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Full Length Research Paper

Transcript accumulation of putative drought responsive genes in drought-stressed chickpea seedlings

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Differential display reverse transcriptase PCR was used to identify cDNA sequences induced by drought in chickpea seedlings. The sequences of differentially expressed cDNAs: 192, 214, 219 and H1 showed high similarities at the protein level to known drought-inducible genes encoding for alanine aminotransferase, βAKIN1, a proteine kinase from the SnRK1 complex, lipid binding protein and COR protein, respectively. No significant similarity was found with the candidate sequence 277. Semi-quantitative multiplex PCR was used to verify that differentially amplified cDNAs were derived from differentially expressed genes. Relative quantification of the candidate sequences in the controls (well-watered seedlings) of drought-tolerant cv. ICCV2 and drought-susceptible cv. ILC3279 confirmed their induction upon drought in both cultivars. The transcript accumulation of the highly induced sequences, 219 and H1, was more important in the tolerant than the susceptible cultivar. These sequences are likely to be associated with drought tolerance in chickpea seedlings in contrast to sequences 214 and 277 which showed no variation in the mRNA accumulation between cultivars. The effect of ABA treatment on the mRNA accumulation of the isolated sequences 192, 214, 219 and H1 was analyzed. Sequences H1 and 192 were up-regulated by ABA treatment whereas sequences 214 and 219 were not, indicating an ABA-dependent and ABA-independent pathways in signal transduction in response to drought stress in chickpea seedlings.

Key words: Differential display, DDRT-PCR, *Cicer arietinum*, drought stress.

INTRODUCTION

Molecular studies of drought stress in plants have been performed for many different species subjected to a wide range of water and chilling stresses. Genes with a potential role in drought tolerance have been identified either by reference to physiological evidence or by differential screening, thus establishing major molecular responses in model species to be established (Cushman and Bonhert, 2000; Shinozaki et al., 2003; Valliyodan and Nguyen, 2006). Although many drought responsive genes have been discovered, it is still of great importance to analyse drought-inducible genes and their expression in drought-tolerant crops. Chickpea is a leguminous crop widely grown in semi-arid regions because of its tolerance to drought. Moreover, many cultivars with different levels of tolerance to drought conditions have been bred. Particularly, the ability of Indian cultivars to adapt to drought and high temperature make them ideal for the study of the molecular mechanisms of drought tolerance (Boominathan et al., 2004).

Several approaches have been used to identify drought responsive genes in plants. Among these suppression subtractive hybridization, DNA microarrays and cDNA amplified fragment length polymorphism have provided researchers with essential tools to examine genes responding to drought (Rensink and Buell, 2005; Ouborg and Vrizen, 2007). In the case of chickpea, about 100 expressed-sequence tags (EST) are available in public databases (Boominathan et al., 2004). EST sequences

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from related species such as *Medicago truncatula* allow the identification of genes regulated during nodulation, embryo development and desiccation (http:// www.ncbi.nlm.nih.gov;http://www.ebi.ac.uk/arrayexpress), but may not contain drought-regulated sequences that are the target of this study.

The differential-display reverse transcription-PCR (DDRT-PCR) technique, developed by Liang and Pardee (1992), has been widely used in plants to isolate genes that are differentially expressed in response to various stresses (Malatrasi et al., 2002; Yamazaki and Saito, 2002; Liu and Baird, 2003; Zhang et al., 2007). This technique is a suitable, low-cost technique to identify differentially displayed genes. It does not require cDNA cloning and blotting and can even be applied in laboratories where radioactive labelling is not available by using the silver staining method that enables the detection and recovery of the PCR products from acrylamide gels (Goldman and Merril, 1982). In this work, we describe the use of DDRT-PCR for the isolation of cDNA fragments induced in chickpea seedlings by drought stress. 7 sequences were isolated; 6 of these were shown to be drought up-regulated using semi-quantitative PCR and 5 were analysed using real-time PCR for their relative expression in tolerant and susceptible genotypes. Most of the genes that respond to dehydration are also responsive to exogenous application of abscisic acid (ABA) (Seki et al., 2002a). Therefore, responses to ABA of 4 of the isolated sequences were monitored.

MATERIALS AND METHODS

Plant material and stress treatment

Seeds of chickpea (*Cicer arietinum* L. cvs ICCV2 and ILC3279) were provided by the interznational center for agricultural research in the dry areas (ICARDA, Aleppo, Syria). These cultivars were used in this study because of their contrasting tolerance to drought (R.S. Malhotra, pers. comm.). Seeds were sown in pots containing a sand/soil mixture and grown at 22 - 18°C (day - night) with photoperiod of 16 h. 3 seeds per pot from the same cultivar were sown and a total of 15 pots per cultivar were used to have 3 replicates at each sampling. Pots were arranged in a randomised complete block design. A dry-down experiment was carried out and drought stress was applied at 12-days-old seedlings by withholding water. After 7 days of dehydration, one pot from each cultivar was rehydrated with water to 100% during 2 days. For control seedlings, watering was maintained daily at RWC 100%. Water potential (ψ) was measured using a pressure chamber (Model 600, PMS instruments) on watered and stressed seedlings at 5 and 7 days after withholding water. Leaves from control and time course dehydrated (3, 4 and 5 days) and rehydrated (1 and 2 days) seedlings, were harvested for RNA extraction after measuring the leaf ψ in each pot and stored at 4°C in the RNA later solution (Ambion, Inc, Austin, TX, USA). For ABA treatment, seedlings of cv. ICCV2 were removed from the soil and roots were dipped for 5 h into water with or without 100 µM ABA.

