Quantitative Expression Analysis through Transcript Profiling for Drought Stress in *Cicer Arietinum* L.

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Abstract

Chickpea, one amongst the major pulse crops, is grown mainly in the arid and semi-arid regions. Drought is one of the very important abiotic stresses that changes plant water status and limits plant growth and development. Expression profiles of genes involved in conferring stress tolerance could be utilized to get improved tolerant varieties. In present study, chickpea accessions having diverse levels of drought-tolerance (SBD 377 and PUSA 1103) were quantitatively analysed for variations in transcript profiles during drought stress. 8 gene sequences from drought stressed EST library were selected for gene expression studies. The sequences were subjected to homology search through a BLAST. Out of which 5 genes were found to be up regulated and 3 genes were down regulated with respect to expression of housekeeping genes Ubiquitin and Actin. The increase and decrease in gene expression varied from 0.22 % to 14.82 % fold.

The maximum gene expression was expressed by DREB gene with the minimum gene expression by aquaporin like water channel protein with the relative fold decrease of 0.22 % during drought stress whereas the proline rich protein expressed 0.28 % relative fold decrease in gene expression. The findings have confirmed involvement of PUSA 1103 derived DREB gene, hypoxia conserved region protein gene, sulferodoxin protein gene, glutathione peroxidase - phgpx gene and serine type protein genes in conferring drought stress to the plant. These gene(s) should be utilized for incorporation of drought tolerance in chickpea and other crop improvement programmes.

Keywords: EST, Drought, Gene Expression, Transcriptome, Proline rich protein.

Introduction

Chickpea is the second most important pulse crop after dry beans grown primarily in the arid and semi-arid regions with production of 14.78 million tons.[7] Drought is the prevalent critical environmental condition that reduces productivity.[8,29] The stress drought alone causes 40–50% reduction in chickpea production.[1] Hence, it is imperative to produce cultivars having tolerance to drought in addition to other abiotic-biotic stresses for sustainable production.

To acquire a comprehensive picture of a response of the plant to stress, it is necessary to study the expressions of all genes of the genome or at least those contributing to stress tolerance.[28] The comparative transcriptomic activities against reactions to designated plant stresses through micro array techniques are evaluated for identified and characterized candidate genes utilizing whole genome sequencing.[6] EST data sets can be used in gene expression and functional genomics studies to identify putative genes with differential expression and to generate gene-based functional molecular markers such as EST-SSRs, EST-SNPs and SFPs - single feature polymorphisms.[28] EST sequences derived from chickpea drought exposed libraries have been reported.[2,3,5,6,25,29]

Jain and Chattopadhyay[15] have also observed variations in transcript profiles of two chickpea cultivars during drought treatment. Gao et al[8] have identified drought genes using two non-normalized cDNA libraries utilizing seedling stage young leaves of a drought-tolerant chickpea cultivar grown under PEG and non-PEG treated ambient. These studies provide opportunities for illuminating the mechanism of drought tolerance in chickpea and unfold the molecular pathways used by the plant as well as the function of the involved candidate gene.

Gene expression analysis of stress induced genes will facilitate the understanding of the molecular mechanisms involved in stress tolerance and will be a boon for breeding of "drought-tolerant" crops. This approach will help molecular plant breeders in improving stress tolerance through gene selection and /or genetic manipulation.[6] Proteins encoded by some of these identified genes have confirmed their tolerance to drought stress and involvement in the signal transduction pathway. Due to taxonomic proximity of model legume genome *Medicago truncatula* with the chickpea and its ability to grow in the soil with relatively low water content, it has a unique advantage to understand how a plant responds to drought stress.[8] Gene expression profiling has allowed the identification of hundreds of induced genes when plants are exposed to stress.[16,17,22,24]

This constitutes a new powerful technology that has already made possible the identification of several unannotated transcripts responsive to abiotic stress.[9,23] The present investigation quantitatively dissects the drought-tolerance expressions of two chickpea varieties having diverse levels of transcript profiles.

