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BIOTIC AND ABIOTIC STRESS

Gene expression changes in response to drought stress in *Citrullus colocynthis*

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Abstract *Citrullus colocynthis* (L.) Schrad, closely related to watermelon, is a member of the Cucurbitaceae family. This plant is a drought-tolerant species with a deep root system, widely distributed in the Sahara-Arabian deserts in Africa and the Mediterranean region. cDNA amplified fragment length polymorphism (cDNA-AFLP) was used to study differential gene expression in roots of seedlings in response to a 20% polyethylene glycol-(PEG8000) induced drought stress treatment. Eighteen genes which show similarity to known function genes were confirmed by quantitative relative (RQ) real-time RT-PCR to be differentially regulated. These genes are involved in various abiotic and biotic stress and developmental responses. Dynamic changes with tissue-specific pattern were detected between 0 and 48 h of PEG treatment. In general, the highest induction levels in roots occurred earlier than in shoots, because the highest expression was detected in roots following 4 and 12 h, in shoots following 12 and 48 h of drought. These drought-responsive genes were also affected by the plant hormones abscisic acid (ABA), salicylic acid (SA), or jasmonic acid (JA), indicating an extensive cross-talk between drought and plant hormones. Collectively, these results will be useful to explore the functions of these multiple signal-inducible genes for unveiling the relationship and crosstalk between different signaling pathways.

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Introduction

Drought is the major abiotic stress that has adverse effects on growth and productivity of crop plants. Plant cells have evolved to perceive different signals from their surroundings, to integrate them and respond by modulating appropriate gene expression. The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction (Bartels and Sunkar 2005; Zhu 2001). A combination of biochemical and physiological changes at the cellular and molecular levels, such as an increase in the plant stress hormone ABA, accumulation of various osmolytes and proteins coupled with an efficient antioxidant system are known to result in stress tolerance. These gene products are classified into two major groups (Shinozaki and Yamaguchi-Shinozaki 1997): (1) those that protect plant cells against stress, such as heat shock proteins, late embryogenesis abundant (Lea) proteins, osmoprotectants, antifreeze proteins, transporters, detoxification enzymes, and free-scavengers; (2) those that function in signaling cascades and transcriptional control, such as kinases, phospholipases, and transcriptional factors.

Plant hormones ABA, JA, and, SA have a broad effect on plant physiology, including developmental processes and stress responses (Wasternack 2007; Fujita et al. 2006). The complex regulatory and interaction network occurring between hormone-signaling pathways allows the plant to activate the responses to different types of stimuli (Bari and Jones 2009; McSteen and Zhao 2008). ABA is involved in

responses to many stresses. The ABA-signaling system has developed in complexity, because it enables plants to respond to and adapt to stresses efficiently (Hirayama and Shinozaki 2007). ABA may be required for overall plant resistance. Many ABA-inducible genes are induced after drought, high salinity and cold stress treatments (Seki et al. 2002). ABA affects JA biosynthesis, suggesting that ABA precedes JA in the activation of defenses against some pathogens (Adie et al. 2007). SA induces pathogenesis-related (PR) genes and activates local and systemic-acquired resistance in a wide variety of plant species (Ryals et al. 1996). The analysis of SA-deficient mutants showed that SA plays an important role in modulating redox balance and protects plants from oxidative stress (Yang et al. 2004). SA-treatment induces a sharp accumulation of ABA, which in turn is an inducer of various anti-stress reactions in plants (Sakhabutdinova et al. 2003). There is a negative interaction between SA and JA-signaling pathways. In wild type Arabidopsis plants, JA levels increased slightly in response to pathogen infection. However, in mutant plants impaired in SA signal transduction, JA accumulated to 25-fold higher levels, suggesting that in wild-type plants JA formation was suppressed by endogenously accumulating SA. Furthermore, pathogen-induced SA accumulation is associated with the suppression of JA-responsive gene expression (Spoel et al. 2003).

Although genetic variation for drought tolerance has been observed in crop plants, its molecular dissection has mainly been investigated in the model species *Arabidopsis* and is still not fully understood in many crop plants. Expression profiling has become an important tool to understand the plant's response to environmental changes. Currently, several techniques, such as differential display reverse transcription-polymerase chain reaction (DDRT-PCR), serial analysis of gene expression (SAGE), suppression subtractive hybridization (SSH), cDNA-AFLP and cDNA microarray are available for transcriptome analysis. Among these, cDNA-AFLP has been widely used to identify genes whose expression has been altered under different environmental conditions because of its efficiency, technical simplicity, and lack of requirement of previous genomic information of the species of interest (Baisakh et al. 2006; Rodríguez et al. 2006; Umezawa et al. 2002).

Citrullus colocynthis (L.) Schrad is closely related to domesticated watermelon *C. lanatus* (Thunb) Matsum & Nakai var. *lanatus* and wild watermelon *C. lanatus* var. *citroides*. The species, commonly known as bitter apple or bitter gourd, is a non-hardy drought-resistant herbaceous perennial vine. It has a rich history as an important medicinal plant and as a source of valuable oil and is widely distributed in the Sahara-Arabian region of northern Africa and the Mediterranean (Dane et al. 2006). It can survive arid environments by maintaining its water content without wilting of the leaves or desiccation under severe stress conditions. Drought tolerance studies of wild watermelon (Akashi et al. 2001; 2004; Yokota et al. 2002) indicated that under drought conditions in the presence of strong light, high concentrations of citrulline, glutamate, and arginine accumulate in watermelon leaves. The accumulation of citrulline and arginine might be related to the induction of DRIP-1 (drought-induced peptide), a homolog of acetylornithine deacetylase (ArgE) in *E. coli*. One of the isolated genes, *CLMT2*, shares significant homology with plant type-2 metallothionein (MT) sequences which has an extraordinarily potent activity for scavenging hydroxyl radicals. CLMT2 induction contributes to the survival of wild watermelon under severe drought/high light stress conditions.

