1	5e-78
|XP_002463955.1|	Hypothetical protein SORBIDRAFT_01g009520 [*Sorghum bicolor*]	2	2e-43
|BAD97437.1|	Hypothetical protein MTR_7g011450 [*Medicago truncatula*]	1	8e-43
|XP_003601178.1|	Hypothetical protein MTR_3g076860 [*Medicago truncatula*]	1	8e-39
|CAJ13713.1|	Hypothetical protein [*Capsicum chinense*]	1	5e-49
|XP_003609992.1|	Hypothetical protein MTR_4g125100 [*Medicago truncatula*]	1	5e-107
|NP_001238451.1|	Uncharacterized protein LOC100306156 [*Glycine max*]	1	1e-35
|XP_003542110.1|	Uncharacterized protein LOC100797659 [*Glycine max*]	1	2e-57
|BAJ93493.1|	Predicted protein [*Hordeum vulgare*]	1	9e-13
	Other Functions		
|XP_003541070.1|	21 kda protein-like [*Glycine max*]	1	2e-16
|XP_003620940.1|	Thioredoxin-like protein [*Medicago truncatula*]	1	4e-17
|XP_002279126.1|	Inactive rhomboid protein 1 isoform 2 [*Vitis vinifera*]	1	1e-15
|NP_001236263.1|	40S ribosomal protein S27-2-like [*Glycine max*]	1	4e-56
|XP_002866394.1|	Atgcn1 [*Arabidopsis lyrata*]	1	1e-33
|BAD18384.1|	Type 1 metallothionein [*Lablab purpureus*]	1	9e-20
|XP_003624386.1|	Non-specific lipid-transfer protein [*Medicago truncatula*]	1	2e-22
|NP_001148622.1|	Ubiquilin-1[*Oryza sativa*]	1	3e-34
|XP_003628200.1|	MLP-like protein [*Medicago truncatula*]	2	4e-67
|XP_002323748.1|	2-oxoglutarate/malate translocator [*Arabidopsis thaliana*]	2	9e-84
|XP_002534730.1|	Protein binding protein [*Ricinus communis*]	1	1e-15
|AAA87049.1|	Zn-induced protein [*Oryza sativa*]	2	9e-40
|XP_002281282.1|	Charged multivesicular body protein 1[*Vitis vinifera*]	1	4e-75
|NP_001235842.1|	Nodulin-C51 precursor [*Glycine max*]	1	1e-103

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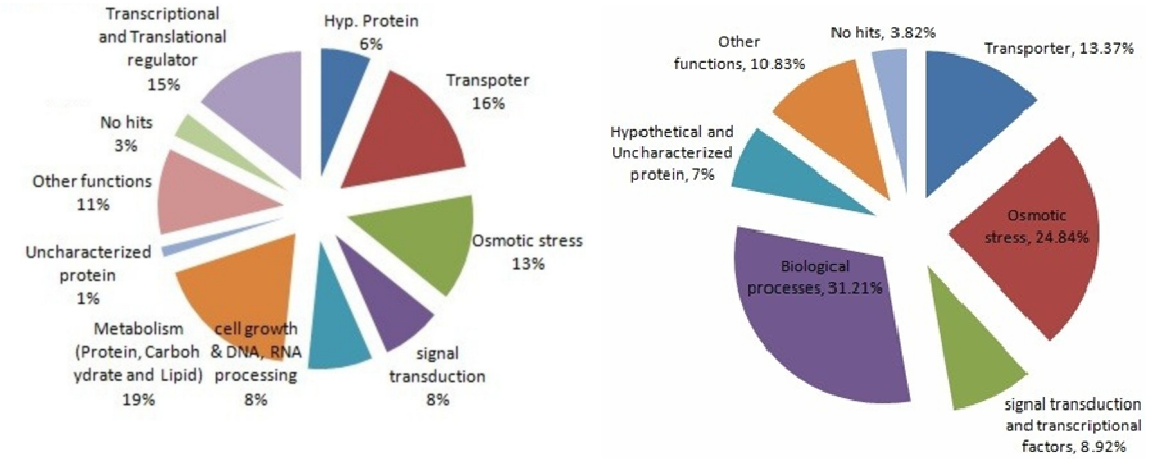

Figure 2. Functional classification of pigeonpea ESTs obtained from water deficit cDNA library based on homologous proteins from non-redundant protein sequence database.

JK973747|, |JK973892|), early response to drought (Gen Bank ID | JK973890|), cyclophilin (Gen Bank IDs | JK973816|, | JK973852|, | JK973854|, | JK973873|), zinc finger (Gen Bank IDs | JK973750|, | JK973759|, | JK973762|, | JK973763|, | JK973872|), F-box (Gen Bank IDs |JK973760|, | JK973765|, | JK973859|), and proline rich protein (Gen Bank IDs | JK973770|, | JK973774|, | JK973813|) were observed, which are thought to be involved in defense mechanisms in response to water deficit. The increased expression of glutathione transferase, peroxidase (Yong *et al.,* 2011), aldehyde dehydrogenase (Wang *et al.,* 2011) alcohol dehydrogenase (Bray, 2004) and water-induced cysteine protease (Harrak *et al.,* 2001) were also reported under water deficit stress.