Material and Methods

Plant materials and stress treatments: Chickpea genotypes PUSA 1103 and SBD 377 were grown in 3-liter size pots with composite soil (peat compost to vermiculite, 1:1) for 1 month. The pots were irrigated with 200 ml water every day. After one month of germination, stress experiments were carried out up to 12 days i.e. 0, 3, 6 and 12 days. 0 represents the last time when a plant was given a defined amount of water. Soil moisture content was checked at each stage. During the investigation the soil moisture content decreased approximately from 50% to 15% at the end of 12 days. As control, certain plants were kept under the defined static condition during the investigation period with watering. Leaves were collected at each stage of stress experiment and were frozen in liquid nitrogen.

Estimation of relative water content (RWC): The leaf tissues of chickpea were collected and weighed immediately [fresh weight, FW]. The tissues were rehydrated in water for 24 hours and reweighed until fully turgid and surface-dried [turgid weight, TW] followed by reweighing after oven drying at 80°C for 48 hours [dry weight, DW].

The following formula was used for RWC calculation:

RWC (%) = (FW-DW/TW-DW) ×100.

The experiment was repeated thrice.

RNA isolation and cDNA synthesis: Total RNA was isolated from direct cell using c-DNA kit as per protocol instructions (Ambion).

qRT-PCR analysis: EST derived 8 SSR sequences obtained from drought stressed the EST library had been selected for gene expression studies. The sequences were subjected to homology search through a BLAST. In order to see quantitative gene expression analysis of "candidate", EST derived SSR sequences, we performed real-time PCR using the SYBR green technique. This method is based on measuring PCR products in the logarithmic phase of the reaction by determining the Ct value, the threshold cycle at which the fluorescence emission reaches the log phase of product accumulation. Samples were analyzed in 10 µl volume using the Bio-Rad Light Cycler. Reactions were performed in triplicate using cDNA templates obtained from stress treated and normal plant samples for each gene.

A 10 µl master mix of RT-PCR reaction contained DNA 20 ng, forward primer 0.5 µl, reverse primer 0.5 µl, Taq polymerase 0.5 µl, Taq buffer 1 µl, SYBR green 3.5 µl, $MgCl_2$ 0.73 µl, H_2O 1.77 µl and dNTP mix 0.5 µl. Reactions were carried out under the following conditions: one cycle of denaturation at 95°C for 2 min followed by 45 cycles of 95°C for 20 sec (denaturation) and 60°C for 50 sec (annealing and elongation). Actin gene (Accession No: AJ012685) was used as control. Gene-specific EST derived SSR primers were designed utilizing primer express (version 3.0) software (Table 1). The genes identified have been known to be up regulated or down regulated to confer drought resistance to the plant.[5,11,14] Relative quantitative gene expression of each candidate sequence in drought stressed leaves as compared to control was analyzed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Average Ct value was calculated over 3 replicates. Ct values were calculated for the housekeeping actin gene and each candidate gene sequence in control as well as stressed cDNA samples.

ΔCt was determined by subtracting the average actin Ct value from the average candidate sequence Ct value. The ΔΔCt was determined by subtraction of ΔCt value of the irrigated one from ΔCt value of the stressed sample. Each "candidate" gene sequence was expressed as a fold difference in stressed conditions as compared to non-stressed conditions by calculating $2^{-\Delta\Delta Ct}$.

Results

RWC Content: Leaf relative water content (RWC) varied from 98 % to 55%. The decrease in relative water content reflects drought stress in a plant that indicates water status in plants and reflects the balance between the water supply to a leaf tissue and transpiration rate.[19]

qPCR analysis: EST derived candidate gene sequences (Table 1) were selected for qPCR analysis because the capability of genotypic identification was more precise than that of genomic-SSR.[27] Out of 10 selected primers for real time PCR, 5 primers were found to be up regulated and 3 primers were down regulated with respect to two housekeeping genes (Actin and Ubiquitin). Apart from a housekeeping gene (Actin and Ubiquitin), all the sequences reflected induction of drought. The relative quantification of the candidate gene in both the cultivars as compared to that of control (well-watered condition) is presented in table 2.