Domesticated watermelons have been selected for their productivity and quality. Domestication of crop plants and plant breeding has dramatically eroded allelic variations of crop species, which has contributed to an increasing susceptibility of crop plants to environmental stresses, diseases, and pests (Tanksley and McCouch 1997). Wild germplasm has been used with great success in breeding for simply inherited resistance to diseases and insects. *C. colocynthis* has specific mechanisms to combat water deficits. If we can understand these mechanisms, we can improve the ability of domesticated watermelon to grow under stress conditions. In order to identify drought-responsive genes and to gain a better understanding of drought stress responses in this drought-tolerant cucurbit species, genome-wide investigation of drought-responsive genes was performed in *C. colocynthis* using cDNA-AFLP. Expression patterns of these drought-responsive genes under drought and hormone treatments show the promise for improvement of drought tolerance in plants.

Materials and methods

Plant material and treatments

C. colocynthis seeds (Accession 34256, from Israel) were shown in Turface (Profile Product LLC, Buffalo, IL) in the greenhouse with a 14-h photoperiod at temperatures ranging from about 22–30°C, and ambient relative humidity. A 1/2 strength Hoagland's nutrient solution (PhytoTechnology Laboratories, Shawnee Mission, KS) was used to daily irrigate plants after germination. Seedlings at 5- to 6-leaf stage (2–3 weeks old) were transplanted into 20% PEG 8000 solution for drought induction. Shoot and root samples were collected at 0, 4, 8, 12, 24, and 48 h and stored immediately at −80°C.

For plant hormone treatment, seedlings at 5- to 6-leaf stage were treated with 100 μM ABA, 1 mM SA, and

50 μM JA via spraying and irrigation. Shoots and roots were harvested 8 h following ABA treatment, 4 h following SA, or 4 h following JA treatment. Shoots and roots from untreated seedlings (0 h) were harvested as control.

Plant water status

Relative water content (RWC) was measured (in triplicate) during PEG treatment using 1 cm leaf discs. Leaf discs were placed immediately in pre-weighed vials, sealed and reweighed to derive their fresh weight (FW). They were rehydrated by floating for 4 h on distilled water to obtain their turgid weight (TW). Their dry weight (DW) was obtained after oven-drying at 85°C for 24 h. RWC was calculated according to the formula: $RWC = [(FW - DW)/(TW - DW) \times 100]$ (Smart and Bingham 1974).

RNA isolation and cDNA-AFLP

RNA was extracted from roots or shoots according to RiboPure kit protocol (Ambion, Austin, TX). To eliminate the remaining genomic DNA, RNA was treated with DNase I (Ambion) according to the manufacturer's instruction. The concentration of RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY). The quality of RNA was checked using 7% formaldehyde agarose gel electrophoresis.

RNAs from root at 0 h and 8 h PEG treatment were used in cDNA-AFLP analysis (Bachem et al. 1996). cDNA was synthesized using RETROscript™ (Ambion) according to the manufacturer's instructions, and digested using the *Mse*I/*Eco*RI enzyme combination. AFLP analysis was conducted according to the protocol of AFLP kit from Li-COR (Li-COR Biosciences, Lincoln, NE). Sequences of the adapters and primers used for cDNA-AFLP analysis were provided by Li-COR. The selective amplication products were run on 6% polyacrylamide sequencing gel containing urea at 80 W for 5 h. The cDNA bands were visualized by silver staining according to the Silver Sequence™ DNA Sequencing System Technical Manual (Promega, Madison, WI).

Cloning and sequence analysis of DNA fragments

Differentially expressed transcript-derived fragments (TDFs) were extracted from the gel, and used as template for re-amplification by PCR. TDFs were ligated directly into the pGEM-T Easy Vector (Promega, Madison, WI), and then transformed into competent *Escherichia coli* (Promega). Plasmids were isolated using Plasmid Mini Kit (Bio-Rad laboratories, Hercules, CA).

Fragments were sequenced with ABI 3100 DNA sequencer (AU Genomics Lab, Auburn, AL) using T7 and SP6 primers (Promega). Analysis of nucleotide sequence of fragments was carried out using NCBI BLAST search tool.

RQ real-time RT-PCR

RQ real-time RT-PCR was carried out using an ABI 7500 RealTime PCR System and 7500 System software version 1.2.3 (Applied Biosystems, Foster City, CA). The *C.colocynthis*-specific actin gene (GH626171), used as reference gene, was amplified in parallel with the target gene allowing gene expression normalization and providing quantification. Detection of real-time RT-PCR products was done using the SYBR® Green Universal Master mix kit (Applied Biosystems) following the manufacturer's recommendations. Five microliters of cDNA (equivalent of 25 ng total RNA) were used as template for PCR. PCR cycling conditions comprised an initial cycle at 50°C for 2 min, one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. For each sample, reactions were set up in triplicate to ensure the reproducibility of results. To distinguish specific product from nonspecific products and from primer dimers, a melting curve was generated immediately after amplication, Following a denaturation step at 95°C at starting temperature of 60°C and an end temperature of 95°C, with a temperature increase of 0.1°C/s. Ten microliters of each sample was run in 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

PCR efficiencies of target and reference genes were determined by generating standard curves. The method currently used to determine PCR efficiency is partly automated and based on serial dilutions prepared from cDNA templates. Subsequently, the C_T values were plotted against the log of the known starting concentration value and from the slope of the regression line (y). The amplification efficiency was estimated according to the equation: $E = [(10^{-1/y}) - 1] \times 100$.

