STUDIES ON DROUGHT-RESPONSIVE GENES

IN *CITRULLUS COLOCYNTHIS*

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STUDIES ON DROUGHT-RESPONSIVE GENES IN *CITRULLUS COLOCYNTHIS*

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STUDIES ON DROUGHT-RESPONSIVE GENES

IN *CITRULLUS COLOCYNTHIS*

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VITA

Ying Si, daughter of Peixin Si and Junling Zhang, was born on November 16, 1973, in Wuhan city, Hubei Province, People's Republic of China. She graduated from Hubei College of Traditional Chinese Medicine in Pharmacy in 1995. She worked as research assistant in Hubei Institute of Traditional Chinese Medicine for 3 years, and entered the Graduate School of Wuhan Institute of Botany, Chinese Academy of Sciences. She earned a Master of Science degree in Botany in July 2001. From 2001-2004, she worked as a research assistant professor in Wuhan Institute of Botany, Chinese Academy of Sciences. In August 2004, she enrolled in Auburn University to pursue a Doctor of Philosophy Degree in the Department of Horticulture. She married Ping Zhou in December 2002. They have a daughter, Siyu (Ariel) Zhou.

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IN *CITRULLUS COLOCYNTHIS*

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Citrullus colocynthis (L.) Schrad, closely related to watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai.), 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 isolate drought responsive genes in roots of seedlings following a 20% polyethylene glycol (PEG8000) treatment to induce drought stress. Eighteen genes which show similarity to known function genes were confirmed by relative quantitative (RQ) real-time RT-PCR to be differentially regulated.

The expression of these genes was quantified in root and shoot tissues at five time points (4, 8, 12, 24, and 48 h, respectively) following PEG treatment. In general, the highest induction levels in roots occurred earlier than in shoots, since the highest expression levels were detected in roots following 4 h and 12 h, in shoots following

12 h and 48 h of drought. Some genes showed tissue specific expression patterns and were induced in shoots, but suppressed in roots. Seedlings were treated with salicylic acid (SA), jasmonic acid (JA) or abscisic acid (ABA) and analyzed using RQ real-time RT-PCR. A complex interplay between hormone signaling pathways regulate plant gene expression during adaptive responses to abiotic stress.

The full length *Ccrboh* gene, encoding respiratory burst oxidase protein, was cloned using RACE (Rapid Amplication cDNA Ends). Sequence comparisons showed that the Ccrboh protein contains a Ca^{2+}-binding motif of the EF-hand loop type at the N-terminal, and cytosolic FAD-and NADPH-binding domains at the C-terminal, characteristic of the RBO protein family. Southern blot analysis indicated that this gene exists as one or two copies in *C. colocynthis*. The subcellular location of Ccrboh was investigated by transient expression of Ccrboh::GFP fusion protein in protoplasts, and the result confirmed that Ccrboh is located at the plasma membrane.

RQ Real-time RT-PCR analysis showed that expression of *Ccrboh* was rapidly and strongly induced by abiotic stress imposed by PEG, ABA, SA, and JA treatment in *C. colocynthis*, but it did not change in domesticated watermelon (*C. lanatus* var. *lanatus* CLL) under these treatments. Grafting of *C. colocynthis* (CC) and CLL was conducted. *Ccrboh* gene expression in CLL scion with CC as a rootstock was induced, but induction levels were less as compared to non-grafted CC plants. *Ccrboh* gene levels did not change in CC scion grafted on CLL rootstock. Gene expression changes also occurred following vegetative growth and root growth. Our data suggest that *Ccrboh* plays a broader role during stress and in plant development, and may hold great promise for improving stress tolerance of other cucurbit species.

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I LITERATURE REVIEW

INTRODUCTION

Plants are sessile and thus have to endure abiotic and biotic challenges such as cold, drought, salinity, high temperature, wounding, and many different pathogens. Among these, drought is a major environmental factor that adversely affects plant growth and crop productivity in North America and worldwide (Boyer 1982). As water resources are likely to decline in the coming decades, crop production will be increasingly threatened by water availability. Plants have developed complex strategies to cope with water shortages, which vary in timing and severity from place to place, and season to season (Pennisi 2008). Adaptation mechanisms conserve water or optimize its acquisition to survive the adverse conditions, and drought avoidance is due to specific growth habits to avoid stress conditions. Stress-tolerant plants have evolved adaptive mechanisms to display different degrees of tolerance, which are largely determined by genetic plasticity. Thus, a better understanding and control of the mechanisms that enable a plant to adapt to drought and maintain processes involved in growth, development and production have been an aim of breeding for drought resistance.

In the field, crops and other plants are routinely subjected to a combination of different abiotic stresses. Plants have to cope with the interaction of other stresses such as salinity, heat, and low temperature that often arise concomitantly with drought, and ultimately involve oxidative stress because of excess production of reactive oxygen

species (ROS). ROS cause oxidative damage to membrane lipids, proteins and nucleic acids. Although drought, salt and cold stresses are clearly different from each other in their physical nature and each elicits a specific plant response, they also activate some common reactions in plants. All these stresses lead to cellular dehydration, which causes osmotic stress. Plant responses to these stresses involve nearly every aspect of plant physiology and metabolism. It is logical to assume that the simultaneous exposure of a plant to different abiotic stress conditions will result in the co-activation of different stress-response pathways. These might have a synergistic or antagonistic effect on each other. In addition, dedicated pathways specific for the particular stress combination might be activated (Mittler 2006; Zhu 2001; Simpson 1981).