A noticeable increase in the transcript accumulation was seen on 12th day of dehydration. In PUSA 1103, the transcript accumulations of these up regulated genes were more prominent than the drought sensitive cultivar. The relative % fold of increase and decrease in gene expression of 8 EST SSR genes across the drought stress tolerant and sensitive varieties varied from 0.22 % to 14.82 % fold.

The maximum gene expression was demonstrated by DREB gene during drought stress with the relative fold increase of 14.82 % with the minimum gene expression by "Aquaporin like", water channel protein with the relative fold decrease of 0.22 %, whereas the proline rich protein expressed 0.28 % relative fold decrease in gene expression. The fold changes in transcript accumulations after drought stress relative to the irrigated conditions in both the cultivars are presented in fig. 1 and fig. 2.

Discussion

8 EST sequences of candidate genes selected encode different transcripts and enzymes which play a significant

role in drought tolerance pathways. Sequence 2(EX303744.1) corresponds to the DREB (Dehydration responsive element binding), which is a transcript and adheres to the drought receptive cis acting element. It represents to the ERF/AP2 family and consists of 2 subclasses i.e. DREB-1/CBF and DREB-2 induced by cold and dehydration stress.[26] DREB-2 is induced by dehydration stress and may activate other related genes involved in drought stress tolerance.[18]

Ito et al[14] have also reported that the DREB -1 gene improves drought and chilling tolerance in rice. In present study, DREB gene expression increased up to 14 folds in drought tolerant variety. Another gene sequence 4(GI|116183024) showed homology with hypoxia conserved region / hypoxia induced protein. It is known to be up-regulated by stresses of the micro environment such as low oxygen or low glucose conditions. It was found to be upregulated with 3.70 % increase in relative fold of gene expression. Hypoxia-inducible factors (HIFs) are a group of basic helix–loop–helix Per-ARNT-Sim transcript factors expressed in response of hypoxic conditions to maintain oxygen homoeostasis.

Table 1
EST derived candidate gene sequences selected for qPCR analysis

S.N.	Gene	Sequence	Gen Bank Accession Number	Blast Homology
1. Sequence 1	Tonoplast Intrinsic Protein	F GTGCTAACATTTTGGTTGGA R GTGTGGCTAATGAAGACGAC	AJ489613.1	AJ243309, AF020793
2. Sequence 2	Dreb (phaseolus)	F AGATTGCTGTTCCTCCAACT R CCCACTTCCTCATCCTTATT	EX303744.1	AK244651.1
3. Sequence 3	Aquaporin like Water channel protein	F AGGTGACATTGATTGGGG R ATCCAGAGTGGGGAAGATAG	FL512354.1	HM803185.1
4. Sequence 4	Hypoxia Conserved Region	F AGGGACTAAGAGGCATAGGG R GTTGGTCTCAAATTCCAAGC	GI|116183024	HM178929.1
5. Sequence 5	Sulferodoxin	F CATGCTCAGGCTCTTACACT R CTATTATGCCAAGCTGCG	EG359332.1	BT146268.1
6. Sequence 6	Glutathione peroxidase (phgpx gene)	F AAGGTTGTGGACAGATATGC R TGCATAACCCAAATACACAA	AJ487466.1	XM003630921.1
7. Sequence 7	Serine type protein	F ATATGCAGCCAGCAAAACTG R GGTTCGGATTGTCACTTGCT	AJ225026.2	AB1627051
8. Sequence 8	Proline rich protein	F AGGTGACATTGATTGGGG R ATCCAGAGTGGGGAAGATAG	FL512352	X97354.1, AJ233399.1
9. Sequence 9	Actin	F GTAACATTGTGCTCAGTGGTGG R ACGACCTTAATCTTCATGCTGC	CAC10126	XP004070770
10. Sequence 10	Ubiquitin	F GCTACTCCCAATCCCACTC R ATACTTCATTTCCATCCTGTCC	CAC12987	CAC12987.1

