Long Term Transcript Accumulation during the Development of Dehydration Adaptation in *Cicer arietinum*[1]

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Cool season crops face intermittent drought. Exposure to drought and other abiotic stresses is known to increase tolerance of the plants against subsequent exposure to such stresses. Storage of environmental signals is also proposed. Preexposure to a dehydration shock improved adaptive response during subsequent dehydration treatment in a cool season crop chickpea (*Cicer arietinum*). We have identified 101 dehydration-inducible transcripts of chickpea by repetitive rounds of cDNA subtraction; differential DNA-array hybridization followed by northern-blot analysis and analyzed their responses to exogenous application of abscisic acid (ABA). Steady-state expression levels of the dehydration-induced transcripts were monitored during the recovery period between 2 consecutive dehydration stresses. Seven of them maintained more than 3-fold of expression after 24 h and more than 2-fold of expression level even at 72 h after the removal of stress. Noticeably, all of them were inducible by exogenous ABA treatment. When the seedlings were subjected to recover similarly after an exposure to exogenous ABA, the steady-state abundances of 6 of them followed totally different kinetics returning to basal level expression within 24 h. This observation indicated a correlation between the longer period of abundance of those transcripts in the recovery period and improved adaptation of the plants to subsequent dehydration stress and suggested that both ABA-dependent and -independent mechanisms are involved in the maintenance of the messages from the previous stress experience.

Plants are often exposed to various environmental stresses when grown in field and within a physiological tolerance limit. A mild abiotic stress induces an adaptive response in the plant, allowing it to grow with a greater tolerance to the same or different stresses (Siminovitch and Cloutier, 1982; Lang et al., 1994; Mantyla et al., 1995; Knight et al., 1998). Pretreatment with thermal or chemical shock induced a substantial chilling tolerance in germinated cucumber seeds (Jennings and Saltveit, 1994). Wilted excised cabbage leaves recovered turgor in absence of water uptake when allowed to lose water at a slow rate (Levitt, 1986). Plants express a number of genes in response to water deficit. At the cellular level, a part of this response results from cell damage, whereas the others correspond to adaptive processes. Adaptation to water deficit brings about changes in the metabolic processes and perhaps in the structure of the cell that allows the cells to continue metabolism at low water potential (Ingram and Bartels, 1996). Dehydration and other stresses cause rapid elevation in the cytosolic free calcium ion ($[Ca^{+2}]_{cyt}$) concentration (Knight et al., 1991). As an adaptive response, the subsequent stresses show altered magnitude and kinetics of $[Ca^{+2}]_{cyt}$, depending on the nature and intensity of the previous stress even after a 48-h deacclimation period, indicating existence of a signal storage mechanism. Different stress-exposure alters cytosolic calcium-signature and calcium-regulated gene expression differently in the following stresses (Knight et al., 1996, 1998), suggesting that plants are able to discriminate different stimuli and then store the impression of individual stimuli in a unique way.

Accumulation of abscisic acid (ABA) in the plant organs corresponds to many physiological and metabolic changes that occur during dehydration stress (Lee et al., 1993; Moons et al., 1995). Most of the genes that respond to dehydration are also responsive to exogenous application of ABA (Seki et al., 2002a). The involvement of ABA in dehydration signaling and hence in tolerance was illustrated using ABA-deficient plants (Xiong et al., 2002; Zhu, 2002). Arabidopsis mutants deficient in or insensitive to ABA are impaired in dehydration-induced freezing tolerance and demonstrated the role of ABA in stress-adaptation (Mantyla et al., 1995). However, there are cases of evidence suggesting existence of additional mechanisms (Trejo and Davies, 1991; Griffith and Bray, 1996).

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Drought-induced accumulation of endogenous ABA does not correspond to drought-tolerance in sunflower (*Helianthus annuus*) or drought-induced development of freezing tolerance in Arabidopsis (Lang et al., 1994; Cellier et al., 1998).

We have observed an improved dehydration-tolerance in chickpea (*Cicer arietinum*) seedlings pre-exposed to dehydration shock, even when a recovery phase was allowed between two subsequent stresses. This observation was in accordance with the hypothesis that experiences of previous encounters with stress define the response to the following stresses (Knight et al., 1998). Studies with RNA synthesis inhibitors have suggested an important role of transcription regulatory mechanisms in the long-term memory consolidation in animal systems (Andrews, 1980; Korzus, 2003). We have identified 101 dehydration-inducible genes in chickpea and analyzed their responses to exogenous application of ABA. In order to detect any long-term impression of a stress on gene expression after the removal of the stress, we followed the steady-state expression of those transcripts at 2 different time points during the recovery period using nylon filter arrays. We have detected that some of the transcripts maintained their expression levels more than 2-fold compared to unstressed condition even after 24 h of recovery phase. We have carefully checked, by northern analysis, the expression level of some of the selected transcripts that maintain high expression during recovery after the dehydration shock. In this paper we report a correlation between the long term abundance of some of the dehydration-inducible transcripts after the removal of stress and adaptation to subsequent stress in chickpea seedlings.

RESULTS

Dehydration Stress and Relative Water Content

For dehydration-shock experiments, stressed and control seedlings were sampled at the same time to avoid diurnal variation. The control seedlings were removed similarly from the soil and immediately planted in the same pot and kept under the same condition as the stressed samples to normalize the shock due to uprooting and changes in the environment. During this treatment relative water content (RWC) of the stressed leaves decreased from about 80% to about 55% after 5 h of dehydration and wilted, whereas the same for the unstressed samples did not change. RWC of the samples treated with ABA did not change appreciably in the period of treatment (not shown). To test their tolerance to the indicated condition, some of the plants were replanted in watered soil after 5 h of stress, returned to normal growth condition, and observed for a week. All of them recovered turgor within 4 h and resumed growth with the development of new leaves (not shown). RWC in this period returned to about 74% (average) after 5 h and became about 81% (average) after 24 h of replantation. For recovery, all the samples (control, dehydrated, and ABA treated) were replanted in the same pot containing water-saturated soil to maintain equal soil moisture content. After 24 h of recovery, all of them were subjected to a second dehydration as before. Again RWC of the leaf samples were determined at different time points as a measure of stress-adaptation as it (RWC) accounts for osmotic adjustment, which is considered as one of the most important mechanisms of plants for adaptation in water-limited environment (Turner, 1986; Munns, 1988). The control samples, as expected, continued to lose water reaching to 61.02% (±1.41%) after 3 h and to 55.95% (±1.92%) after 5 h of dehydration and wilted. The rate of decrease in RWC for the ABA pretreated plants was much slower (67.5% [±1.65%]) up to 3 h and then quickly reached 59.73% (±2.02%) after 5 h, showing some degree of tolerance. Some of the lower positioned, matured leaves showed wilting after 5 h. In contrast, the plants, which wilted after 5 h during the first exposure to dehydration, retained turgor throughout the treatment and showed much higher RWC, 69.24% (±1.2%) and 64.71 (±1.53%), after 3 and 5 h of dehydration, respectively, compared to the control or ABA pretreated samples (Fig. 1, top).