Data analysis

The quantification of the relative transcript levels was performed using the comparative C_T (the threshold cycle) method (Livak and Schmittgen 2001). The transcript levels of the target genes were normalized against the β-actin gene transcript levels as described in the ABI PRISM 7500 Sequence Detection System user bulletin #2 (Applied Biosystems). The induction ratio (IR) was calculated as recommended by the manufacturer and corresponds to $2_T^{-\Delta\Delta C}$, where $\Delta\Delta C_T = (C_{T,\ \text{Target gene}} - C_{T,\ \text{actin}})_{\text{stressed}} - (C_{T,\ \text{Target}} - C_{T,\ \text{actin}})_{\text{control}}$. Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in target sample versus reference samples (Pfaffl 2001).

Results and discussion

The measurement of leaf relative water content

Leaf RWC of *C. colocynthis* was measured to define the induction of the drought condition following PEG treatment (Fig. 1). The leaf RWC in control plants was 90%. When drought stress started, leaf RWC decreased rapidly to 75% within 12 h and then gradually adjusted its leaf RWC to 82% and maintained a RWC of 80% during treatment. This indicates that *C. colocynthis* has some mechanism to cope with drought stress.

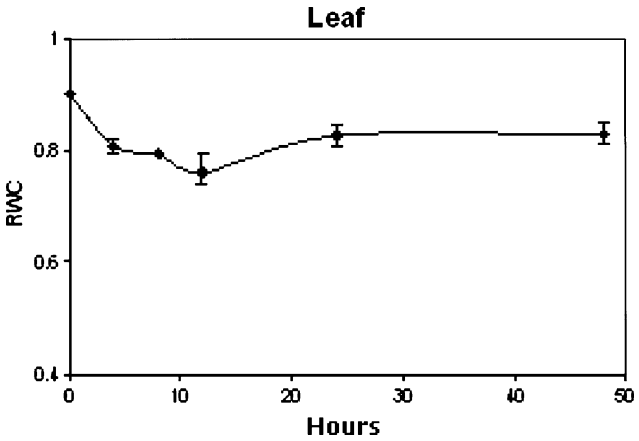

Fig. 1 Relative water content (%) of *C. colocynthis* leaves during PEG treatment

Identification of drought-related transcripts

The cDNA-AFLP was used to study the response of *C. colocynthis* to drought stress. A total of 32 different primer combinations were used, and over 84 putative TDFs were cloned and sequenced. Sequence comparison of the cloned TDFs using BLASTx revealed 40 fragments homologous to genes with known functions, whereas the rest of fragments showed homology to either unknown proteins or unclassified proteins. Eighteen clones which were confirmed as differentially expressed have been submitted to the NCBI database and are presented in Table 1 with GenBank Accession numbers. These drought responsive genes were selected for expressional analyses and can be classified into two groups (Table 1). The first group includes functional proteins, or proteins that function in stress tolerance such as heat shock protein (HSP) 70 (CC4), HSP22 (CC85), grpE like protein (CC47), pathogen related (PR)-protein (CC-61), synaptobrevin-related protein (CC48), translocon outer envelope of chloroplast (TOC)34-1 (CC24), ABC transporter (CC16), VIRE2-INTERACTING PROTEIN (VIP) 2 (CC38), anaphase promoting complex (APC) subunit 11 (CC26), respiratory burst oxidase homolog (RBOH) D (CC37), pyruvate kinase (CC32), beta-amylase (CC64), alpha7 proteasome subunit

Table 1 Homology of transcription-derived fragment (TDF) sequences isolated from root of *C. colocynthis* following 8 h of PEG treatment

TDFs	Accession number	Sequence homology	Organism	*E* value
Functional proteins				
CC4	FK707354	Heat shock protein 70	*Cucumis sativus*	9e−42
CC85	GH626170	Heat shock 22 kDa protein, mitochondrial	*Glycine max*	3e−08
CC47	GH626164	grpE like protein	*Arabidopsis*	6e−22
CC61	GH626166	Putative pathogenesis-related protein	*Cucumis sativus*	3e−43
CC26	GH626159	APC11 (anaphase promoting complex subunit 11)	*Arabidopsis*	0.009
CC36	GH626162	Putative alpha7 proteasome subunit	*Nicotiana tabacum*	2e−12
CC37	EU580727	RBOHD (respiratory burst oxidase)	*Arabidopsis*	3e−23
CC38	GH626163	VIP2 (VIRE2-INTERACTING PROTEIN2)	*Arabidopsis*	2e−13
CC16	FK707355	ABC transporter-like protein	*Arabidopsis*	1e−45
CC48	GH626165	Synaptobrevin-related protein	*Pyrus pyrifolia*	9e−27
CC24	GH626158	Toc34-1 (translocon outer envelop of chloroplast)	*Zea mays*	0.79
CC64	GH626167	Beta-amylase	*Prunus armeniaca*	4e−18
CC32	GH626161	Pyruvate kinase	*Arabidopsis*	1e−10
CC75	GH626168	TIP1 (TIP GROWTH DEFECTIVE 1)	*Arabidopsis*	1e−27
Regulatory proteins				
CC19	GH626156	Leucine-rich repeat transmembrane protein kinase	*Arabidopsis*	2e−11
CC23	GH626157	Protein kinase	*Fagus sylvatica*	5e−12
CC27	GH626160	Hairy meristem	Petunia x hybrida	1e−19
CC76	GH626169	NAC 2	*Glycine max*	8e−59

(CC36), and TIP GROWTH DEFECTIVE (TIP) 1 (CC75). The second group is involved in signaling cascades and in transcriptional control, such as protein kinases (CC23 and CC19), and transcriptional factors GRAS (CC27) and NAC (CC76). The differential expression of these TDFs under PEG treatment in roots and shoots was confirmed by RQ real-time RT-PCR (Fig. 2).