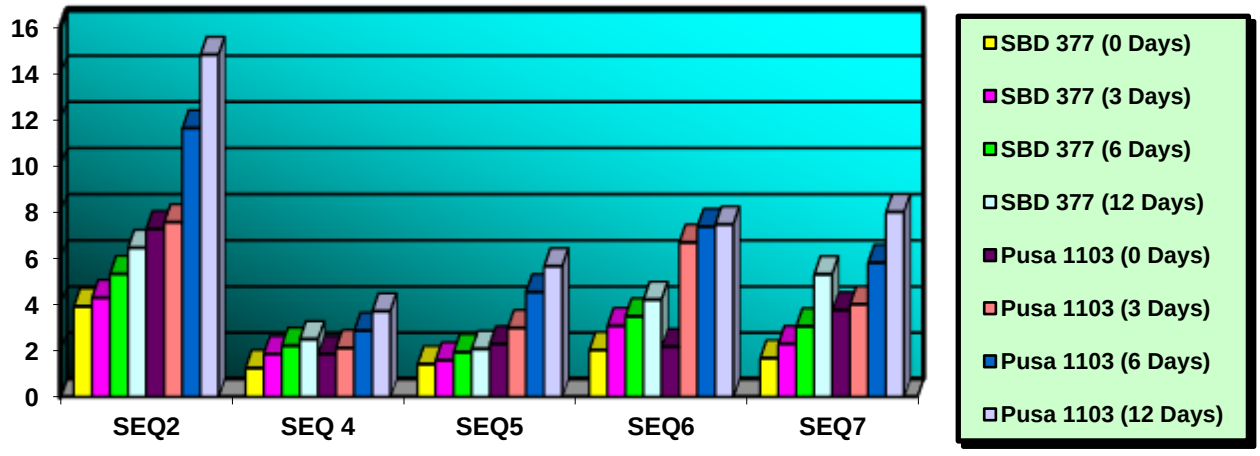

Figure 1*:* Upregulated genes - The fold change in transcript accumulation after drought stress relative to the irrigated conditions in both the cultivars

Table 2
The relative quantification of the candidate gene in both the cultivars as compared to that of Control