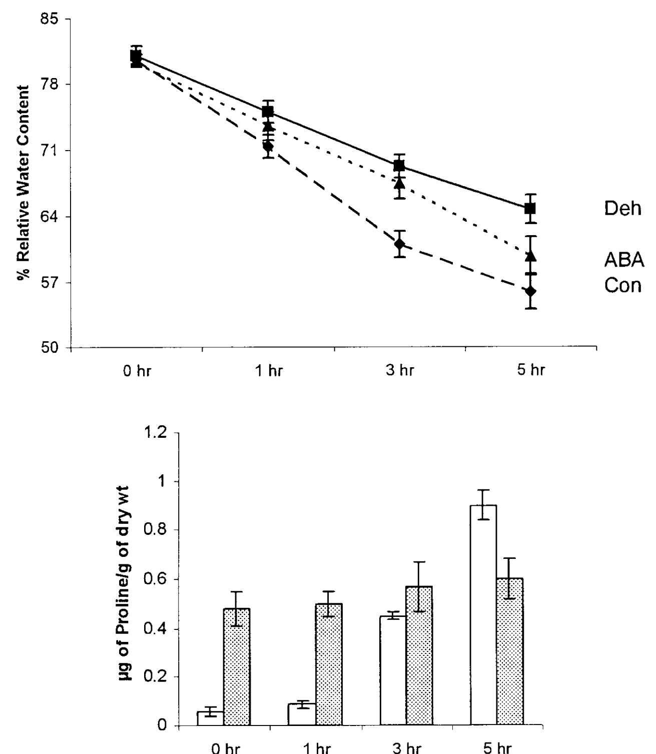

Figure 1. Top, Effect of preexposure to dehydration or exogenous ABA on the RWC during the subsequent dehydration treatment to chickpea seedlings. RWC of leaves in response to dehydration stress (described in "Materials and Methods") at different time points applied 24 h after the end of no treatment (control); pretreatment with dehydration shock (5 h) or 100 μM of ABA (5 h). The traces shown are the averages of four replicates (20 fully expanded leaves each) with SEM. Bottom, Accumulation of Pro in response to dehydration in control (as above; white bar) and dehydration pretreated (as above; gray bar) chickpea seedlings.

Stress-dependent accumulation of Pro has been observed in many plants (Yoshiba et al., 1997). However, the importance of Pro in stress-adaptation is contradictory; its accumulation is considered as one of the indicators of adaptive response (Delauney and Verma, 1993). We have monitored the accumulation of Pro in control and in dehydration pretreated chickpea seedlings during subsequent dehydration treatment. In the control seedlings, Pro started accumulating within 3 h and increased more than 15-fold within 5 h of the treatment. In contrast, the pretreated seedlings showed high constitutive level of Pro (8-fold of the unstressed) from the beginning of subsequent stress and maintained the level throughout the period of stress (Fig. 1, bottom).

Identification of Dehydration-Inducible Transcripts

Transcripts accumulated during dehydration stress were identified by a combination of approaches. Six-day-old chickpea seedlings were subjected to dehydration treatment with proper control as mentioned above and sampled at the same time. A subtracted cDNA library was constructed with poly(A^+) RNA isolated from dehydrated and control seedlings by repetitive rounds of subtractive hybridizations as described in "Materials and Methods." The length of the subtracted cDNA products visibly ranged from 200 bp to 1,200 bp (not shown). The whole library was represented by about 3,000 clones. A population of expression sequence tags (EST) representing dehydration-induced transcripts was identified by screening 1,000 randomly picked clones. PCR amplified cDNAs were spotted in duplicate on nylon membranes in 96-format. Drought-inducible ESTs were identified by differential hybridization with radiolabeled first strand cDNA probes prepared using poly(A^+) RNA isolated from control and stressed samples (Fig. 2). Each DNA element was tested at least three times with different sets of cDNA probes from three independent dehydration experiments to verify reproducibility. Expression ratio was calculated according to Seki et al. (2001). Normalized signal intensity of each spot was determined after subtracting the local background intensity. Signals, with at least five times more intensity than that of the negative control (NPTII) in the dehydrated samples, were included in the analysis (about 97%). Effective signal intensities of the spots were calculated by subtracting the normalized intensity of the negative control. Fold induction was presented as the expression ratio (dehydration to unstressed) of each EST to that of actin. Actin transcript level was shown to be unchanged by water-stress and ABA treatment in Arabidopsis (Xiong et al., 1999) and in chickpea (Fig. 3 and not shown).

$$\text{Fold Induction} = \frac{\text{Effective signal intensity in dehydration}}{\text{Effective signal intensity in control}} \div \frac{\text{Intensity of Actin in dehydration}}{\text{Intensity of Actin in control}}$$

ESTs (total 377 spots) showing at least 2-fold of induction (average of 3 independent experiments) were selected for sequencing and annotated by BLASTX homology search. There were a number of redundant clones, indicating their abundances in dehydrated sample. Among the most notable are β-amylase (21 clones), myoinositol-1-phosphate synthase (MIPS; 17 clones), albumin (14 clones), leuco anthocyanidine dioxygenase (8 clones), polygalacturonase inhibiting protein (8 clones), 9-cis-epoxycarotenoid dioxygenase (NCED; 6 clones), etc. Different sequences showing highest homology to the same accession number were also registered. Excluding the redundant clones, a list of 101 unique transcripts along with their annotations, average fold inductions, e-values, and SDs are presented in Table I. Most of the induced transcripts were subjected to northern-blot analysis to validate the differential dot-blot hybridization results and all of them have shown more than 2-fold of induction. RNA gel analysis of 10 selected ESTs confirming dot-blot data is shown in Figure 3.

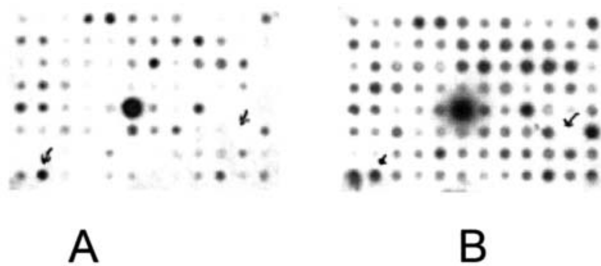

Figure 2. An example of DNA array hybridization of subtracted cDNA clones immobilized on nylon membrane. Segments of representative identical nylon membranes containing cDNA spots from subtracted cDNA library of chickpea after hybridization with ^{32}P-labeled cDNA probes prepared from equal amount of poly(A^+) RNA of control (A) and dehydration treated (5 h; B) seedlings. Arrows indicate actin and NPTII spots.

Response of Dehydration-Inducible Transcripts to Exogenous Application of ABA

Dehydration stress resulted in accumulation of ABA in chickpea seedlings within 2 h and continued to accumulate up to 5 h, when the last sample was assayed (1.087 μg/g dry weight in control to 13.99 μg/g dry weight at 5 h). Similar kinetics and magnitude of ABA accumulation in response to dehydration was reported in another legume crop, cowpea (*Vigna unguilata*; Iuchi et al., 2000). Majority of the drought-inducible genes are induced by exogenous application of ABA in Arabidopsis (Seki et al., 2002a). Labeled cDNA probes prepared from control and ABA-treated samples were used for differential hybridization. Drought-inducible genes (61 among 101) showed

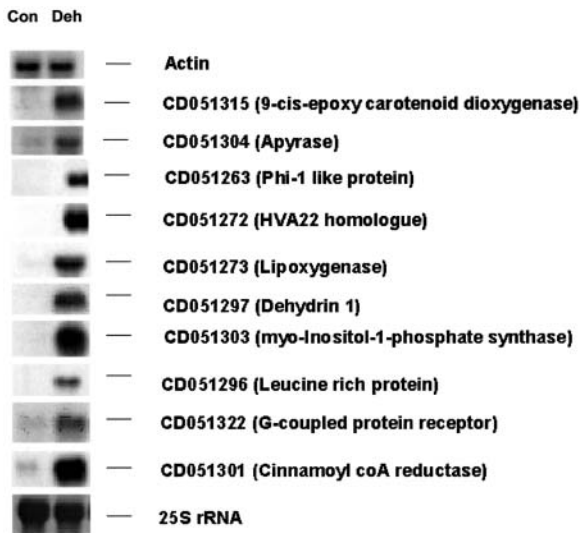

Figure 3. RNA-blot analysis of selected ESTs confirmed increased accumulation of the transcripts as determined by nylon filter array analysis. Twenty micrograms of total RNA isolated from unstressed (Con) and stressed (Deh) chickpea seedlings were analyzed by northern hybridization with α^{32}P-dCTP labeled probes corresponding to indicated EST clones. A PCR product of chickpea actin cDNA (see "Materials and Methods") was used as internal control and 25S ribosomal RNA was shown as loading control. Annotations assigned by BLASTX homology search were mentioned.

more than 2-fold of induction by exogenous ABA treatment (Table I). We compared our results to a similar report in Arabidopsis (Seki et al., 2002a). Previously reported drought-inducible transcripts totally corroborated ABA-mediated gene expression in Arabidopsis, which indicates that probably similar mechanisms for exogenous ABA mediated response operate in Arabidopsis and chickpea. We have revalidated our results by RNA blots with selected ESTs (not shown).