RNA extraction and purification

Total RNA was isolated from leaf tissue of control and stressed seedlings with RNA pure^{TM} reagent (GenHunter Corp., Nashville, TN, USA) as follows: 1.5 ml of RNA pure^{TM} reagent was added to 25 mg of leaf tissue and homogenized. After incubation on ice, the lysate was extracted with 150 µl of chloroform. After centrifugation, the RNA in the upper phase was precipitated with an equal volume of isopropanol. RNA was washed with 70% cold ethanol and resuspended in 50 µl of nuclease free water. DNA contaminants were removed with the message clean kit (GenHunter Corp.). RNA concentration was measured by reading the absorbance at 260 nm. RNA was stored at 20°C after adding 1 U of RNAase ribonuclease inhibitor (RNAasin; Promega).

Reverse transcription and differential mRNA display

Reverse transcription of mRNA was performed as described in the RNA image kit (GenHunter Corp.). Each reverse transcription reaction was conducted in 20 µl total volume with 2 µg of total RNA, 1x RT buffer, 2 µM dNTP, 0.2 µM of anchor primer ($T_{11}A$ or $T_{11}G$ or $T_{11}C$) and 100 U of MMLV reverse transcriptase. RT reactions were performed at 37°C for 60 min followed by incubation at 75°C for 5 min. PCR amplifications were performed as described in the RNA image kit in 20 µl total volume containing 2 µl of RT mix, 2 µM of dNTP, 0.2 µM of the same anchor primer as in the RT reaction, 0.2 µM of an arbitrary primer (from HAP9 to HAP22, GenHunter Corp.), 0.5 U of Taq polymerase (Promega, Madison, WI, USA) with its own buffer. PCR amplifications were performed in a thermocycler (Perkin Elmer 7500) for 40 PCR cycles. Each cycle consisted on a denaturation step at 94°C for 30 s, annealing at 40°C for 2 min and extension at 72°C for 30 s. A final extension step of 5 min at 72°C followed. For each PCR 3.5 µl of products were mixed with 2 µl of loading dye (GenHunter, Corp), denatured for 2 min at 94°C and placed immediately on ice before loading on a 6% acrylamide gel.

After electrophoresis at 75 W for 135 min, the acrylamide gels were stained using the silver staining protocol described by Hamza et al. (2004). PCR bands up-regulated by drought stress were excised and eluted after incubation for 45 min at 90°C in 30 µl of a solution containing 10 mM Tris pH8 and 1 mM EDTA. Amplification of 1 µl of the eluted DNA was performed as above using the same set of primers.

Cloning and sequencing

Of the re-amplified product, 0.5 µl was used for cloning into pGEM-T easy vector according to the manufacturer's protocol (Promega). Plasmids were extracted from white colonies and amplified with T7 and SP6 primers. The size of PCR products was verified by agarose gel electrophoresis. A recombinant plasmids containing an insert with size matching the size of the amplified fragment was retained for sequencing. T7 and SP6 primers were used for amplification with the ABI prism dye terminator kit and the ABI model 373 automated DNA sequencer (Applied Biosystem). Nucleotide sequences or the deduced amino acid sequences were compared with DNA and protein sequences from NCBI database using the basic alignment search BLASTX.

Semi-quantitative polymerase chain reaction

The differential expression of each clone was confirmed by semi-quantitative PCR using 2 sets of primers. Based on the nucleotide sequence of each insert, specific primers were designed by beacon designer 04 software (version 1.0, Biorad) for each sequence, except for H3 (Table 1). A second set of primers was designed by beacon designer software to amplify a fragment of 168 bp from ubiquitin (Genbank accession No. CAC12987) or 100 bp from actin (Genbank accession No. CAA10126) genes of *C. arietinum* (Table 1). Semi-quantitative PCR was conducted both with primers specific

Table 1. Sequences of specific primer pairs used for semi-quantitative and real-time PCR analysis.

Sequence designation	Primer pair	Sequence (5' - 3')	PCR product size (bp)
181	1621RTF	CCTTCGTACCTTCCTTCTTCC	130
	1621RTR	TGAACCCGTAATACCACTAAGC	
192	1622RTF	GGTTTGAGAAGCAGGGTATG	85
	1622RTR	TAGAGGTTATTCAAGGTTGTAATG	
219	219RTF	GCTGTGACTATCCCTAAACG	96
	219RTR	GCAATAACCTCAACTCTATCAAG	
214	24214RTF	TATTTGTAGCCTCAGTCTGTAAAG	76
	24214RTR	GACACAAGACCTTCATATACCATC	
H1	H1RTF	CTATGCCTTGGATGATGAGC	79
	H1RTR	CACATGCCCTTCTTTAGCC	
H3	*H3F	CGCTGCAAAATACGGCG	176
	*H3R	TCAAACTGGAATCCCT	
277	277RTF	CTGTCTTCTGGAATTTCTAACG	96
	277RTR	TATTATTACACAACTCACTTGGTC	
Actin	ActinF	GGTAACATTGTGCTCAGTGGTGG	100
	ActinR	AACGACCTTAATCTTCATGCTGC	
Ubiquitin	UbiquitinRTF	GCTACTCCCAATCCCACTC	168
	UbiquitinRTR	AATACTTCATTTCCATCCTGTCC	