Primer sequence No & Protein Gene	Plant at number of dehydration Days	Relative % fold of increase or decrease in Gene Expression in relation to housekeeping genes
1. Sequence1 (Tonoplast Intrinsic Protein)	SBD 377 (0 Days)	1.61
	SBD 377 (3 Days)	1.11
	SBD 377 (6 Days)	0.70
	SBD377(12 Days)	0.64
	Pusa 1103 (0 Days)	0.71
	Pusa 1103 (3 Days)	0.69
	Pusa 1103 (6 Days)	0.64
	Pusa 1103 (12 Days)	0.58
2. Sequence 2 (Dreb phaseolus))	SBD 377 (0 Days)	3.91
	SBD 377 (3 Days)	4.28
	SBD 377 (6 Days)	5.32
	SBD 377 (12 Days)	6.45
	Pusa 1103 (0 Days)	7.26
	Pusa 1103 (3 Days)	7.56
	Pusa 1103 (6 Days)	11.63
	Pusa 1103 (12 Days)	14.82
3. Sequence 3 (Aquaporin like Water channel protein)	SBD 377 (0 Days)	0.77
	SBD 377 (3 Days)	0.53
	SBD 377 (6Days)	0.39
	SBD377(12 Days)	0.22
	Pusa 1103 (0 Days)	0.90
	Pusa 1103 (3 Days)	0.87
	Pusa 1103 (6 Days)	0.90
	Pusa 1103 (12 Days)	0.32
4. Sequence 4. (Hypoxia conserved region)	SBD 377 (0 Days)	1.24
	SBD 377 (3 Days)	1.85
	SBD 377 (6 Days)	2.21
	SBD377(12 Days)	2.49
	Pusa 1103 (0 Days)	1.85
	Pusa 1103 (3 Days)	2.11
	Pusa 1103 (6 Days)	2.86
	Pusa 1103 (12 Days)	3.70
5. Sequence 5 (Sulferodoxin)	SBD 377 (0 Days)	1.41
	SBD 377 (3 Days)	1.58
	SBD 377 (6 Days)	1.93
	SBD377(12 Days)	2.07
	Pusa 1103 (0 Days)	2.28
	Pusa 1103 (3 Days)	2.98
	Pusa 1103 (6 Days)	4.53
	Pusa 1103 (12 Days)	5.65
6. Sequence 6 (Glutathione peroxidase - phgpx gene)	SBD 377 (0 Days)	2.02
	SBD 377 (3 Days)	3.07
	SBD 377 (6 Days)	3.48
	SBD377(12 Days)	4.20
	Pusa 1103 (0 Days)	2.16
	Pusa 1103 (3 Days)	6.68
	Pusa 1103 (6 Days)	7.36
	Pusa 1103 (12 Days)	7.46
7. Sequence 7 (Serine type protein)	SBD 377 (0Days)	1.67
	SBD 377 (3 Days)	2.29
	SBD 377 (6Days)	3.05

	SBD 377(12 Days)	5.30
	Pusa 1103 (0 Days)	3.75
	Pusa 1103 (3 Days)	4.00
	Pusa 1103 (6 Days)	5.80
	Pusa 1103 (12 Days)	8.00
8. Sequence 8 (Proline rich protein)	SBD 377 (0 Days)	0.70
	SBD 377 (3 Days)	0.65
	SBD 377 (6 Days)	0.53
	SBD 377(12 Days)	0.42
	Pusa 1103 (0 Days)	0.52
	Pusa 1103 (3 Days)	0.42
	Pusa 1103 (6 Days)	0.34
	Pusa 1103 (12 Days)	0.28

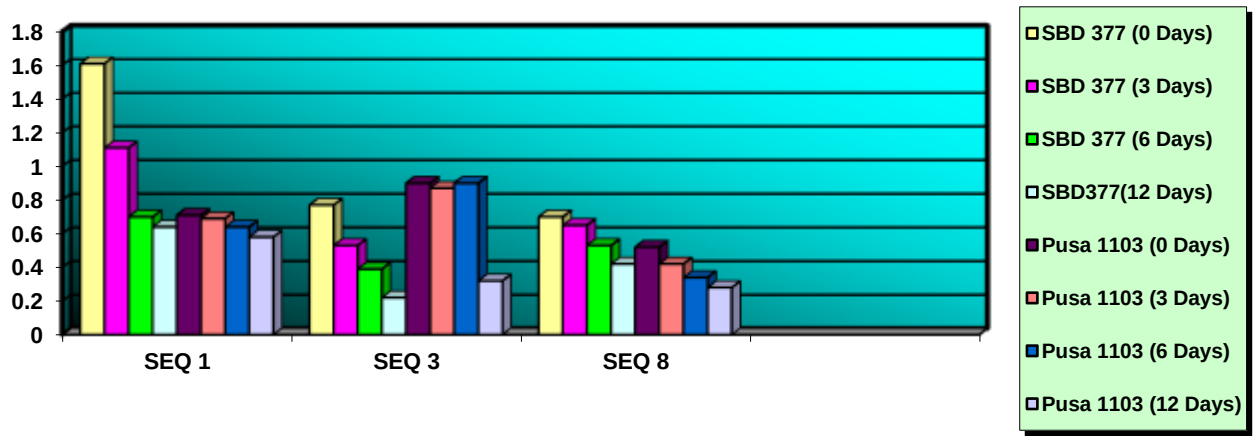

Figure 2: Down regulated genes- The fold change in transcript accumulation after drought stress relative to the irrigated conditions in both the cultivars

Since, it is an adaptive response activated during hypoxic condition, its role may be further studied to reveal new insights about its potential role in inferring drought tolerance to the plants.