Steady-State Abundances of Dehydration-Inducible Transcripts upon Rehydration

A short-term dehydration shock improved tolerance of chickpea seedlings to the subsequent dehydration treatment when 24 h of recovery period was allowed (Fig. 1). We monitored steady-state expression levels of the dehydration-induced transcripts in the recovery period to detect any long-term impression of the previous stress. Nylon filter arrays were used to determine fold expression at 5 and 24 h time points after replantation of the dehydrated plants in watered soil. As controls, we kept untreated plants (same as the control for dehydration) harvested at the same time point. The average expression ratios from two independent experiments are presented as scatter plots (Fig. 4). Accumulation of most of the transcripts was reduced down to less than 2-fold within 5 h of replantation; however, 15 out of 101 transcripts tested showed more than 2-fold of expression in comparison to the corresponding control after 24 h (Table II). Northern-blot analysis of 6 selected transcripts showed total compliance with the dot-blot data (Table II). Expression of 3 unrelated transcripts, corresponding to chlorophyll a/b-binding protein, Rubisco small subunit, and actin during this period, was unchanged showing that the experimental condition did not cause a global change in transcript abundance (not shown).

We have analyzed by northern blot the expression of 7 transcripts showing more than 3-fold of expression at 24 h time point (Table II). Their expressions were monitored up to 72 h during recovery phase (Fig. 5). Other than CD051303 (myoinositol-1-phosphate synthase) and CD051297 (Dehydrin1), steady-state abundances of all the transcripts gradually decreased in this period. The kinetics of CD051303 were surprising. Its constitutive expression in the control sample was almost undetectable, however, accumulated more than 20-fold upon dehydration (Fig. 3). Upon rehydration, its expression totally diminished to basal level within 5 h, however, again started accumulating and maintained above the basal level up to 72 h (Fig. 5). Two explanations can be given for this unique expression pattern. The rate of degradation of the accumulated transcripts upon rehydration was more than the rate of expression early in the recovery phase and/or expression of a new factor(s) was required for reaccumulation in the latter part. The transcript corresponding to Dehydrin1 (CD051297) continued to express at high level as the dehydrated sample up to 24 h of replantation (Fig. 5). Five other transcripts were accumulated more than 3-fold over the control after 24 h as observed in the nylon filter hybridization (Table II; Fig. 5). Expression levels of 5 of the 7 high expressing transcripts were maintained at above 3-fold even after 72 h when the last sample was harvested (Fig. 5). Longer periods of abundance of these transcripts over the others can be explained by higher intrinsic stability of these mRNAs. Alternatively, preexposure to dehydration may have selectively increased their stabilities or extended the duration of their expression.

Expression of all the seven transcripts mentioned above was inducible by exogenous application of ABA (Table I). To verify whether the characteristic long-term abundance of these transcripts is specific to dehydration or general for any stress, the chickpea seedlings were exposed to 100 μM of ABA for 5 h and were allowed to recover as before. Steady-state accumulation of those transcripts in this period was monitored similarly by northern analysis up to 24 h. As expected, all the seven transcripts were accumulated upon exposure to ABA, in some cases accumulated more than that in dehydration treatment (Fig. 5, dotted lines). The transcript CD051303 (MIPS) followed the same kinetics as before. Its steady-state amount decreased abruptly at 5 h and reaccumulated at 24 h time point indicating that an ABA-dependent or a general stress-regulated mechanism is responsible for this unique pattern. Abundance of the rest of the transcripts decreased sharply to reach the basal expression level within 24 h; in some cases within 5 h

Table I. *Up-regulated transcripts in chickpea seedlings in response to dehydration stress (5 h) with the fold-expression values*

Standard deviations (SD±) are calculated from three independent experiments. Fold-expression values of the transcripts in response to exogenous application of ABA are also presented with SDs. The transcripts are listed according to their possible functions.

Accession No.	GenBank Match	Annotation	E Values	Deh	SD(±)	ABA	SD(±)
Metabolism							
CD051266	NP_189034.1	β-Amylase	1e-89	4.06	1.69	1.20	0.70
CD051265	NP_199040.1	Alcohol dehydrogenase	4e-12	7.00	4.10	1.04	1.08
CD051303	AAK69514.1	Myoinositol-1-phosphate synthase	1e-53	7.32	1.44	5.33	1.32
CD051329	AAB81011.1	Asn synthetase (*Medicago sativa*)	1e-36	4.27	1.35	4.72	1.09
CD051345	CAA09040.1	Glyceraldehyde 3-phosphate dehydrogenase	4e-42	2.90	0.61	3.94	1.49
CD051358	CAA89019.1	Cobalmine-independent Met synthase	4e-12	2.61	0.64	2.53	0.29
CD051350	S68358	Delta 8 sphingolipid desaturase	4e-08	2.43	0.51	2.31	0.93
CD051347	Q9SM59	Phosphoglucomutase	1e-34	2.36	0.34	2.50	0.14
CD051311	BAA97584.1	Rubisco activase	2e-12	2.89	0.65	1.19	0.33
CD051301	CAC07424	Cinnamoyl CoA reductase	2e-58	6.34	1.91	4.26	1.03
CD051310	Q01517	Fru biphophate aldolase 1, chloroplast precursor	1e-48	3.26	0.92	1.42	0.04
CD051304	AAK15160.1	Putative Apyrase	8e-48	2.95	0.75	2.49	0.58
CD051352	NP_1733761	Very long chain fatty acid condensing enzyme CUT1	e-116	3.17	0.82	1.35	0.51
CD051279	AAK27718	ADP-Glc pyrophosphorylase (L1)	1e-33	5.50	1.98	0.84	0.75
CD051285	NP_197299.1	Phosphoribosylanthranilate transferase-like protein	2e-17	3.41	0.82	2.63	0.79
CD051305	H71447	Trehalose-6-phosphate synthase homolog	2e-25	2.24	0.64	2.42	0.32
CD051278	BAB11549	Leucoanthocyandin dioxygenase-like protein	2e-36	4.50	1.21	0.39	0.11
CD051342	CAA07519	Trans-cinnamate-4-monooxygenase	e-125	2.93	0.66	2.35	0.69
Signal Transduction							
CD051357	AAF04915	JA2	2e-19	3.87	1.22	2.72	0.90
CD051343	NP_564584.1	Putative protein kinase	2e-41	4.01	1.34	4.55	2.12
CF340748	AAM83095.1	Ser/Thr protein kinase homolog	e-111	4.84	1.03	3.97	1.77
CD051322	AAF75794.1	7-transmembrane G-protein-coupled receptor	2e-65	2.80	0.60	2.36	1.19
CD051324	NP_191788.1	ADP-ribosylation factor-like protein	2e-09	2.98	0.70	0.86	0.67
CD051317	CAA67554.1	Protein kinase (*Trifolium repens*)	1e-05	3.17	0.88	0.72	0.71
CD051274	AAM65034	Putative protein kinase	3e-34	2.28	0.25	6.20	2.58
CD051264	NP_196473.1	WD repeat protein-like protein	4e-26	3.18	0.72	2.52	0.06
CD051312	AAD17804.1	Protein phosphatase type 2C	1e-84	3.12	0.71	2.49	0.44
CD051261	NP_564656	Membrane protein	1e-54	4.97	1.88	9.18	4.81
CD051323	NP_180081.1	Putative Ser/Thr protein kinase	2e-77	2.56	0.45	1.40	0.70
CD347670	NP_201489.1	Putative Ser/Thr protein kinase	2e-54	4.44	1.44	1.36	0.56
Cellular Organization							
CD051268	CAB71135.1	Imbibation protein	2e-38	2.66	0.73	4.31	0.07
CD051271	O49816	LEA-1	6e-16	4.04	1.63	4.24	0.06
CD051272	A48892	HVA 22	3e-06	8.13	1.15	7.21	2.57
CD051277	P46519	LEA 14 homolog	5e-18	3.70	0.87	3.15	0.07
CD051290	CAB53509.1	Histone 2A	2e-22	3.55	1.76	0.55	0.11
CD051297	AAN77521.1	Dehydrin1	3e-25	6.24	1.96	4.30	1.58
CD051339	T09820	Fiber protein 1 [imported], upland cotton	5e-33	2.10	0.48	2.46	0.54
CD051320	NP_198008.1	Chaperonin a-chain-like protein	3e-33	3.17	0.85	3.93	0.43
CD051326	O49817	Late embryogenesis abundant protein 2	6e-37	2.81	0.76	3.08	0.67
CD051288	AAL02402	Late embryogenesis abundant protein 4	4e-31	3.46	0.97	2.27	0.75
CD051295	AAM61711	Prolyl-4-hydroxylase, α-subunit	e-100	3.44	1.03	2.53	1.39
Translation							
CD051286	AAD28753.1	60S ribosomal protein L 37 A	1e-46	3.28	0.77	1.45	0.56
CD051292	NP_193053.1	Ribosomal protein L 13a-like protein	2e-30	3.40	0.99	2.38	1.04