Characterization of drought responsive genes

Heat shock proteins and osmolytes function in protecting macromolecules and membranes from dehydration. TDFs CC4, CC85 and CC47, with significant homology to HSP70, HSP22, and grpE proteins, respectively (Diefenbach and Kindl 2000; Padidam et al. 1999), were induced during drought treatment. HSPs are shown to be involved in protecting macromolecules and membranes, and present extensive overlap between heat and non-heat stress response pathways (Sun et al. 2002; Swindell et al. 2007). Differences in the specificities of Hsp70s toward interacting cochaperones, such as GrpE, probably account for their functional diversity. GrpE proteins play a crucial role in the regulation of nucleotide exchange as cochaperones and therefore control the Hsp70 chaperone cycle (Groemping and Reinstein 2001). HSP22 induction by extreme temperature and oxidative stress in different species is well documented (Stupnikova et al. 2006; Banzet et al. 1998; Tanaka et al. 2000), and showed plant cell protection and adaptive mechanism. In this study, transcripts CC4 and CC47 were up-regulated to 8-fold during drought, while CC85 increased to over 100-fold (Fig. 2). This has also been observed in maize under heat stress (Lund et al. 1998). Maize HSP70 did not change during stress, but maize HSP22 increased dramatically during stress and decreased after the stress was relieved. This may imply that HSP70 and grpE are constitutively expressed for protein folding or they may be expressed in excess to afford protection during the stress event. In contrast, HSP22 may not be necessary for constitutive protein folding, and appear to be expressed only during stress (Lund et al. 1998). Transcript CC64 has high homology to a beta-amylase, known for its function in starch breakdown to produce maltose. β-amylase may act as a vegetative storage protein or a stress-related protein. β-amylase induction during heat shock at 40°C and cold shock at 5°C in *Arabidopsis* can lead to starch-dependent maltose

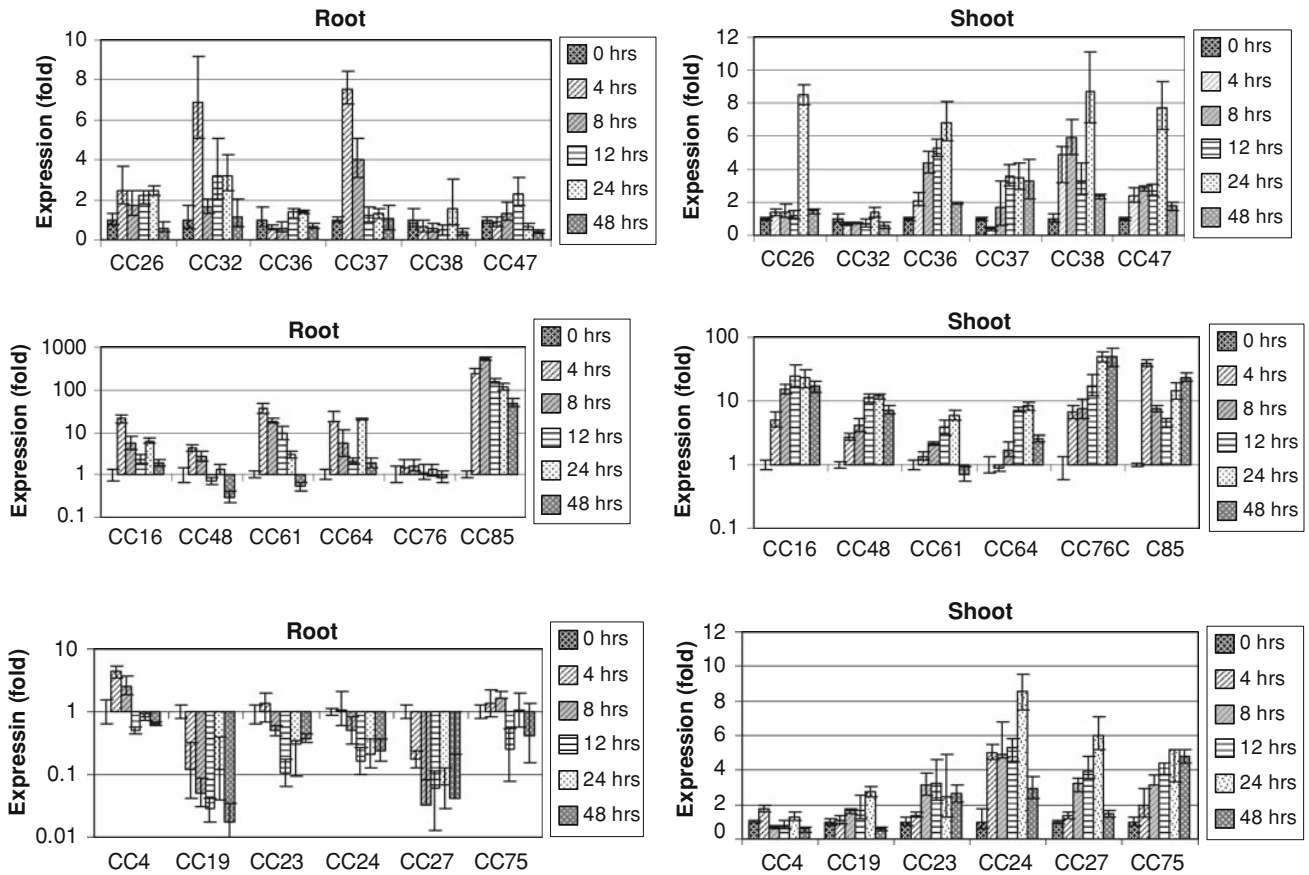

Fig. 2 The relative expression level of genes in shoot and root during 0, 4, 8, 12, 24, and 48 h of drought (PEG) treatment; gene expression was normalized by comparing ΔΔCt to control (0 h)

accumulation. Maltose has the ability to protect proteins, membranes, and the photosynthetic electron transport chain. Therefore, β-amylase induction and the resultant maltose accumulation may function as a compatible solute-stabilizing factor in the chloroplast stroma in response to acute temperature stress (Kaplan and Guy 2004; 2005). The CC64 clone was strongly induced both in root and shoot upon drought and hormone treatment. The results indicate that β-amylase is a stress-related protein that can function in osmotic protection (Fig. 2).