Sequence 5 (EG359332.1) and 6 (AJ487466.1) showed homology with glutathione peroxidase and sulpherodixin respectively and are ROS scavenging genes. Abiotic stress like drought tolerance leads to the ROS accumulation which may lead to the toxic levels and harm the plant system. According to the real time PCR data, these sequences were found to be up regulated during stress condition with an increase of 7.46 % fold and 5.65 % fold respectively. This was in close association with the observation made by Mahdavi Mashaki et al[21] during drought stress in chickpea. Sequence1 (AJ489613.1) and sequence 3(FL512354.1) genes have been observed to be down regulated with a decrease of 0.64 % fold and 0.23 % fold of expressions and showed homologies with the tonoplast intrinsic proteins (TIPs) and aquaporin (AQPs like water channel proteins). Water movement through cellular membrane is largely controlled by tonoplast intrinsic and aquaporin proteins.

Various environmental stimulants have been observed to control the behaviour of both TIPs and aquaporin in various ways and levels.[11] He et al[13] too reported a significant role for *PgTIP1* by its overexpression in the growth and development of *A. thaliana*. Ma et al[20] using RNA interference investigated the physiological role of AtTIP1:1 in plants and found a strong down-regulation of *AtTIP1:1* led to plant death and suggested an essential physiological role of AtTIP1: 1.

Sequence 7 (AJ225026.2) expressed homology with the serine incorporator like protein in literature but not directly associated with drought tolerance. However, as per our study, this sequence 7 gene may be associated with serine incorporator like protein stress as it is up-regulated with 8% relative increase in gene expression during drought conditions. The *PRP* gene - Sequence 8 (FL512352) was found to be down regulated with a decrease of 0.34 % fold of gene expression and corresponds to the proline rich cell wall proteins in plants.[5]

Plant *PRP*s are known to be regulated in response to many external abiotic stress factors. Harrak et al[12] had identified a gene encoding a Thr-, Pro- and Gly-rich protein (PTGRP) in case of *Lycopersicon chilense*, which was negatively regulated by drought and also observed as down regulation of the PTGRP gene in desiccated cell suspensions of

Lycopersicon chilense. Our study is also supported by identification of down regulated response to salt and drought stress of *SbPRP* gene in soybean by He et al.[13]

Conclusion

The drought tolerant variety PUSA 1103 expressed abundance of sequence 2 containing DREB gene, sequence 4 (GI|116183024) containing hypoxia conserved region protein gene, sequence 5(EG359332.1) containing sulferodoxin protein gene, sequence 6 (AJ487466.1) containing glutathione peroxidase - phgpx gene, sequence 7(AJ225026.2) containing serine type protein gene indicating a correlation between transcript abundance and drought tolerance. The hypoxia conserved region protein has been observed to be linked with activation for tolerance of drought during hypoxic conditions, but as per our study it has also expressed a relative increase in gene expression during drought conditions.

Similarly, till date serine type protein has not been investigated for gene expression studies during drought stress. However, we have found a relative increase in gene expression during drought conditions. These observations confirm their involvement in conferring drought stress to the plant. Thus, the chickpea variety PUSA 1103 derived DREB gene, hypoxia conserved region protein gene, sulferodoxin protein gene, glutathione peroxidase - phgpx gene and Serine type protein genes may be utilized for induction of drought tolerance in chickpea and other crop improvement programmes.

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