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Accession No.	GenBank Match	Annotation	E Values	Deh	SD(±)	ABA	SD(±)
CD051346	P17093	40S Ribosomal protein S11	2e-32	3.11	0.70	2.46	0.99
CD051338	AAM63913	40S ribosomal protein S7 homolog	4e-17	2.34	0.58	2.38	0.81
CD051333	AAM67061.1	Ribosomal protein S2	1e-50	2.01	0.23	2.61	0.64
CD051300	CAA11705	Putative translation elongation factor (Arabidopsis)	1e-25	4.26	1.44	3.32	0.31
CD051284	NP_172256.1	Ribosomal protein S 15	2e-58	4.32	0.90	2.97	0.23
CD051267	NP_175268.1	Ribosomal protein L18	4e-16	8.91	4.09	3.10	1.68
CD051351	NP_172752	Eukaryotic peptide chain release factor subunit 1	1e-30	2.67	0.51	1.31	0.64
CD051287	P24921	Eukaryotic translation initiation factor 5A-1	3e-59	3.41	0.87	1.33	0.22
Transcription							
CD051308	AAC 49770.1	RAP2.4	9e-09	2.73	0.54	1.56	0.46
CD051355	AAC36019	RAP 2.6	2e-17	2.53	0.38	2.5	0.26
CD051360	AAN77051.1	DREB 2(1)	1e-06	3.71	0.65	1.1	0.64
CD051361	CAB93939.1	DREB 2(2)	3e-23	3.60	0.51	1.1	0.80
CD051330	CAB96991.1	Putative zinc finger protein (*Cicer arietinum*)	4e-22	2.66	0.60	3.04	0.77
CD051282	CAA09196.1	RNA helicase (RH3)-like protein	1e-21	2.28	0.21	0.67	0.60
CF074502	AAP47161.1	AP2 domain containing putative transcription factor	1e-29	3.52	1.07	1.19	0.44
Protein Degradation							
CD051293	CAA51821.1	Ubiquitin conjugating enzyme E2	9e-56	2.79	0.69	1.48	1.06
CD051336	S24602	Cysteine proteinase tpp	1e-52	3.53	1.03	2.42	0.35
CD051341	P35100	ATP-dependent clp protease ATP-binding subunit	8e-73	2.52	0.48	1.43	0.60
Hormone Biosynthesis							
CD051262	P50302	S-adenosyl Met synthetase	2e-22	7.98	3.40	1.2	0.21
CD051273	CAC43237.1	Lipoxygenase	7e-24	5.37	1.81	0.72	0.76
CD051299	S56655	Lipoxygenase (EC 1.13.11.12)	2e-57	4.09	1.84	1.20	0.26
CD051315	BAC10549.1	9-cis-epoxycarotenoid dioxygenase1	2e-51	3.51	0.95	0.65	0.44
Cell Defense							
CD051353	Q39458	Metallothionin-1	1e-39	2.52	0.34	2.66	0.12
CD051270	AAB19212.1	Polygalacturonase-inhibiting protein (apple)	8e-44	2.78	1.43	1.49	0.14
CD051289	BAA29056.1	Polygalacturonase-inhibiting protein (citrus)	7e-47	3.93	1.18	2.33	0.60
CD051291	NP_172076	Chitinase family 19	1e-34	4.29	1.35	2.61	0.52
CD051296	BAB01963.1	Leu rich protein:polygalacturase inhibitor-like protein	3e-10	3.35	0.89	2.47	0.79
CD051321	O23758	Nonspecific lipid-transfer protein precursor	4e-53	3.09	0.79	0.92	0.12
Cell Transport							
CD051335	NP_192428.1	Kinesin-like protein A	2e-52	3.59	1.05	2.73	0.69
CD051276	C85065	Kinesin-like protein	5e-50	4.11	2.30	1.42	0.79
CD051281	AAB60858.1	Vacuolar assembly protein (VPS 41 p)	2e-43	4.86	1.63	2.88	0.46
Energy Metabolism							
CD051307	P29450	Thioredoxin F type, chloroplast precursor	1e-40	3.51	0.94	2.84	0.60
CD051283	CAB71010.1	Mitochondrial uncoupling protein	2e-18	3.68	1.08	2.61	0.96
CD051309	CAB50768.1	CytochromeP 450	8e-24	3.51	1.07	1.33	0.25
CD051327	NP_084850.1	NADH dehydrogenase subunit 1	4e-27	3.71	1.30	2.29	0.27
CD051325	AAG14961.1	Cytochrome p450-dependent mono-oxygenase	8e-62	2.73	0.65	2.58	0.17
CD051280	CAC29436.1	P type H+ ATPase	1e-11	3.51	1.09	2.29	0.17
Unclassified							
CD051269	NP_175875.1	Hypothetical protein	6e-42	3.03	0.37	1.22	0.06
CD051275	NP_190940.1	Putative protein	8e-40	3.41	0.91	4.53	1.40