Drought led to the induction of ubiquitin-protein ligase and proteasome involved in protein fate in *C. colocynthis*. Transcript CC26, which corresponds to APC11 (anaphase promoting complex subunit 11), is homologous to a RING-H2 finger protein which plays a key role in the ubiquitylation reaction (Gmachl et al. 2000). The anaphase-promoting complex or cyclosome (APC/C) is a cell-cycle-regulated ubiquitin-protein ligase that mediates metaphase to anaphase transition and exit from mitosis (Capron et al. 2003a, b). Most Arabidopsis APC/C protein subunits are a single-copy gene in Arabidopsis (Capron et al. 2003a, b). In expression analysis of 11 putative *AtAPC* genes (Eloy et al. 2006), only two genes were highly expressed in organs (roots or flower bud) with active proliferation. In contrast, the other *AtAPC* genes including *AtAPC11* are preferentially expressed in organs (siliques or leaves) with low overall cell division. The results suggested that the arrangement of the complex executes distinct functions required for growth and environmental adaptation in specific tissues and/or cellular compartments (Capron et al. 2003a, b). This conclusion is supported by our study since CC26 did not change in root, but was upregulated over 8-fold in shoot after 24 h of drought treatment (Fig. 2). Transcript CC36 is homologous to putative alpha7 proteasome subunit (Dahan et al. 2001) in tobacco. Thirteen α (α1, α3, α4, α5, α6 and α7) and β (β1-*tcI* 7, β2, β3, β4, β5, β6 and β7) 20 S proteasome subunits were cloned in tobacco, in which only β1-tcI 7, α3, and α6 subunits encoding genes were up-regulated by cryptogein, a proteinaceous elicitor of plant defense reactions. The CC36, a putative α7 subunit, was induced in shoot after 8 h of drought condition. The results indicated that the activation of these subunits might induce a specific proteolysis involved in the defense reaction (Dahan et al. 2001). Proteolysis is expected to play an important part in such processes by replacing the protein set characteristic of the old phase with a set needed to establish new cellular identity. Currently, a number of developmental stages and environmental responses has been linked with or shown to depend on significant changes in proteasome abundance or activity (Kurepa and Smalle 2008).

Drought induction in *C. colocynthis* resulted in expression changes in transporter- and membrane-related proteins of various functions. Transcript CC16 showed similarity to ABC transporter (Sato et al. 1997). The hallmark of ABC transporters is their ability to derive energy from ATP hydrolysis to transport molecules through membranes. This family depends on the presence of one or two ATP-binding cassette (ABC) domains. More than 130 ABC transporter encoding genes have been found in Arabidopsis, and 128 in rice. Their functions in the excretion of potentially toxic compounds, in transport of peptides, secondary metabolites, heavy metals and ions, and in regulation of ion channels have been well reviewed (Schulz and Üner Kolukisaoglu 2006; Stacey et al. 2002; Yazaki 2006). Some ABC transporters have been associated with various host-pathogen interactions. The pathogen-responsive expression of *AtPRD12* gene was confirmed after inoculation with compatible and incompatible pathogens and exposure to the defense associated chemical signals, SA, ethylene, and methyl jasmonate (MeJA) (Campbell et al. 2003). The CC16 transcript was significantly up-regulated under drought conditions and during treatment with ABA, JA, and SA (Table 2), suggesting that this gene is a stress-response gene and responds to stress hormone signals. Transcript CC24, which shows homology to Toc34-1 (translocon outer envelop of chloroplast 34-1), which is a GTP-binding regulatory component of protein import machinery within the outer envelop of plastids (Hirohashi and Nakai 2000). Toc 34 forms a stable import complex with Toc 159. Two different Toc34 homologs which are AtToc33 and AtToc34 exist in Arabidopsis (Jarvis and Soll 2001). Two AtToc34 knockout mutants *ppi3-1* and *ppi3-2* were identified and characterized (Constan et al. 2004). Aerial tissues of the ppi3 mutants appeared similar to the wild type throughout development and contained structurally normal chloroplasts. While in the roots, significant growth defects were observed in both mutants, indicating that the AtToc34 is relatively more important for plastid biogenesis in roots. The failure to develop a double homozygote lacking AtToc34 and AtToc33 by crossing the ppi3 mutants with ppi1, an atToc33 knockout mutant, indicated that the function provided by AtToc33/AtToc34 is essential during early development. Transcript CC48 shows homology to the synaptobrevin-related protein, a small integral membrane protein, which belongs to the superfamily SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors). Besides SNAREs interaction to draw vesicle and target membrane surfaces together for fusion of the bilayers, some are involved in stomatal movements, gravity sensing, pathogen resistance and signal transduction and response (Pratelli et al. 2004). A tobacco syntaxin (Nt-Syr1) is a key element in the ABA-signaling cascade which mediates the ABA control of guard cell ion channel activity (Leyman et al. 1999). The mutant *osml* (Atsyp61) was isolated from

Arabidopsis. It was found that OSM1/SYP61 functioned in stomatal movement, the development, and growth sensitivity of roots to osmotic stress (Zhu et al. 2002). Expression of Nt-Syr1 in leaves was promoted by ABA, salt, drought, and wounding, but not by cold, auxin, kinetin, or gibberellic acid. In contrast, Nt-Syr1 levels in the root were unaffected by ABA (Leyman et al. 1999, 2000). The rice OsNPSNs transcript was significantly activated by the treatment of H_2O_2, but down-regulated under NaCl and PEG6000 treatment (Bao et al. 2008). The CC48 transcript was highly induced in shoot following drought. These results indicated that some SNAREs may be involved in stress-related signaling pathways. Transcript CC75 shows homology to TIP1 (TIP GROWTH DEFECTIVE 1). An Arabidopsis mutant (*tip1*) displayed defects in both root-hair and pollen-tube growth (Schiefelbein et al. 1993).The predicted TIP1 protein contains an N-terminal region with six ankyrin repeats, four transmembrane domains, and a DHHC Cys-rich domain (DHHC-CRD). The TIP1 regulates root hair growth by acting as an S-acyl transferase, and overexpression of TIP1 in Arabidopsis led to longer root hairs (Hemsley et al. 2005).