*Primer pair was not designed using beacon designer 0.4 software.

to each candidate sequence and with ubiquitin or actin specific primers in a separate PCR reaction. Based on the constitutive expression of actin or ubiquitin genes, equal intensity of their respective PCR product in control and drought-stressed samples will testify to the induction of the candidate sequences if their respective PCR products show different intensities. RT-PCR reactions were performed as previously described for the reverse transcription. PCR amplifications were performed in 25 µl total volume containing 1 to 2 µl of the cDNA, 200 µM of dNTP, 1 µM of each primer, 1 U of Taq polymerase (Promega) and 1x PCR buffer containing 1.5 mM $MgCl_2$. After preheating at 94°C for 2 min, 30 PCR cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s were performed and followed by a final extension step at 72°C for 10 min. PCR products were analysed in 2% agarose gel after staining with ethidium bromide.

Relative quantification

In order to quantify cDNA of the candidate sequences, we performed reverse transcription and 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 C_t value (Livak and Schittgen, 2001); C_t is the threshold cycle at which the fluorescence emission reaches the log phase of product accumulation. RT-PCR reactions were conducted as above in 25 µl total volume using 2 µg of total RNA extracted from watered (control) and stress treated seedlings. The real time PCR was done in a total volume of 25 µl containing 5 µl of 1/20 diluted cDNA, 12.5 µl of SYBR green master mix with Rox (Biorad) and 300 nM of forward and reverse primers (Table 1). The ABI prism 7700 sequence detection system (with version 1.9.1 software, PE Applied Biosystems) was used for thermal cycling. PCR reactions were conducted for 40 cycles, each cycle consisted of a denaturation at 94°C for 15 s and annealing extension at 60°C for 1 min. PCR efficiency of each primer pair was determined by evaluating the C_t value for 5 dilution series (10^{-1} to 10^{-5}) of cDNA. C_t values were used to calculate a linear regression line. The slope of standard curves was used to determine the PCR efficiency (EFF) in conformity with $EFF=10^{[-1/slope]-1}$. All the optimized PCR had an EFF ranging of 95 - 98%.

Relative mRNA accumulation of each candidate sequence in stressed (drought or ABA) seedlings was compared to the control by using the comparative C_t ($2^{-\Delta\Delta Ct}$) method. Average C_t value was calculated over 3 replicates. C_t value was calculated for the housekeeping actin gene and for each candidate sequence in controlled and stressed cDNA samples. ΔC_t was determined by subtracting the average actin C_t value from the average candidate sequence C_t value and $\Delta\Delta C_t$ involves subtraction of ΔC_t of the irrigated from ΔC_t of the stressed samples. For each candidate sequence, results were finally expressed as a fold difference in stressed conditions compared to non stressed conditions by calculating $2^{-\Delta\Delta Ct}$. To assess the relative expression in tolerant and sensitive cultivar 2 independent experiment for each sequence were conducted and standard error of $2^{-\Delta\Delta Ct}$ value was determined over these 2 independent experiments. The means of $2^{-\Delta\Delta Ct}$ were compared and separated using the least significant difference (LSD) test at 5% level using SAS 9.1. For ABA treatment, the upper and lower range limit of $2^{-\Delta\Delta Ct}$ of a given sequence relative to the control were respectively $2^{-\Delta\Delta Ct+s}$ and $2^{-\Delta\Delta Ct-s}$ with s, the standard deviation calculated using the following formula $s = \sqrt{s_1^2 + s_2^2}$; s_1 and s_2 are standard deviation of C_t values the candidate sequence and actin respectively (Chemistry Guide, Sequence Detection Systems, Applied Biosystems).

RESULTS

Differential display and sequence homology

DDRT-PCR technique was used to identify drought responsive sequences in cv. ICCV2 seedlings. An

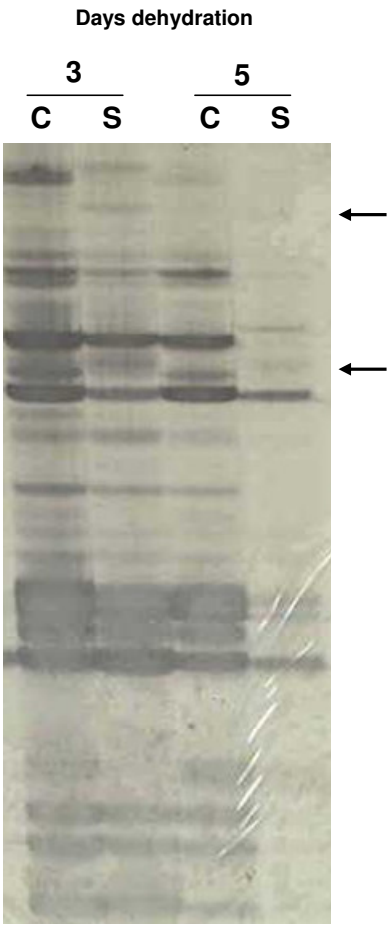

Figure 1. Time course PAGE electrophoresis of differentially displayed PCR products in watered (C) and drought stressed (S) seedlings of cv. ICCV2; arrows indicate differentially displayed bands

example of a differential display pattern using RNA isolated from leaves of time course drought-treated seedlings is shown in Figure 1. Some of the up-regulated DDRT-PCR fragments appeared more than once during the dry-down experiment, showing long time-eliciting of the sequence after stress. A particular care in excising these bands from the gel was taken to avoid the isolation of artefact bands. By screening 45 primer combinations, we observed 31 potential differential expressed PCR products. However, only seven PCR products were cloned after re-amplification. The remaining excised bands provided more than one PCR product after re-amplification and were discarded.