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Accession No.	GenBank Match	Annotation	E Values	Deh	SD(±)	ABA	SD(±)
CD051298	NP_199239.1	Putative protein	4e-21	5.07	1.24	0.78	0.18
CD051306	AAM64945.1	PDI-like protein	7e-07	3.03	1.90	2.36	0.63
CD051313	ZP_00006053	Hypothetical protein	7e-32	3.38	0.84	2.37	0.30
CD051314	BAC42399.1	Unknown protein (Arabidopsis)	1e-11	3.34	0.95	1.42	0.73
CD051316	NP_187912.1	Unknown protein (Arabidopsis)	2e-23	3.57	1.05	2.38	0.76
CD051332	NP_194708.1	Putative protein (Arabidopsis)	1e-11	3.05	0.75	1.2	0.71
CD051334	NP_566737.1	Expressed protein	3e-26	2.68	0.57	2.60	0.44
CD051337	NP_567643.1	Expressed protein (Arabidopsis)	2e-38	2.30	0.99	3.72	1.22
CD051344	CAC04249.1	PPF-1 protein (*Pisum sativum*)	2e-48	2.50	0.58	1.26	0.93
CD051348	NP_564617.1	Expressed protein (Arabidopsis)	3e-08	3.15	0.83	2.37	0.56
CD051349	NP_188633.1	Hypothetical protein	2e-15	10.17	3.75	1.18	0.25
CD051354	AAM65383.1	Unknown protein	1e-05	3.85	1.04	4.82	0.95
CD051340	AAM67211.1	Ser rich protein (Arabidopsis)	3e-18	3.18	0.84	1.41	0.42
CD051331	AAM62421	Drm3	5e-17	3.20	0.93	2.56	0.66
CD051294	AAA16929.1	Cold induced alfalfa gene (CAS 15)	9e-14	4.18	1.12	1.33	0.56
CD051302	NP_201140.1	Unknown protein protein (Arabidopsis)	6e-07	3.14	1.87	2.41	0.71
CD051263	NP_201231.1	Phi-1-like protein	1e-51	4.75	0.64	2.18	1.14
CD038847	P08688	Albumin (*Pisum sativum*)	6e-21	6.20	2.31	10.36	3.09
CD051328	AAL60005	Unknown protein	1e-29	3.32	0.87	1.41	0.49

of replantation (Fig. 5), suggesting that long-term abundance of these transcripts after removal of dehydration stress was not due to higher intrinsic stability of mRNAs.

Accumulation of myoinositol in Actinidia seedlings in response to salt stress has been reported and its (myoinositol) level was shown to be maintained above the control level after removal of stress (Klages et al., 1999). In our study, 1 of the 7 transcripts (CD051303) maintaining higher expression level after removal of stress encodes for MIPS, a key enzyme for synthesis of myoinositol. We have assayed myoinositol content in the chickpea seedlings during dehydration treatment and subsequent recovery period. Myoinositol accumulated more than 4-fold from the control after 5 h of dehydration (6.48 ± 0.64–28.5 ± 2.03 mg/g dry wt); however, it declined to a level 2-fold of the control after 24 h of recovery (13.15 ± 0.97 mg/g dry wt) and maintained up to 72 h (11.8 ± 1.15 mg/g dry wt).

DISCUSSION

Dehydration-Inducible Genes in Chickpea

We have selected chickpea, the third most consumed legume crop (Graham and Vance, 2003) for this study because it requires less irrigation and adapts well in a water-limited environment (Turner et al., 2001). As a part of the chickpea genomics program, in this paper we have reported an EST database of dehydration-inducible transcripts. Temperate grain legumes such as pea, fava bean, lentil, chickpea, and others share similar gene arrangements (VandenBosch and Stacey, 2003). Therefore, we expect that our database will benefit the study of other legume plants by comparing the expression patterns of the transcripts.

Small sugar molecules are believed critical to maintain osmotic balance of cells in stress (Bray et al., 2000; Lee et al., 2003). Dehydration in chickpea induced a number of transcripts for the enzymes associated with degradation of starch such as β-amylase, Fru-1,6-biphosphate aldolase, phosphoglucomutase, and synthesis of small sugars such as trehalose-6-phosphate synthase and myoinositol-1-phosphate synthase (MIPS), enzymes that promote synthesis of trehalose and pinitol, respectively, (Nelson et al., 1998; Lee et al., 2003). Pinitol accumulates in the leaves of legumes and halophytes in salt and drought stress (Pattanagul and Madore, 1999). In *Mesenbryanthemum crystallinum*, salinity, but not drought or exogenous ABA, causes accumulation of MIPS transcript (Vernon et al., 1993), whereas Arabidopsis does not show induction in MIPS gene expression in response to excess salt (Ishitani et al., 1996). However, in our study, both dehydration and exogenous ABA caused accumulation of MIPS transcripts (Table I; Fig. 3). In several leguminous plants role of apyrase, an enzyme involved in nucleic acid metabolism, has been implicated in legume-rhizobia symbiosis. However, in *Medicago truncatula*, expression of apyrase is not regulated symbiotically, rather it is induced by wounding or stress associated with harvesting in an ethylene-independent manner (Navarro-Gochicoa et al., 2003). Expression of apyrase in chickpea in response to dehydration indicates either that the dehydration treatment mimicked the stress associated with harvesting or that dehydration regulates expression of apyrase through a different pathway.

The CBF/DREB family of transcription factors are an integral part of early regulation of water stress-mediated response (Liu et al., 1998). CBF regulon comprises a part of total genes expressed in cold stress

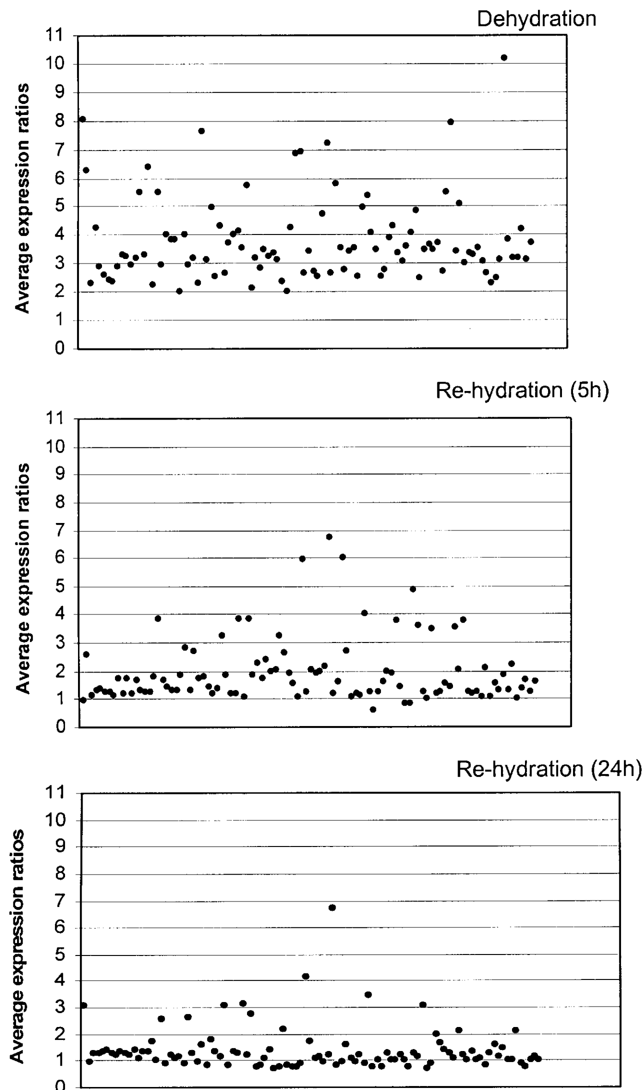

Figure 4. Scatter plots of expression ratios of dehydration-induced ESTs during recovery phase. Average of the ratios of normalized intensities of each EST during the recovery phase after a dehydration stress to the corresponding control (see "Materials and Methods") from two independent experiments is plotted. Scatter plots of dehydration stress, rehydration (5 h), and rehydration (24 h) are shown.

(Jaglo et al., 2001; Fowler and Thomashow, 2002). A number of genes expressed by overexpression of CBF homologs do not contain DRE/C-repeat in their promoters. It has been postulated that RAP2.1, a CBF-inducible, AP2-family protein in turn may activate transcription of the CBF target genes in cold-response pathway (Fowler and Thomashow, 2002). Expression of transcripts encoding DREB2, RAP2.4, and RAP2.6 indicates that a similar subregulon may function in dehydration-stress response. Simultaneous up-regulation of DREB2, RAP2.4, and RAP2.6 transcripts during mechanical wounding in Arabidopsis (Cheong et al., 2002) reinforces this concept.