Other stress-related genes with different functions were induced following drought. Transcript CC37 shows homology to RBOHD (respiratory burst oxidase D). In *Arabidopsis*, ten *Atrboh* genes (A-J) have been isolated (Sagi and Fluhr 2006). The *Atrbohd* and A*trbohf* mutants largely eliminate reactive oxygen intermediate (ROI) accumulation during disease-resistance reactions of *Arabidopsis* to avirulent *Pseudomonas syringae* and *Peronospora parasitica*. Hence, *Atrboh*D and *Atrboh*F are responsible for ROI accumulation during defense responses in *Arabidopsis* (Torres et al. 2002; Simon-Plas et al. 2002). ROS act as secondary messengers, which trigger diverse stress tolerance responses, and may cross-talk with plant hormones, such as ABA and ethylene, to regulate plant physiological response such as stomatal closure (Desikan et al. 2006). The CC37 transcript levels were induced in both root and shoot following drought treatment which might result in the increase of ROS production and then regulation of gene expression in plant stress responses. Also, plants are equipped with some mechanism to balance ROS production. NtrbohD in tobacco was negatively regulated by defense induced (din) subunit of proteasomes. The defense-induced proteasomes might serve to restrict the ROS burst, to limit cell death, and help retain responsiveness to pathogen invasion (Lequeu et al. 2005). Transcript CC38 is a homolog of *Arabidopsis thaliana* VIRE2-INTERACTING PROTEIN2 (VIP2) with a NOT2/NOT3/NOT5 domain that is conserved in both plants and animals. The transcriptome analysis of wild-type Arabidopsis and mutant *Atvip2* identified 4241 differentially expressed genes across different functional groups. 2,157 genes had more transcript abundance in *Atvip2* compared

Table 2 Differential gene expression of TDFs isolated from *C. colocynthis* in response to different treatments (ABA, SA, JA, and PEG)

TDFs	Sequence homology	Root				Shoot			
		ABA	SA	JA	PEG	ABA	SA	JA	PEG
CC4	HSP70	N	N	N	++	N	++	++	N
CC85	HSP22	++	+++	++	+++	++	++	++	++
CC47	grpE	−−	−	−	+	+	+	++	+
CC61	PR-related	++	+++	+++	+++	++	+++	+++	+++
CC26	APC11	+	N	N	+	+++	+	++	++
CC36	α7proteasome subunit	N	N	N	+	++	+	+	++
CC37	RBOHD	N	+	N	+	+	++	N	++
CC38	NOT2/NOT3/NOT5	N	+	N	N	++	+	+	+
CC16	ABC-transporter	+	++	++	++	+++	+++	++	+++
CC48	Synaptobrevin	N	N	N	+	+	++	++	++
CC24	Toc34-1	N	N	N	N	++	+	+	+
CC64	β-amylase	+	+	+	++	+	+	N	+
CC32	Pyruvate kinase	N	N	N	++	+	+	+	N
CC75	TIP1	N	N	−	+	+++	++	+	++
CC19	Leucine-rich protein kinase	−	−−	−−	−−	+	N	+	+
CC23	Protein kinase	−	N	−	N	++	N	+	+
CC27	Hairy meristem	−−−	−−	N	−	++	++	+++	++
CC76	NAC2	+	+	+	N	+++	+++	+++	+++

'N' no change, '+' to '+++' strong up-regulation, '−' weak to '−−−' strong down-regulation

with Col-0, whereas 2,084 genes had more transcript abundance in Col-0 compared with *Atvip2*. These data support the hypothesis that VIP2 plays a direct or indirect role in transcription regulation of many genes (Anand et al. 2007). The NOT proteins are an integral component of the CCR4 (carbon catabolite repression) transcriptional complex, where the complex serves as a regulatory platform to control several cellular machines (Collart 2003). In yeast, this platform senses nutrient levels, stress and possibly other signals to coordinately regulate these machines. It seems evident that such a regulatory platform plays an essential role to maintain cells in the appropriate state, and be able to alter this state as efficiently as possible in response to extracellular signals. Transcript CC32 showed high similarity to pyruvate kinase (PK). PK is an important regulatory enzyme of the glycolytic pathway that catalyzes the essentially irreversible transfer of Pi from phosphoenolpyruvate to ADP through binding of the substrate, phosphoenolpyruvate (PEP), and one or more allosteric effectors, yielding pyruvate and ATP. PK has been reported to have a role in the plant defense signal transduction pathway (Kim et al. 2006). The expression of *Capsicum annuum cytosolic pyruvate kinase 1* (*CaPKc1*) gene was induced in hot pepper plants during the incompatible interaction of the plants and viral pathogen, *Tobacco mosaic virus* (TMV). *CaPKc1* gene was also triggered not only by various hormones such as SA, ethylene, and MeJA, but also NaCl and wounding. The CC32 was up-regulated in roots during drought, which also indicated that PK plays a role in abiotic stress in plant. Transcript CC61 corresponds to a putative pathogenesis-related protein from cucumber, which is strongly induced in benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) treated and *Colletotrichum lagenarium* inoculated leaves (Bovie et al. 2004). This protein contains a SnoaL-like polyketide cyclase region. Besides its function in biotic stress, this protein is also involved in abiotic stress, since CC61 was highly induced over 50-fold in root under drought (Fig. 2).