The 7 PCR products were cloned into pGEM-T easy vector and were sequenced using T7 and SP6 primers. Table 2 lists the clones obtained and summarises the homology search. The deduced amino acid sequence of clone 192 showed 92% similarity to an alanine aminotransferase (AlaT1) of *Vitis vinifera*. The deduced amino acid sequence of clone 219 showed 76% similarity to a lipid binding protein of *Arabidopsis thaliana*. The deduced amino acid sequence of clone 214 showed 80% similarity to AKINβ1 of *M. truncatula* with low E score value (0.057). E score value increased to 2e-38 when the nucleotide sequence was compared (data not shown). The deduced amino acid sequence of clone H1 showed 63% similarity to a cold regulated protein of *T. aestivum*. A low similarity of sequence H3 was found with sulfiredoxin-like protein (E score value 0.50) and no significant similarity was found with the candidate sequence 277.

Verification of differential expression

To verify that the candidate sequences were really induced by drought stress, semi-quantitative PCR was conducted on cDNA of cv. ICCV2 at 5 days of drought stress, using primers designed from the candidate sequences and from the constitutive ubiquitin and actin genes (Table 1). Except for the sequence 181 which showed constitutive expression between irrigated and stressed seedlings, the candidate sequences 192, 214, 219, 277, H1 and H3 showed similar pattern to the one observed in the original differential display gels (Figure 2A). In fact, the 6 candidate sequences showed differential expression between drought-stressed and well-watered seedlings compared to the constitutive expression of the housekeeping gene.

To analyse the mRNA accumulation pattern of the sequences 192, 214, 219, 277 and H1, a time course was conducted by semi-quantitative RT-PCR on cDNAs prepared from a tolerant and sensitive seedlings at 3, 4 and 5 days of dehydration, and at 1 and 2 days rehydration. The mRNA accumulation of sequences 192, 219 and H1 increased with the duration of drought stress on cv. ICCV2 (Figure 2B). A weak induction of the sequences 214 and 277 was observed only at 5 days of drought stress and it was detectable by real time PCR. No difference in the transcript accumulation pattern was found between the tolerant and sensitive cultivars except for sequence 192 on cv. ILC3279 which showed constitutive accumulation in both watered (control) and dried conditions. Upon 1 and 2 days rehydration, the mRNA accumulation of sequences H1 and 219 dropped whereas it was maintained for the rest of the sequences.

Real-time PCR assessment

Real-time PCR was conducted on 5 candidate sequences (192, 214, 219, 277 and H1) to further confirm their response to drought and to compare their mRNA accumulation pattern between the drought tolerant (ICCV2) and susceptible (ILC3279) cultivars. In this

Table 2. Homology search of DDRT-PCR sequences.

DDRT-PCR clone designation (Genebank accession No)	Size (bp)	Homology search results (BLASTX)		
		Genbank match (Genebank accession No)	% identity (No of amino-acid)	E value
181 (**EG359328**)	414	Hypoxia induced protein conserved region [*M. truncatula*](ABE80615.1)	90% (90)	4e-29
192 (**EG359329**)	384	*alanine aminotransferase AlaT1 [*V. vinifera*] (AAZ43369.1)	92% (57)	1e-24
214 (**EG359331**)	241	*Akinβ1, *M. truncatula* (AAO61676.1)	80% (21)	0.057
219 (**EG359330**)	264	*Lipid binding [*Arabidopsis thaliana*] (NP 190966.1)	76% (34)	3e-09
277 (**EG359327**)	264	No significant similarity found		
H1 (**EG359333**)	326	*Cold regulated gene, *T. aestivum* (BAC41494.1)	63% (71)	1e-20
H3 (**EG359332**)	176	sulfiredoxin-like protein [*O. sativa*] (AAZ52795.1)	87% (16)	0.50

* Protein known to be induced by osmotic stress (Seki et al., 2002; Buitink et al., 2003).