Transcripts encoding ribosomal proteins and translation elongation factor are expressed during the initial phase of salt stress in a salt tolerant rice variety but not in a salt-sensitive one. It was speculated that part of the salt-tolerance may be conferred by the ability to translate the components of the response circuit early in the stress that lacked in the sensitive variety (Kawasaki et al., 2001). In chickpea, we have observed induction of transcripts encoding a number of ribosomal structural proteins and the proteins involved in translational initiation, elongation, and termination. Generation of reactive oxygen molecules accompanied by protein degradation are the consequences of dehydration stress (Ingram and Bartels, 1996). Mitochondrial uncoupling protein and metallothionin-like proteins may decrease and detoxify such species. A number of transcripts encoding protein-degrading enzymes were also identified (Table I).

Long-Term Expression of Some Dehydration-Inducible Genes during Recovery

All the genes expressing more than 2-fold at 24 h after removal of stress were inducible by ABA. However, this cannot be explained by residual high ABA concentration, because at that time point endogenous ABA level had returned to normal level (1.136 μg/g dry weight). Other ABA-inducible genes also returned to basal level of expression at this time point (Fig. 4). In another legume crop, cowpea, expression of the gene for 9-cis-epoxycarotenoid dioxygenase, the rate limiting enzyme for ABA biosynthesis, was also shown reduced down to basal level within 10 h of rehydration (Iuchi et al., 2000).

Two of the transcripts expressing in the recovery period code for myoinositol-1-phosphate synthase and trehalose phosphate synthase are involved in synthesis of pinitol and trehalose, respectively. Concentration of pinitol was reported to be higher in the halophytic plants and the plants that are adapted to drought (Vernon and Bohnert, 1992). We also have observed a high constitutive level of myoinositol in the dehydration pretreated chickpea seedlings. Interestingly, myoinositol concentration in Actinidia leaves increased more than 2-fold at harvest time when a short water-stress was given in early summer (Miller et al., 1998).

Late-embryogenesis abundant (LEA) groups of proteins are thought to provide protection to the cellular macromolecular structures against desiccation-mediated damages. A transcript (CD051297) encoding dehydrin1 (LEA D11) continued to express at the same level as in the dehydrated sample up to 24 h after the removal of dehydration stress. Accumulation of dehydrin (both dhn1 and dhn2) transcripts was shown to correlate drought-adaptive response in a relatively tolerant variety of sunflower; however, accumulation of ABA was not responsible for the varietal difference in drought tolerance (Cellier et al., 1998). Alteration in the kinetics of dehydrin transcript accumulation during recovery from dehydration from that after

Table II. *A list of ESTs and their fold expressions with different treatments (as described) determined by nylon filter hybridization. Averages of the ratios from two independent experiments (described in Fig. 3) with ±SD values were mentioned. Fold expressions of some of the transcripts were compared with RNA-blot results*

Accession No.	Annotation	Dehydration	Rehydration	Rehydration
		5 h	*5 h*	*24 h*
CD051263	Phi-1-like protein	3.59 (±0.53)	1.60 (±0.45)	1.00 (±0.10)
	(RNA blot)	7.62	1.20	1.30
CD051266	β-Amylase	4.97 (±1.00)	1.21 (±0.20)	1.32 (±0.23)
	(RNA blot)	8.32	1.10	1.10
CD051360	DREB2(1)	3.40 (±0.38)	2.07 (±0.18)	0.78 (±0.24)
	(RNA blot)	4.05	2.90	1.10
CD051272	HVA22 homolog	7.95 (±2.17)	3.56 (±0.79)	2.14 (±0.10)
	(RNA blot)	22.20	2.60	2.30
CD051348	LEA-1	5.51 (±1.20)	3.85 (±0.06)	2.58 (±0.32)
	(RNA blot)	12.3	3.46	2.25
CD051305	Trehalose-6-phosphate synthase	3.86 (±0.67)	2.21 (±0.27)	2.13 (±0.53)
	(RNA blot)	3.26	2.24	2.36
CD051348	Expressed protein	3.19 (±0.88)	3.21 (±0.54)	2.17 (±0.09)
CD051280	P-H^+ ATPase	4.03 (±0.52)	2.81 (±0.15)	2.64 (±0.14)
CD051284	Ribosomal protein S15	5.72 (±0.19)	3.85 (±0.52)	2.75 (±0.02)
CD051296	Leu rich protein	3.68 (±0.22)	3.49 (±0.21)	2.09 (±0.43)
CD051303	Myoinositol-1-phosphate synthase	8.06 (±1.71)	0.99 (±0.04)	3.08 (±0.05)
CD051312	Protein phosphatase 2C	4.13 (±0.24)	3.82 (±0.17)	3.16 (±0.13)
CD051323	Putative Ser/Thr protein kinase	4.29 (±0.42)	3.22 (±0.05)	3.09 (±0.44)
CF340748	Ser/Thr protein kinase homolog	4.98 (±1.40)	4.05 (±0.33)	3.44 (±0.47)
CD051301	Cinnamoyl CoA reductase	6.91 (±1.50)	5.93 (±1.15)	4.14 (±0.98)
CD051354	Unknown protein	4.86 (±0.84)	3.57 (±0.73)	3.07 (±0.09)
CD051297	Dehydrin1	7.02 (±0.57)	6.73 (±1.10)	6.72 (±0.26)

ABA-exposure supports the observation in sunflower and suggests that additional factors other than ABA might be involved in the expression of dehydrin during stress-adaptation. In Arabidopsis, expression of rab18 gene in response to low temperature was shown to be ABA dependent. However, a small, but reproducible accumulation of rab18 mRNA persisted during low temperature adaptation in Arabidopsis even after endogenous ABA level decreased to the basal level (Lang et al., 1994). RAB18 protein expression was maintained almost comparable to the induced level up to 3 d of deacclimation (Mantyla et al., 1995).

Two transcripts (CD051343 and CF340748) encoding 2 putative Ser/Thr kinases that maintained higher expression for 72 h after stress-removal resemble closely CBL-interacting protein kinases (CIPK) 6 and 16, respectively, of Arabidopsis. CBL-interacting protein kinases are cytosolic calcium-regulated autophosphorylating enzymes (Halfter et al., 2002; Hardie, 1999). Calcium was shown to be involved not only in the early events of signal transduction (Knight et al., 1991; Bush, 1995), but also in the signal storage and retrieval (Verdus et al., 1997). A signal storage mechanism involving an autophosphorylating kinase has been proposed (Lisman, 1985) and involvement of calcium-dependent protein kinases in long-term maintenance of seed embryo dormancy was suggested (Trewavas, 1986). The transcript CD051312 possesses high homology to ABI1, a protein phosphatase. ABI1 locus regulates a wide spectrum of Abscisic acid-mediated responses. ABI1 protein contains a Ca^{2+} binding site in its N-terminal region and interacts strongly with one of the CIPKs (PKS18), indicating a possibility that this protein may connect ABA and Ca^{2+}-mediated signaling pathways (Leung and Giraudat, 1998; Ohta et al., 2003). Cytosolic calcium also regulates plasma membrane H^+-ATPase (CD051280; Kinosita et al., 1995; Qui et al., 2002). All these cases of evidence indicate that ABA and cytosolic calcium probably play major role in the maintenance of the transcript messages from the previous stress. Possible involvement of cell wall has been implicated as a reservoir of stored signal concerning the fate of the cell during development (Brownlee and Berger, 1995). Changes in the cell wall structure were also proposed in drought acclimation (Levitt, 1986). Cinnamoyl coA reductase, encoded by CD051301, is one of the enzymes involved in lignin biosynthesis for cell wall (Ruelland et al., 2003).