In the present investigation, expression of a key enzyme involved in the proline biosynthesis pathway, namely, ornithine aminotransferase, was significantly enhanced under water deficit stress, and the up-regulation of ornithine aminotransferase was confirmed through Northern blot analysis (Figure 3), which indicates that the ornithine aminotransferase pathway may potentially play a role in proline biosynthesis under stress. A significant increase in proline concentrations has been reported in plants responding to water

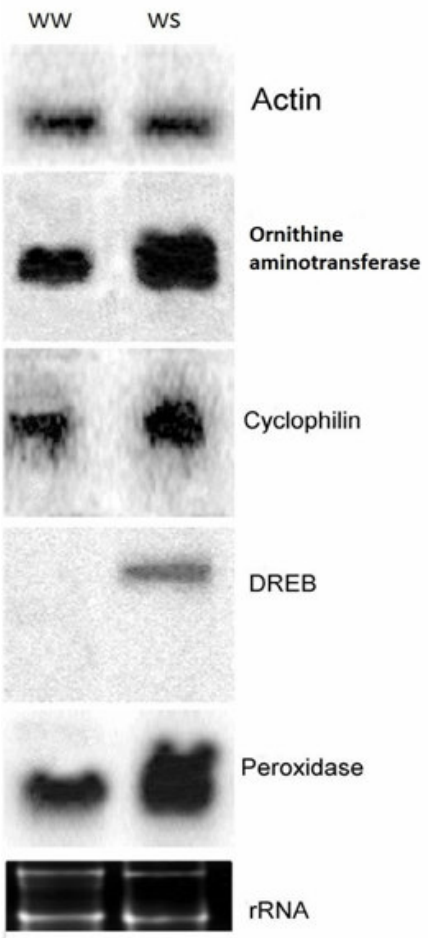

Figure 3. Northern blot analysis of genes under non-stressed and stressed conditions. Actin served as the control (WW: Well Watered/Non-stressed; WS: Water deficit Stressed).

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deficit, and accumulation of proline is considered as an indicator of stress-adaptive response of plants (Deokar *et al.,* 2011; Kumar *et al.,* 2011a). Priyanka *et al.* (2010a) reported the presence of hybrid proline rich protein from pigeonpea under water stress, which seems promising as a prime candidate gene to fortify crop plants with abiotic stress tolerance, especially since it acts in various processes of plant growth and development in a tissue- and cell-specific manner. The presence of an alternative proline biosynthesis pathway, i.e. ornithine/arginine pathway, was also reported in higher plants growing on high nitrogen source (Kavi Kishor *et al.,* 2005).

The Northern blot analysis suggests the up regulation of cyclophilin under stress (Figure 3). The up regulated expression of cyclophilin was previously reported to occur under drought stress by Sharma and Kaur (2009). Cyclophilins occur in diverse organisms ranging from bacteria, fungi, and plants to animals and humans (Wang and Heitman, 2005). The implicit role of cyclophilin in conferring multiple abiotic stress tolerance at whole-plant level in pigeonpea is also reported (Sekhar *et al.,* 2010).

The presence of transcripts encoding osmotin, DREB, zinc finger, and homeobox proteins indicates a direct role of the underlying genes in water stress. The transcriptional factors regulate the expression of particular sets of genes that activate or repress the pathways related to drought tolerance in plants (Recchia *et al*., 2013). The upregulation of DREB was also observed in Northern blot analysis (Figure 3). Drought Response Element Binding (DREB) factors present on the promoters of many stress response genes are one of the most characterized groups of transcription factors in the water deficit stress response (Lata and Prasad, 2011; Matsukura *et al.,* 2010). Zinc finger protein (ZAT12), is induced by water stress, as well as many other abiotic and biotic stress conditions and this protein appears to be a positive transcriptional regulator in osmotic stress tolerance. However, it is not known if it acts in an ABA-dependent or ABA-independent pathway (Davletova *et al.,* 2005).

The upregulation of peroxidases were also proven through Northern blotting (Figure 3) which implies a vital role of these antioxidative enzymes. They are involved in detoxifying oxidative radicals and repairing damage under various stresses, suggesting the enzymatic removal of toxic H_2O_2. As plants are well endowed with antioxidant molecules and scavenging systems which establish a link between tolerance to water stress and rise in antioxidant enzyme concentration in photosynthetic plants, superoxide dismutase is thought to provide the first line of defense against the toxic effects of reactive oxygen radicals by catalyzing conversion of O_2^- to H_2O_2 and O_2. Peroxidases have a high capacity for the decomposition of H_2O_2.