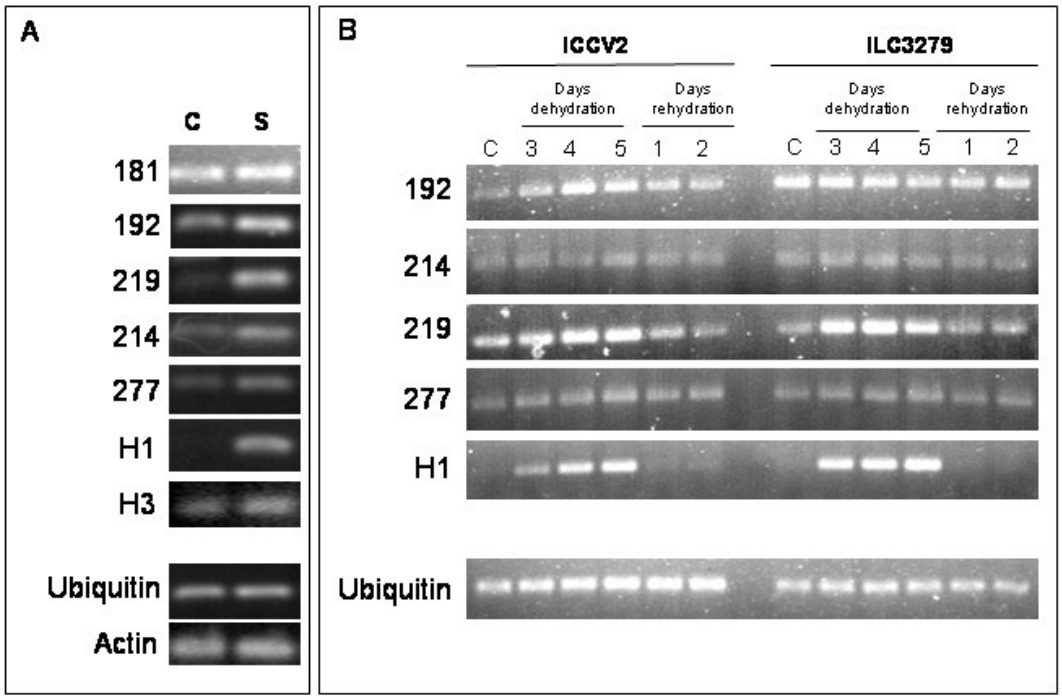

Figure 2. Semi-quantitative RT-PCR with A. cDNA of watered (C) and 5 days dehydrated (S) seedlings of cv. ICCV2 using ubiquitin or actin primers and specific primers of candidate sequences (Table 1) and with B. cDNA of time course dehydrated (3, 4 and 5 days) and rehydrated (1 and 2 days) seedlings of tolerant (ICCV2) and sensitive (ILC3279) cultivars.

context, to ensure that both cultivars were exposed to the same internal stress conditions before sampling, leaf ψ was measured for stressed seedlings at different sampling dates during the application of drought and for well-watered control seedlings. Sample leaves were attempted from seedlings with similar ψ in both cultivars (Table 3).

The relative quantification of the candidate sequences

Table 3. Leaf water potential of 2 chickpea cultivars in well-watered and 5 and 7 days dehydrated seedlings.

Cultivar	ψ (Mpa) at sampling date			
	Stressed		Well-watered	
	Date 1	Date 2	Date 1	Date 2
ICCV2	-1.53	-2.03	-0.50	-0.53
ILC3279	-1.52	-2.00	-0.50	-0.50

Date 1: 5 days of dehydration.
Date 2: 7 days of dehydration.

as compared to the control (well-watered seedlings) is shown in Figure 3. All 5 sequences were confirmed to be induced by drought. However, increases varied from 235.2-fold (sequence H1; cv. ICCV2) to 2.1-fold (sequence 214; cv. ILC3279). For the highly induced sequences (219 and H1) a significant difference ($P < 0.05$) in transcript accumulation between the tolerant and sensitive cultivar was observed at 7 days dehydration. In the tolerant cultivar, the transcript accumulation of these sequences increased significantly with drought pressure whereas in the sensitive cultivar it remained stable. Weakly induced sequences (277 and 214) showed no variation in the mRNA accumulation between cultivars and drought duration.

Expression pattern in response to ABA

Real-time PCR was conducted on four candidate sequences to study their relative mRNA accumulation to irrigated condition in response to ABA treatment (100 µM) in cv. ICCV2. Sequences H1 and 192 were up-regulated by ABA whereas sequences 214 and 219 were not (Table 4).

DISCUSSION

The DDRT-PCR technique generates a high proportion of false positive sequences (Liu and Baird, 2003). Under our conditions, this technique was very efficient since 6 of the 7 isolated bands were found to be induced by drought. The success of the experiment might be the result of particular attention to excising only bands that appeared at least twice in the time course experiment.

Of the 7 cloned sequences, semi-quantitative PCR showed 6 were up-regulated by drought. The induction of 5 of these sequences was further confirmed by quantitative PCR. Sequence H3 was omitted from this analysis due to the fact that beacon designer 04 software could not provide suitable primers for real time PCR.

Sequence 214 was homologous to AKINβ1 protein of *M. truncatula* with low score value (0.057) after BLASTX. However, the homology had a much higher score (2e-38) after BLASTN (data not shown), confirming that

Table 4. Relative transcript accumulation of the candidate sequences upon to ABA treatment in cv. ICCV2.