Prolonged gene expression can be explained by two mechanisms: increased mRNA stability and/or slow mRNA synthesis for longer period. Salt-regulated increased mRNA stability has been demonstrated for the transcript accumulation of a cell wall targeted alfalfa protein MsPRP2, which contain a sequence for mRNA destabilization at the 3′- untranslated region (Deutch and Winicov, 1995). It will be interesting to check whether these transcripts have such a common sequence at their 3′-UTR. Unlike dehydration, exposure to ABA had no appreciable effect on RWC of the

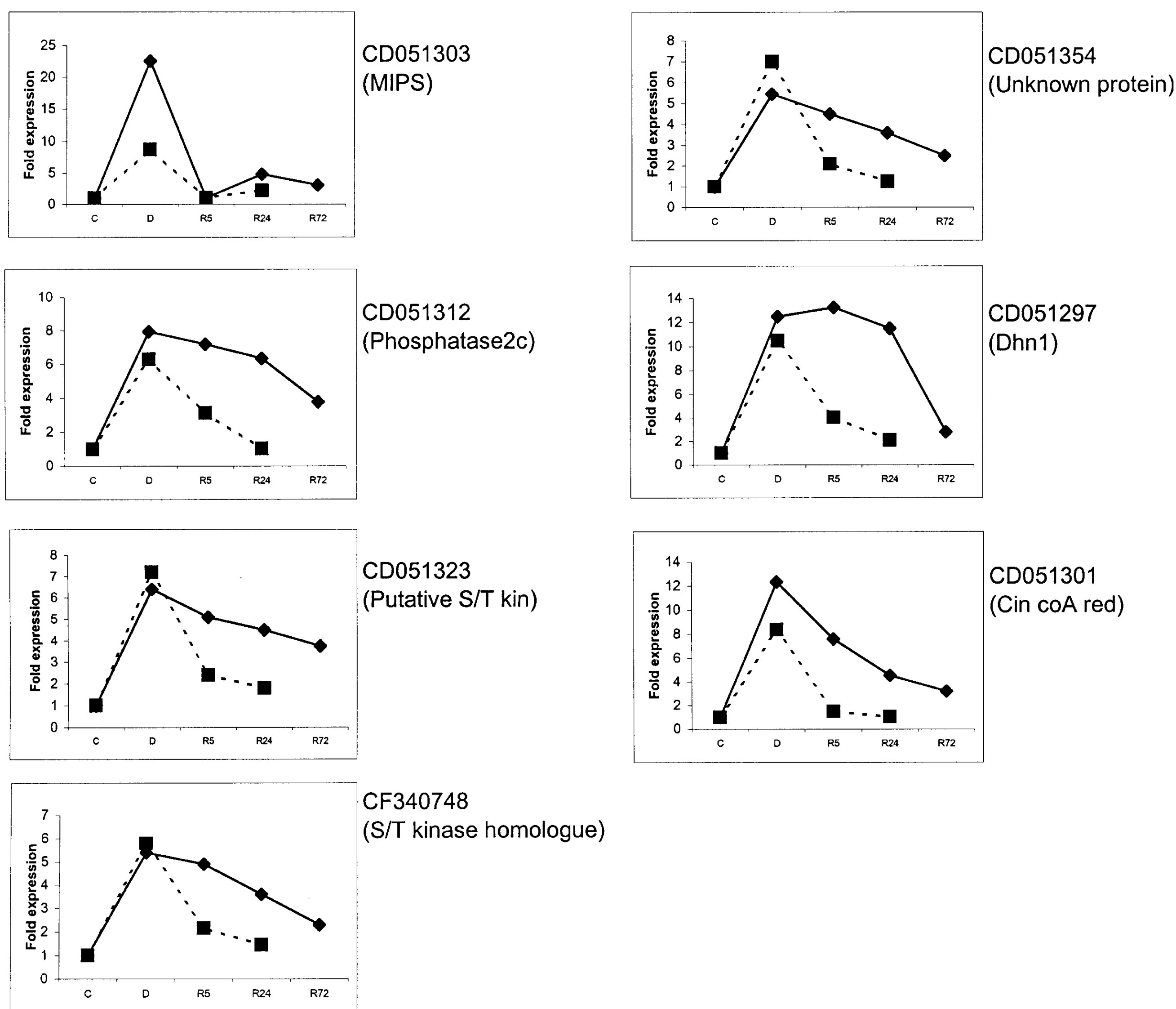

Figure 5. Steady-state abundances of the transcripts corresponding to ESTs, CD051303 (MIPS), CD051312 (Protein Phosphatase 2C), CD051323 (putative Ser/Thr kinase), CF340748 (Ser/Thr kinase homolog), CD051297 (Dehydrin1), CD051301 (Cinnamoyl CoA reductase), and CD051354 (unknown protein) were compared during the recovery phase after dehydration stress (solid line) and after exogenous application of ABA (100 μM; dashed line). Twenty micrograms of total RNA from unstressed (C), stressed (D), and replanted seedlings at 5 h (R5), 24 h (R24), and 72 h (R72) after replantation were analyzed by northern hybridization and quantitated by phosphorimager scanner. Fold expressions at different time points as compared to the control were plotted.

leaves; and in contrast to dehydration, preexposure to ABA did not prolong the expression of six transcripts during recovery. Therefore, it is possible that some dehydration-regulated, but ABA-independent factor(s) is responsible for the increased stability of those mRNAs. Alternatively, unlike ABA-exposure, water-limitation may cause a permanent alteration in the cell structure and in the signaling pathways, resulting in sustained expression of those transcripts.

Preexposure to ABA, though it did not prolong the expression of 6 transcripts in the recovery phase as dehydration pre-exposure, improved water retention ability in the chickpea leaves to some extent in the following dehydration treatment (Fig. 1). ABA pretreatment maintains the expression levels of MIPS and dehydrin1 transcript more than 2-fold up to 24 h during recovery. However, this may not be the only reason. We have analyzed the expressions of only 7 genes after ABA pretreatment. Detail expression analysis of more genes in the recovery period after ABA exposure is required to address this issue. Finally, it cannot be excluded that storage of previous stress signals does not result only from the maintenance of some gene expression; rather it is the manifestation of cumulative changes in the structural and functional relationships of multiple pathways involved in stress metabolism.

MATERIALS AND METHODS

Plant Materials and Stress Treatment

Seeds of chickpea (*Cicer arietinum* L. cv BGD72), provided by IARI (New Delhi, India), were germinated, sown in composite soil (peat compost to vermiculite, 1:1), and grown for 6 d after germination at 18°C to 22°C day/10°C to 15°C night/50% relative humidity with a photoperiod of 10 h with appropriate watering. For dehydration treatment, seedlings were carefully removed from the pot and subjected to dehydration for 5 h on 3 MM paper (Whatman, Clifton, NJ) at room temperature under dim light. For control, plants were removed from the soil and immediately replanted in the same pot and kept under the same condition for the same period. For stress-recovery, seedlings exposed to dehydration for 5 h were replanted in water-saturated soil and kept for the indicated period in normal growth condition. As controls for stress recovery, seedlings were removed from the soil and replanted immediately as earlier and kept for the same period of time to avoid diurnal variation. For ABA treatment, plants were removed from the soil as before, and the roots were dipped into aerated deionized water with or without 100 μM of ABA for 5 h. For the recovery from ABA-stress, seedlings were similarly treated as in the case of dehydration. RWCs of the leaf samples were measured as described (Levitt, 1986). Four samples each of 20 fully expanded leaves were taken for each of the time points.