The presence of plant aquaporins (Gen Bank IDs |JK973779|, |JK973804|, |JK973806|, |JK973826|, |JK973830|, |JK973833|, |JK973889|) also suggests their possible role in coping with water stress. Aquaporins are members of a large super-family of membrane spanning proteins and highly conserved across different plant species. The down regulation of the aquaporins gene expression results in a reduced hydraulic conductivity of roots and further induces the extra growth of root systems (Martre *et al*., 2002). Although aquaporins play a cardinal role in water relations of roots, leaves, seeds, and flowers, they have also been linked to plant mineral nutrition and carbon and nitrogen fixation (Maurel *et al.,* 2008).

In addition, few novel ESTs transcripts (Gen Bank IDs |JK973775|, |JK973793|, |JK973799|, |JK973832|, |JK973859|, |JK973898|), uncharacterized (Gen Bank IDs | JK973856|, | JK973857|, | JK973863|), and hypothetical proteins (| JK973772|, | JK973782|, | JK973789|, | JK973793|, | JK973800|, | JK973810|, | JK973821|, | JK973870|), which seem to play prime roles in water deficit stress tolerance in

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pigeonpea, were identified in the subtracted library under stress.

CONCLUSIONS

The present investigation provides a repertoire of water deficit stress related genes in pigeonpea root tissues on the basis of SSH experiments, which correspond to results of similar studies in other plants. These differentially expressed ESTs identified from root tissues of pigeonpea could be regarded as candidate targets potentially associated with drought tolerance. In addition to known drought-induced genes, some of the identified differentially expressed genes were unknown, whose functional roles therefore remain unclear and require further investigation. The present study was also directed towards understanding the expression pattern of selected drought induced genes which revealed the up-regulation of ornithine aminotransferase, peroxidase, DREB and cyclophilin. Our present work contributes to a basic understanding of the molecular mechanisms of water-stress tolerance and facilitates the genetic manipulation of stress tolerance in pigeonpea and other legumes. The present report may be exploited to overcome water deficit stress related problems limiting production in drought sensitive legumes and, subsequently, in breeding advanced cultivars giving better yields under rain-fed conditions.

Abbreviations

ESTs= Expressed Sequenced Tags, PEG: PolyEthylene Glycol, SSH= Suppression Subtractive Hybridization.

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بررسی مجموعه آر.ان.ای های ریشه لوبیای سودانی (Pigeonpea) در شرایط کمبود آب با روش تجزیه فرونشا نندگی دو رگه گیری افتراقی

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چکیده

لوبیای سودانی (Pigeonpea) از لگوم ها متحمل خشکی است که در مناطق خشک و نیمه خشک جهان در سطح وسیعی کاشت می شود و دارای ریشه عمیق و گسترده ای است که به فعالیت های فیزیولوژیکی و متابولیکی در سازگاری با خشکی کمک می کند. کار برد روش های موجود ژنومیکس کارکردی برای بهبود محصول دهی در شرایط کمبود آب نیازمند شناخت بهتر سازوکارهای دخیل در واکنش لوبیای سودانی به تنش ناشی از کمبود آب است. به منظور شناسایی ژن های مرتبط با کمبود آب در این گیاه، یک مخزن ژنی (**library**) با روش تجزیه فرونشا نندگی دو رگه گیری افتراقی **cDNA** از بافت ریشه جوان لوبیای سودانی که تحت تنش آبی ناشی از پلی اتیلن گلیکول بود ساخته شد و با توالی یابی ۳۰۰ همسانه تصادفی، ۱۵۷ **EST** با کیفیت بالا به دست آمد که منجر به ۹۵ تک ژنی (**unigene**) شامل ۳۷ کانتیگ و۵۸ سینگلت (**singlet**) شد. تجزیه خوشه ای **EST** ها اشکار ساخت که بیشتر ژن ها به پروتئین های شناخته شده و موجود در پایگاه داده ها شباهت زیادی داشتند و با آنها پروتئین هایی منحصر به فرد و فرضی یا نا مشخص همراه بود. در ادامه، ویژگی های **EST**های تفریقی و ژن های مربوط به فرایند های فیزیولوژیکی معین شناسایی شد.سپس، تجزیه لکه گذاری نورترن، تنظیم افزونی(**up regulation**)مواد **ornithine aminotransferase** ، **cyclophilin**، **DREB** و پراکسیداز را آشکار ساخت. چنین تصور می شود که توالی های بیان شده به صورت افتراقی به عنوان منبعی مستعد برای ژن های القایی تنش کم آبی در ترنس کریپتوم (مجموعه آر.ان.ای های پیک) عمل می کنند و از این رو می توانند اطلاعات مفیدی برای درک سازوکارهای ملکولی مدیریت کمبود آب در لگوم ها به دست دهند.

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