The second group of drought-responsive genes encodes regulatory proteins that are protein kinases and transcriptional factors involved in further regulation of signal transduction and gene expression in the stress response. Transcript CC23 has high homology to a protein kinase in *Fagus sylvatica*. FsPK1 accumulation increases after ABA treatment when seeds are unable to germinate and disappears when seeds are able to germinate after the addition of gibberellin (GA) or upon stratification. The location of FsPK1 is in the vascular cells of the apical meristem of the embryonic axis, the region of cell proliferation for root growth. Therefore, these results suggested that FsPK1 controls embryo growth mediated by ABA and GA during the transition from dormancy to germination in *F. sylvatica* seeds, probably by interfering with the phloem function, which is critical to feed the growing cells near the root apex (Reyes et al. 2006).

Two transcriptional factors were differently expressed during drought treatment. Transcript CC27 shows high homology to *HAIRY MERISTEM (HAM)* in *Petunia* (Stuurman et al. 2002). *HAM* encodes a putative transcription factor of the GRAS (*G*AT, *R*GA and *S*CR) family. This gene family has diverse functions in plant growth and development such as gibberellin signal transduction, root radial patterning, axillary meristem formation, phytochrome A signal transduction, and gametogenesis (Bolle 2004), and also in the plant stress response (Czikkel and Maxwell 2006). Comparative sequence analysis showed that HAM falls into a group with the putative Arabidopsis proteins AtSCL6 (SCARECROW-like) and AtSCL15 (Stuurman et al. 2002). HAM might signal cell fate decisions in the shoot apex, promoting the undifferentiated state as a distinct cellular identity (Stuurman et al. 2002). Transcript CC76 shows high homology to GmNAC2 (*N*AM, *A*TAF and *C*UC2) from *Glycine max* (Meng et al. 2007). Six *NAC*-like genes were characterized in soybean, which showed different expression patterns, especially during seed development. *GmNAC2* fell into the ATAF subgroup which shares a conserved role in response to stress stimuli (Hegedus et al. 2003), such as wounding, cold, dehydration and pathogen attack. Totally, 105 predicted NAC proteins are present in *A. thaliana* (Ooka et al. 2003) and have been implicated in various aspects of plant development. Microarray analysis of transgenic plants overexpressing ANAC019, ANAC055, or ANAC072 in Arabidopsis, and OsNAC6 in rice (Tran et al. 2004; Nakashima et al. 2007) revealed that many genes inducible by abiotic and biotic stresses were upregulated. CC76 was highly induced in shoot under drought and following ABA, JA, and SA treatment. Collectively, these results indicate that NACs function as transcriptional activators in response to abiotic and biotic stresses in plants.

Tissue specific expression of cDNA fragments over time under PEG treatment

Tissue specific expression patterns over time (0, 4, 8, 12, 24, and 48 h) were investigated using RQ real-time RT-PCR, one of the most sensitive and reliably quantitative methods for gene expression analysis. Quantitative real time RT-PCR is well suited to quantify transcript levels in plant organs, and to perform expression profiling in response to environmental stimuli (Cantero et al. 2006). Although roots and shoots contain different sets of specialized cells, it is not known to what extent the stress response programs differ between these tissues. *Arabidopsis* roots and leaves have different transcriptome responses to cold, salt, and osmotic

stress (Kreps et al. 2002). Tissue-dependent variations in the expression pattern of transcripts under different abiotic stress between roots and shoots have been documented in many other species (Baisakh et al. 2006; Kawasaki et al. 2001; Liu and Baird 2003).

This study showed that CC4, CC32, CC61, and CC37 with homology to HSP70, PK, PR-protein, and RBOHD, respectively, were significantly up-regulated in roots, but induced at low level or without change in shoots. Other TDFs such as CC26, CC36, CC38 CC47, CC48 CC75, and CC76 with homology to APC11, α7 proteasome subunit, VIP2, greE, synaptobrevin, TIP1, and NAC 2, respectively were up-regulated in shoots, but not in roots. CC19, CC23, CC24, and CC27 encoding protein kinases, Toc 34-1 and GRAS, respectively, were suppressed in roots, but induced or without change in shoots under drought. CC16, CC64, and CC85 encoding ABC-transporter, β-amylase and HSP22, respectively, were highly induced in both root and shoot under drought. Dynamic changes in TDFs occurred following 4, 8, 12, 24, and 48 h drought stress time points. The highest expression levels of most transcripts were found at 4 and 12 h in roots, whereas at 8 and 24 h in shoots (Fig. 3). This may imply that roots sense soil water content 4 h earlier than shoots, and some signals transport from roots to shoots, such as ABA which is known to act as the primary root-to-shoot messenger (Jiang and Hartung 2008). Also evidence indicates that water stressed roots accumulate ABA more quickly and with greater sensitivity than leaves (Zhang and Davis 1989). Kreps et al. (2002) also mentioned that only roots (and not leaves) were in direct contact with salt and mannitol treatments. Regulation of the temporal and spatial expression patterns is an important part of the plant stress response.

Gene expression pattern during plant hormone treatments

The plant hormones ABA, JA, and SA are major endogenous low molecular weight signal molecules involved in regulating defense responses in plants. ABA regulates interacting signaling pathways involved in plant responses to several abiotic stresses, such as drought, salt, cold, as well as plant growth and development (Seki et al. 2002). Genetic analysis of Arabidopsis mutants compromised in ABA biosynthesis or signaling has identified a complex interplay between ABA and various other phytohormone signaling pathways (Anderson et al. 2004). SA induces the production of pathogenesis-related proteins and activates local and systemic acquired resistance (SAR) in a wide variety of plant species. SA is involved in plant defense responses as well as flowering and thermogenesis. SA plays an important role to modulate redox balance and protects rice plant from oxidative stress caused by aging as well as biotic and abiotic stress (Yang et al. 2004). JA is a lipid-derived signaling molecule with functions in plant responses to abiotic and biotic stress, as well as in plant growth and development. JA and its various metabolites alter gene expression positively or negatively in regulatory networks with synergistic and antagonistic effects in relation to other plant hormones such as SA, auxin, ethylene and ABA (Wasternack 2007).