Sequence	Fold increase of transcript accumulation upon addition of ABA (100 µM)
192	1.52 (1.4 to 1.6) U
214	0.88 (0.6 to 1.0) N
219	0.47 (0.4 to 0.5) N
277	0.916 (0.647 to 1.3) N
H1	3.29 (2.73 to 3.77) U

The fold increase corresponding to $2^{-\Delta\Delta Ct}$, which indicates that the fold change in transcript accumulation relative to the control conditions was calculated as described in the material and methods. U: up regulated, N: not regulated, nd: not determined. The values between the brackets are respectively lower and upper limit range of relative transcript accumulation. Induction was considered when the relative mRNA accumulation still over one fold considering the lower limit range.

sequence 214 was homologous to the 3' end untranslated sequence of *Akinβ1* messenger RNA. BLASTX resulted in the alignment of the 5'end of the sequence 214 with the last 31 amino acid of *M. truncatula* AKINβ1 kinase protein, thereby providing a low score value. AKINβ1 is an isoform of SnRK1 complex that is involved in the regulation of carbon metabolism. In plants, SnRK1 has been proposed to be induced by high intracellular sucrose and/or low intracellular glucose levels (Purcell et al., 1998). An adjustment of osmotic pressure to protect cells from dehydration through increased intracellular sucrose level has been described in drought-stressed plants (Mccree, 1986). This phenomenon could possibly be a consequence of induction of the SnRK1 isoform. High score values were obtained for sequences 219, 192 and H1 and all of them were homologous to known drought-responsive genes (Table 2). Sequence H1 was homologous to a gene encoding for a cold regulated protein of *T. aestivum*. *COR/LEA* genes have been shown to be induced both by cold and drought (Seki et al., 2002b; Kume et al., 2005). These genes encode for highly hydrophilic proteins which interact with macromolecules and/or membranes to protect them from dehydration-induced damage, thereby retaining their functional integrity (Kazuoka and Oeda, 1992; Webb and Gilmour, 1996). Other studies have shown that LEA proteins might act as a novel form of molecular chaperone to help preventing the formation of damaging protein aggregates during water stress (Goyal et al., 2005). Sequence 219 is homologous to a lipid binding protein or lipid transfer protein family. These genes were also shown to be induced by drought in *A. thaliana* (http:// www.scri.sari.ac.uk). By having properties of lipid binding and transferring phospholipids between membranes, these proteins repair stress-induced damage by changing the lipid composition of the membrane (Kader, 1996). This may regulate the permeability to toxic ions and the fluidity of the membrane. The sequence 192 showed homology to alanine glyoxylate aminotransferase 2 (*AlaAt*)

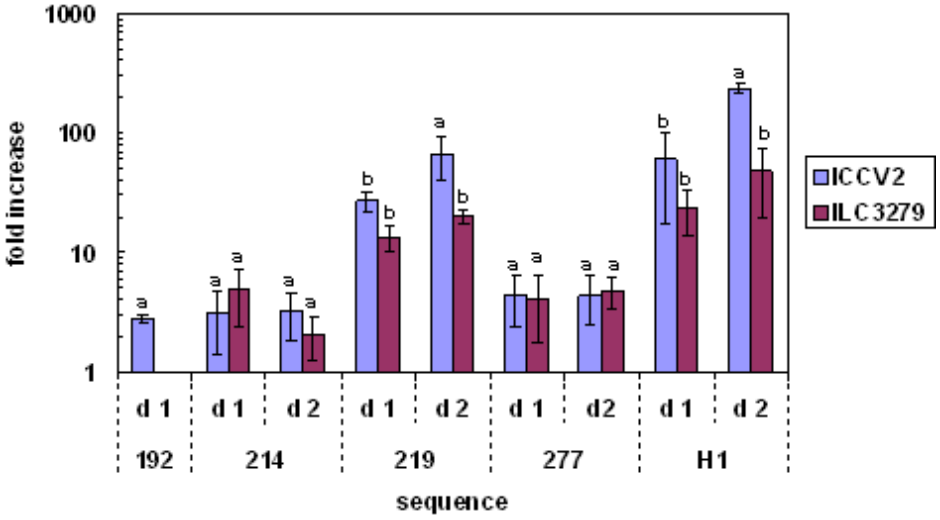

Figure 3. mRNA accumulation profile of the drought responsive sequences (192, 214, 219, 277 and H1) using quantitative real-time RT-PCR in drought tolerant (cv. ICCV2) and susceptible (cv. IL3279) cultivars and for different periods of drought stress (d1 and d2; 5 and 7 days dehydration). Real-time PCR amplifications were conducted on cDNAs prepared by reverse transcription of total RNAs using specific primers to candidates sequences (Table 1). The fold increase, corresponding to $2^{-\Delta\Delta Ct}$, indicates the fold change in transcript accumulation after drought stress relative to the watered conditions. Error bars indicate ± standard error of the mean of 2 independent experiments. For each sequence, vertical bars representing mean values followed by the same letter are not significantly different (P = 0.05) under different period of dehydration treatment (5 and 7 days).

of *A. thaliana*, which has been described as induced by drought stress (http://www.scri.sari.ac.uk). By possessing a peroxisomal glyoxylate aminotransferase activity, this enzyme has probably a central role in photorespiration (Liepman and Olsen, 2003). Therefore, a possible role of this enzyme in stress protection would be the production of glycine which is a glutathione pre-cursor. Since glutatione is a component of the anti-oxidative system in plants, the induction of *AlaAt* may provide additional protection against oxidative stress damage under high light conditions by supplying glycine (Wingler et al., 2000). In addition, stress conditions reduce rates of photosynthetic CO_2 assimilation thus an increase in photorespiration could serve as an energy sink preventing the over reduction of the photosynthetic electron transport and photoinhibition (Wingler et al., 2000).