RNA Isolation and Construction of Subtracted cDNA Library

Total RNA was isolated from whole seedling by using TRIzol Reagent (Life Technologies, Rockville, MD), and polyA$^+$ RNA was purified by mRNA isolation kit (Roche Applied Science, Manheim, Germany). Subtracted cDNA library was constructed by using CLONTECH PCR-Select cDNA subtraction kit (CLONTECH Laboratories, Palo Alto, CA) following manufacturer's protocol. In brief, tester (dehydration) and driver (control) double stranded cDNAs were prepared from 2 poly(A$^+$) RNA (2 μg each) samples. Tester and Driver cDNAs were separately digested with *Rsa*I to obtain shorter blunt-ended molecules. Two tester populations were created by ligating two aliquots of diluted tester cDNA with two different adaptors (adaptors 1 and 2R) separately. First hybridization was performed by the following procedure. Each tester population was mixed with an excess of digested driver cDNA. The samples were heat denatured and allowed to anneal at 68°C for 8 h. The two samples from the first hybridization reaction were mixed together, and more denatured driver cDNA was added for further hybridization to enrich differentially expressed sequences. Differentially expressed cDNAs, with different adaptor sequences at two ends, were selectively amplified by PCR and a second PCR was done with nested primers to further reduce the background. The subtracted and enriched DNA fragments were directly cloned into T/A cloning vector (pT-Adv; CLONTECH Laboratories). Competent cells of *Escherichia coli* DH5α were transformed with the ligation mix and plated on Luria-agar plate containing ampicillin, isopropylthio-β-galactoside, and X-gal for blue-white selection (Sambrook and Russell, 2001).

Library Amplification and Preparation of DNA Arrays

Individual clones of the subtracted cDNA library were amplified in a 96-well PCR reaction plate using M13 forward and reverse primers in a 50-μL reaction at an annealing temperature of 60°C for 30 cycles. The products were analyzed in agarose gel to confirm the insert size, quality, and quantity. Purified PCR products were denatured by adding an equal volume of 0.6 M sodium hydroxide. Equal volume of each denatured PCR product (about 100 ng) of ≥300 bp of size was spotted on two Hybond N membranes (Amersham Pharmacia Biotech, Uppsala) using dot-blot apparatus (Life Technologies, Bethesda, MD) in 96 format to make two identical arrays. In addition, a PCR product of chickpea actin cDNA (GenBank accession no. AJ012685) using primer sequences (5′-CCACGAGACAACATTTAACTC-3′ and 5′-TATTCTGCCTTTGCAATCCAC-3′) was spotted as internal control to normalize the signals of two different blots corresponding to stressed and unstressed samples. A PCR product of Neomycin phosphotransferase (NPTII) gene from the vector pCAMBIA 1305.1 (GenBank accession no. AF354045) using primer sequences (5′-TTTTCTCCCAATCAGGCTTG-3′ and 5′-TCA-GGCTCTTTCACTCCATC-3′) was also spotted as a negative control to subtract the background noise. The membranes were neutralized with neutralization buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 3 min, washed with 2× SSC, and cross linked using UV cross linker (Stratagene, La Jolla, CA).

Probe Preparation, Hybridization, and Data Analysis

Control and stress mRNAs were labeled with α^{32}P-dCTP by first-strand reverse transcription. One microgram of mRNA was labeled in a 20-μL reaction volume containing 1× reaction buffer, 2 μg of 5′-(dT)$_{30}$VN-3′ (V = A/G/C and N = A/G/C/T) primer, 2.5 mM dATP, dTTP, dGTP, 0.02 mM dCTP, 5 μL of α^{32}P-dCTP (10 μCi/μL; 3,000 Ci/mmol), and 200 units of reverse transcriptase (Superscript II, Life Technologies, Grand Islands, NY). After incubation at 42°C for 1 h, RNA was removed by incubating with RNase H at 37°C for 20 min. Radio-labeled cDNAs were cleaned by Sephadex G-25 (Amersham-Pharmacia Biotech) and suspended in hybridization buffer (7% SDS, 0.3 M Sod-phosphate pH 7.4, 1 mM EDTA, 10 μg of sonicated salmon sperm DNA). Nylon membranes were prehybridized with the same buffer for 3 h at 65°C and hybridized with denatured control and experimental (drought-treated) cDNA probes at the same condition for 24 h. The membranes were washed three times with washing buffer (1× SSC, 1% SDS, 30 min each, 65°C). Images of the membranes were scanned in phosphorimager and signal intensities were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The program allows normalization of the signal against background. Sequencing was done using Big Dye Terminator kit version 3.0 (Applied Biosystems, Foster City, CA) and analyzed with the 3,700 ABI Prizm 96 capillary sequence analyzer. The sequences were submitted to the EST data bank of NCBI for accession numbers.

Northern Hybridization

Twenty micrograms of total RNA from control and treated samples were analyzed in 1.2% agarose gel containing formaldehyde and transferred to Hybond N membrane (Amersham Biosciences, Buckinghamshire, UK) following the method mentioned in Sambrook and Russell (2001). PCR-amplified individual cDNA fragment (with primers corresponding to adaptor 1 and 2R) was purified from agarose gel. Primers for the actin cDNA are mentioned above. Probes were labeled with α^{32}P-dCTP using Megaprime DNA labeling system (Amersham Biosciences) and purified through Sephadex G-25. Northern hybridization was performed and band-intensity was calculated following the procedure described above for nylon membrane array.

Assay of Abscisic Acid, Pro, and Myoinositol

ABA content of chickpea seedlings with or without stress was measured according to Setter et al. (2001). Lyophilized seedlings were crushed in chilled 80% methanol. The extracts were fractionated by C18 reverse-phase chromatography, and the ABA contents were assayed by enzyme linked immunosorbant assay (ELISA). The ABA-content is expressed as microgram of ABA per gram of dry weight. Free Pro content was measured from three different lyophilized samples for each time point according to Bates et al. (1973). Myoinositol was measured according to Miller et al. (1998). Briefly, total soluble sugar was extracted from the lyophilized samples with methanol to chloroform to trichloroacetic acid to water (12:5:1:2). Water to methanol (1:1) was added for phase separation. The aqueous phase was lyophilized to complete dryness. The sample was derivatized with Tri-Sil Z (Pierce, Rockland, Il) and run through gas-liquid chromatography coupled with flame ionization detector with nitrogen as carrier gas. Quantitation was made against similar run with standard myoinositol (Sigma-Aldrich, St. Louis). Samples from two independent seedlings were taken for each time point.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AJ012685, AF354045, CD051266, CD051265, CD051303, CD051329, CD051345, CD051358, CD051350, CD051347, CD051311, CD051301, CD051310, CD051304, CD051352, CD051279, CD051285, CD051305, CD051278, CD051342, CD051357, CD051343, CF340748, CD051322, CD051324, CD051317, CD051274, CD051264, CD051312, CD051261, CD051323, CD347670, CD051268, CD051271, CD051272, CD051277, CD051290, CD051297, CD051339, CD051320, CD051326, CD051288, CD051295, CD051286, CD051292, CD051346, CD051338, CD051333, CD051300, CD051284, CD051267, CD051351, CD051287, CD051308, CD051355, CD051360, CD051361, CD051330, CD051282, CF074502, CD051293, CD051336, CD051341, CD051262, CD051273, CD051299, CD051315, CD051353, CD051270, CD051289, CD051291, CD051296, CD051321, CD051335, CD051276, CD051281, CD051307, CD051283, CD051309, CD051327, CD051325, CD051280, CD051269, CD051275, CD051298, CD051306,

CD051313, CD051314, CD051316, CD051332, CD051334, CD051337, CD051344, CD051348, CD051349, CD051354, CD051340, CD051331, CD051294, CD051302, CD051263, CD038847, and CD051328.

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