ABA, SA, and JA were exogenously applied to *C. colocynthis* seedlings, and the expression pattern of 18 genes was examined by RQ real-time RT-PCR. The results showed that a complex interplay between ABA, JA, and SA-signaling pathways regulates plant gene expression during adaptive responses to abiotic stress (Table 2).

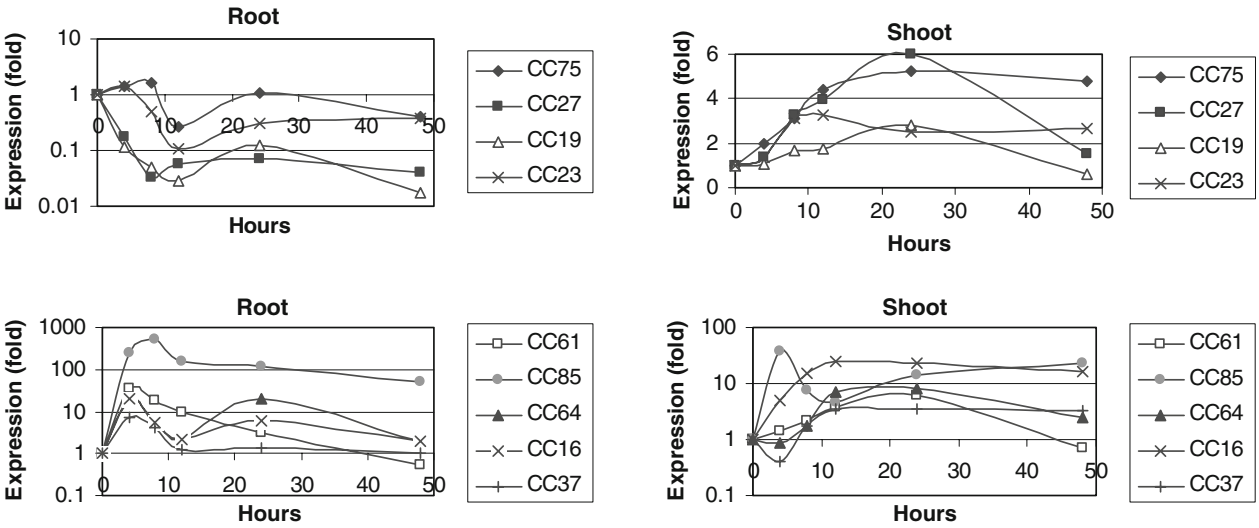

Fig. 3 Comparison of expression profiles of some genes in root and shoot during drought (PEG) treatments. Gene expression was normalized by comparing ΔΔCt to control (0 h)

Almost all drought responsive genes were affected by ABA treatment, supporting that many drought-regulated genes are ABA-responsive genes (Huang et al. 2007, 2008) and there is more crosstalk between drought and ABA responses (Seki et al. 2002). Most of these drought-responsive genes are regulated by ABA in the same direction. They are commonly up- or down-regulated by ABA and drought. This suggests that ABA plays a pivotal role in drought stress responses (Huang et al. 2008). Only one gene CC4 encoding HSP70 was not affected by ABA, suggesting an ABA-independent signaling pathway. Several genes were responsive to more than two hormones also in the same direction as the drought response. It suggested that SA and JA affect or modulate drought responses, and these genes might be the nodes in a regulatory network (Huang et al. 2008). A great number of drought-responsive genes were also regulated by ABA, JA, indole-3-acetic acid (IAA), brassinolide (BL), ethylene, zeatin (CK), and GA in Arabidopsis (Nemhauser et al. 2006; Huang et al. 2008). However, GA, ethylene or auxin may modulate drought stress signaling by acting antagonistically to ABA. The interaction between hormone signaling pathways allows for the physiological and developmental control during the modulation of stress response. A coordinated range of hormones is necessary to achieve the proper response in plant environment interaction (Robert-Seilaniantz et al. 2007). However, most drought-responsive genes were not affected by ABA, SA, or JA in roots of *C. colocynthis* seedlings in this study, which may be a result of cell or tissue specificity. The other explanation is that significant changes of these genes in roots may take place outside the 4–8 h window of the treatment.

Conclusions

In this study, drought-inducible genes were identified for the first time in *C. colocynthis*, and gene expression in roots and shoots during drought stress and different hormone treatments was examined. Most genes encoding proteins are involved in various functions in response to abiotic and biotic stress. This is the first report related to the induction of two genes, PR-related protein (CC61) and protein kinase (CC23), in response to drought stress. Regulating the temporal and spatial expression patterns of specific stress genes is an important part of the plant stress response (Rodríguez et al. 2006; Singh et al. 2002). Tissue-dependent variations in expression pattern of transcripts under different treatments between roots and shoots were observed in *C. colocynthis*. Dynamic changes in genes occurred following 4, 8, 12, 24, and 48 h drought stress time points, indicating roots respond to drought earlier than shoots, and some signals might transport from root to shoot to regulate gene expression in shoot. The drought-responsive genes were regulated by plant hormones. ABA plays a predominant role in gene expression responses to drought. Other plant hormones SA and JA also regulate drought responsive gene expression. Overall, the present study indicates that *C. colocynthis* undergoes a complex adaptive process during drought stress. The overlapping transcriptional regulation between abiotic, biotic, and hormone signaling pathways is important for fine-tuning responses in plants to environmental factors.

The function of most of these genes remains unknown. It is important to investigate the function of the drought-inducible genes not only for further understanding of the molecular mechanisms of stress tolerance and response of the plant, but also for improving drought stress tolerance of crops by gene manipulation. Techniques such as overexpression or silencing of some signaling components may confirm their role in particular pathways. Therefore, we will use the full-length cDNA for further characterization of the encoded proteins by transgenic and biochemical analysis. Furthermore, research is needed to understand the role of each hormone in the stress-response pathway and to elucidate their complex interactions.

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