Sequence H3 showed a low score value (0.5) for its homology to sulfiredoxin-like protein of *Oryza sativa*. This homology was related to only 16 amino acids while the submitted cDNA was 117 bp long. In the literature, sulfiredoxin-like protein has not been described as induced by drought, so it is unlikely that sequence H3 encode for this protein and the isolation of a longer sequence is needed for the correct homology.

In this study, semi-quantitative and real-time PCR were used to monitor the relative mRNA accumulation of the candidate sequences during drought stress in drought-tolerant and susceptible cultivars, in comparison to well-watered conditions, to correlate these sequences with drought tolerance. Semi-quantitative PCR did not show any difference in the mRNA accumulation level between the 2 cultivars. Nevertheless, a clear difference in the mRNA accumulation level of sequences 219 and H1 was revealed by real time PCR. Association of genes involved in stress-tolerance related to different dehydration components (drought, freezing and salinity) has been observed in some crop species (Kiani et al., 2007; Chen et al., 2006). This has been demonstrated through observation of genotypic differences in *dhn* and COR/*LEA* genes expression in response to drought or cold (Zhu et al., 2000; Lopez et al., 2003; Kobayashi et al., 2004; Suprunova et al., 2004). Our observation that H1 (homologous to COR gene) and 219 (homologous to gene encoding lipid binding protein) cDNAs were more expressed in the tolerant cv. ICCV2 than susceptible cv. ILC3279 indicates that these sequences could be associated with drought tolerance in chickpea seedlings. However, this result must be confirmed by observation of differential protein accumulation in susceptible and tolerant cultivars. For weakly expressed sequences there were no differences in relative mRNA accumulation between these cultivars. This also suggested use of quantitative PCR to precisely estimate cDNA copy number.

Most of the genes reported in this work were downstream genes or effectors that have probably a direct function in stress tolerance. In fact, mRNA isolation for DDRT-PCR was conducted long time after the stress was applied (3 and 5 days later). Therefore, genes involved in

abiotic stress regulation or signal transduction were mostly expressed immediately and transiently after stress application would not be identified (Swindelli, 2006). In *Arabidopsis*, expression of transcription factors in response to abiotic stress remained low for several weeks (Zarka et al., 2003). Nevertheless, in the present study a homologue to Mt*Akinβ1*, a gene encoding for a proteine kinase of the SnRK1 complex involved in signal transduction, was isolated. This could be related to its long and high level of expression in vegetative tissue (Buitink et al., 2003).

Sequence H1 and 192 showed similar expression pattern to their homologous *COR* and *AlaAt* genes as they are both induced by drought and ABA (http:// www.scri.sari.ac.uk, Bray, 2002). Sequences 214 and 219 were not regulated by ABA, whereas in the literature, sequences with similar function *AtSnRk3* and lipid binding protein were described as ABA-dependent (http:// www.scri.sari.ac.uk). The presence of genes involved in dehydration, up-regulated or independent of ABA indicates that 2 signal transduction cascades operate between the initial signal of drought stress and the expression of specific genes (Yamaguchi-Shinozaki and Shinozaki, 2005) ABA-dependent and ABA-independent pathways. It would be interesting to isolate the promoter sequence of the isolated genes to identify *cis* acting elements involved in drought stress and ABA response. It would be possible to examine whether they harbour ABA-responsive elements and the dehydration-responsive elements/C-repeats known to be vital part in ABA-dependent and ABA-independent pathways in osmotic and cold stress responses. One way to verify whether *cis* acting elements that respond to drought stress are present is the allelic imbalance assay used in human (Yan et al., 2002), which has now also been used in several plant species (Morgante, pers. comm.).

The drought-responsive sequences identified in this study have shown that the DDRT-PCR technique is useful for isolating drought-induced sequences in drought-tolerant crops. The isolation sequences will allow further investigation such as full cDNA cloning, isolation of promoter sequences and testing for allelic imbalance to better understand the molecular mechanisms of drought tolerance.

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REFERENCES

Boominathan P, Shukla R, Kumar A, Manna D, Negi D, Verma PK, Chattopadhyay D (2004). Long term transcript accumulation during the development of dehydration adaptation in *Cicer arietinum*. Plant Physiol. 135: 1608-1620.

Bray EA (2002). Abscisic acid regulation of gene expression during water-deficit stress in the era of the Arabidopsis genome. Plant Cell Environ. 25: 153-161.

Buitink J, Thomas M, Gissot L, Leprince O (2003). Starvation, osmotic stress and dessication tolerance lead to expression of different genes of the regulatory β and γ subunits of the SnRK1 complex in germinating seeds of *Medicago truncatula*. Plant Cell Environ. 27: 55-67

Chen Y, Chen P, de los Reyes BG (2006). Differential responses of the cultivated and wild species of soybean to dehydration stress. Crop Sci. 46: 2041-2046

Cushman JC, Bonhert J (2000). Genomic approaches to plant stress tolerance. Curr. Opin. Plant Biol. 3: 117-124

Goldman D, Merril CR (1982). Silver staining of DNA in polyacrylamide gels - Linearity and effect of fragment size. Electrophoresis 3: 24-26

Goyal K, Walton LJ, Tunnacliffe A (2005). LEA proteins prevent protein aggregation due to water stress. Biochem. J. 388: 151-157

Hamza S, Ben Hamida W, Rebai A, Harrabi M (2004). SSR-based genetic diversity assessment among Tunisian winter barley and relationship with morphological traits. Euphytica 135: 107-118

Kader JC (1996). Lipid transfer in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 47: 627-654

Kazuoka T, Oeda K (1992). Heat-stable COR (cold-regulated) proteins associated with freezing tolerance in spinach. Plant Cell. Physiol. 33: 1107-1114

Kiani SP, Grieu P, Maury P, Hewezi T, Gentzbittel L, Sarrafi A (2007). Genetic variability for physiological traits under drought conditions and differential expression of water stress-associated genes in sunflower (*Helianthus annuus* L.). Theor. Appl. Genet. 114: 193-207

Kobayashi F, Takumi S, Nakata M, Ohno R, Nakamura T, Nakamura C (2004). Comparative study of the expression profiles of the *Cor/Lea* gene family in two wheat cultivars with contrasting levels of freezing tolerance. Physiol. Plant. 120: 585-594.

Kume S, Kobayashi F, Ishibashi M, Ohno R, Nakamura C, Takumi S (2005). Differential and coordinated expression of *Cbf* and *Cor/Lea* genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. Genes Genet. Syst. 80: 185-197.

Liang P, Pardee AB (1992). Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. Science 257: 967-971.

Liepman AH, Olsen LJ (2003) Alanine aminotransferase homologs catalyze the glutamate:glyoxylate aminotransferase reaction in peroxisomes of *Arabidopsis*. Plant Physiol. 131: 215-227.

Liu X, WV Baird (2003). Differential expression of genes regulated in response to drought or salinity stress in sunflower. Crop Sci. 43: 678-687.

Livak KJ, Schittgen TD (2001). Analysis of relative gene expression data using real time quantitative PCR and the 2^[-delta delta C(T)] method. Methods 25: 402-408.

Lopez CG, Banowetz GM, Peterson CJ, Kronstad WE (2003). Dehydrin expression and drought tolerance in seven wheat cultivars. Crop Sci. 43: 577-582.

Malatrasi M, Close TJ, Marmiroli N (2002). Identification of a putative stress response regulation gene in barley. Plant Mol. Biol. 50: 143-152.

Mccree KJ (1986). Whole-plant carbon balance during osmotic adjustment to drought and salinity stress. Aust. J. Plant Physiol. 13: 33-43.

Ouborg NJ, Vrizen WH (2007). An ecologist's guide to ecogenomics. J. Ecol. 95: 8-16.

Purcell PC, Smith AM, Halford NG (1998). Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose inducibility of sucrose synthase transcripts in leaves. Plant J. 14: 195–202

Rensink WA, Buell CR (2005). Microarray expression profiling resources for plant genomics. Trends Plant Sci. 10: 603–609.

Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002a).

Monitoring the expression pattern of around 7 000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. Funct. Integr. Genomics 2: 282-291.
Seki M, Narusaka M, Ishida J, Nanjo T, Fujita1 M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002b). Monitoring the expression of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J. 31: 279-292.
Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003). Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. 6: 410-417.
Suprunova T, Krugman T, Fahima T, Chen G, Shams I, Korol A, Nevo E (2004). Differential expression of dehydrin genes in wild barley, *Hordeum spontaneum*, associated with resistance to water deficit. Plant Cell Environ. 27: 1297-1308
Swindelli WR (2006). The association among gene expression responses to nine abiotic stress treatments in *Arabidopsis thaliana*. Genetics 174: 1811-1824.
Valliyodan B, Nguyen HT (2006). Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr. Opin. Plant Biol. 9: 189-195.
Webb MS, Gilmour SJ, Thomashow MF, Steponkus PL (1996). Effects of COR6.6 and COR15am polypeptides encoded by COR (Cold-Regulated) genes of *Arabidopsis thaliana* on dehydration-induced phase transitions of phospholipid membranes. Plant Physiol. 111: 301-312.
Wingler A, Lea PJ, Quick WP, Leegood RG (2000). Photorespiration: metabolic pathways and their role in stress protection. Phil. Trans. R. Soc. Lond. 355: 1517-1529.
Yamaguchi-Shinozaki K, Shinozaki K (2005). Organization of cis-acting regulatory elements in osmotic- and cold-stress responsive promoters. Trends Plant Sci. 10: 88-94.
Yamazaki M, Saito K (2002). Differential display analysis of gene expression in plants. Cell. Mol. Life Sci. 59: 1246-1255.
Yan H, Yuan W, Velculescu VE, Vogelstein B, Kinzler KW (2002). Allelic variation in human gene expression. Science 297: 1143.
Zarka DG, Vogel JT, Cook D, Thomashow MF (2003). Cold induction of *Arabidopsis* CBF genes involves multiple ICE (inducer of CBF expression) promoter elements and a cold regulatory circuit that is desensitized by low temperature. Plant Physiol. 133: 910-918.
Zhang J, Tian ZHH, Zhu G, Peng X (2007). Identification of aluminium-responsive genes in rice cultivars with different aluminium sensitivities. J. Exp. Bot. 58: 2269-2278.
Zhu B, Choi DW, Fenton R, Close TJ (2000). Expression of the barley dehydrin multigene family and the development of freezing tolerance. Mol. Gen. Genet. 264: 145-153.