DROUGHT STRESS PHYSIOLOGY

The physiologically relevant integrators of drought effects are the water content and the water potential of plant tissues. They in turn depend on the relative fluxes of water through the plant within the soil-plant-atmosphere continuum. Therefore, apart from the resistances and water storage capacities of the plant, it is the gradient of water vapor pressure from leaf to air, and soil water content and potential that impose conditions of drought on the plant (Cattivelli et al. 2008). Once water potential changes, responses of a wide range of physiological processes are induced. Some of these responses are directly triggered by the changing water status of the tissues while others are brought about by plant hormones that are signaling changes in water status (Verslues and Zhu 2005; Schachtman and Goodger 2008).

Generalized response to water stress

A generalized progression of events can be suggested for the impact of water limitation on most plants (Nilsen and Orcutt 1996). During the initial stages of water limitation, turgor pressure decreases, causing a reduction in cell expansion and a change in the distribution of growth regulators. Stomata close in response to either a decline in leaf turgor and /or water potential or to a low-humidity atmosphere. More evidence shows that stomatal responses are often more closely linked to soil moisture content than to leaf water status. The decreased tissue turgor potential along with an increase in leaf free abscisic acid (ABA) causes stomatal constriction. The stomatal constriction reduces the flow of water through the system and decreases intercellular carbon dioxide (*Ci*) resulting from downregulation of photosynthesis (Chaves et al. 2002). The lower *Ci* can stimulate a reopening of stomata if water availability does not decrease rapidly. However, if turgor continues to decline, stomata will continue to close and photorespiration will increase. Decreased carbon flow into the leaf (large decreases in the rates of photosynthesis) will cause a mobilization of starch and potentially an increase in respiration. Nonstomatal reductions to photosynthesis occur, which limits the *Ci* depletion. Following the adjustment of cell expansion, stomatal aperture, and photosynthesis, further reductions in water potential cause impacts on cytoplasmic physiology such as photoinhibition. Protein synthesis decreases and nonprotein amino acids increase. Only under extreme water limitation will ultrastructural abnormalities occur.

Removal of water from the membrane disrupts the normal lipid bilayer structure and results in the membrane becoming exceptionally porous when desiccated. Stress within

the lipid bilayer may also result in displacement of membrane proteins and this contributes to loss of membrane integrity, selectivity, disruption of cellular compartmentalization and a loss of activity of enzymes, which are primarily membrane based. In addition to membrane damage, cytosolic and organelle proteins may exhibit reduced activity or may even undergo complete denaturation when dehydrated. The high concentration of cellular electrolytes due to the dehydration of protoplasm may also cause disruption of cellular metabolism (Mahajan and Tuteja 2005).

Roles of roots in drought

The numerous functions of roots (Wilkinson 2000), related to water and nutrient uptake, synthesis and translocation of hormones, and respiration processes are sensitive to drought. Roots are critical for plant survival in dry environments. Because of their direct contact with drying soil, roots may mediate drought resistance through various major physiological processes. For example, water uptake is one of the primary functions of roots, which facilitates maintenance of the plant's internal water status. Roots synthesize hormones such as ABA and cytokinins (Schachtman and Goodger 2008), which may act as a chemical messenger relaying stress signals from roots to shoots. Reduction in water loss through the transpirational process can be accomplished by stomatal closure, which is at least partially controlled by chemical signaling sensed by roots in the drying soil. Root hair development and osmotic adjustment may help reduce root dessication and facilitate water uptake and survival of roots in drying soils. Root length density and hydraulic conductivity are related closely to water uptake capacity

when soil moisture is available. A promotion of root growth is then observed in several species (Chaves et al. 2002).

ABA in roots has been found to be the chemical messenger that mediates plant responses to drought. Stomatal closure can be induced by increases in leaf epidermal ABA content. ABA originates in the roots in drying soil. Roots have the capacity to synthesize ABA, although ABA is also produced in shoots. Water-stressed roots accumulate ABA more quickly and with greater sensitivity than leaves. Various studies (Schachtman and Goodger 2008; Jiang and Hartung 2008) have shown that soil drying stimulates a substantial accumulation of ABA in roots, and that ABA synthesized in root tips in response to soil drying can move through the transpiration stream to the leaves, where it induces stomatal closure. Roots seem to be able to "measure" the degree of soil drying and send a chemical message to the leaves where stomatal conductance and transpiration are reduced. Roots act as a primary sensor of soil water content and ABA acts as the primary root-to-shoot messenger. Besides ABA, pH, cytokinins, a precursor of ethylene and microRNA have been implicated as signal molecules from root to shoot under drought (Schachtman and Goodger 2008).

SIGNAL PERCEPTION

The most common model of sensing external stimuli is that of a chemical ligand binding to a specific receptor. However, no plant molecule has truly been identified as osmosensor. Therefore, other factors, such as changes in turgor, membrane strain, or molecular crowding are most probably the primary stimulus detected (Verslues and Zhu 2005). In yeast, hyperosmotic stress is sensed by two types of osmosensors, SLN1 and

SHO1, that feed finally into HOG (high-osmolarity glycerol) MAPK (mitogen activated protein kinase) pathway (Bartels and Sunkar 2005; Maeda et al. 1995; Tamás et al. 2000). The structures and roles of two-component system are well reviewed (West and Stock 2001; Bahn 2008). Two-component systems are structured around two conserved proteins: a histidine protein kinase (HK) and a response regulator protein (RR) that are phosphorylated at His (histidine) and Asp (aspartic acid) residues, respectively. Phosphotransfer from the HK to the RR results in activation of the RR and regeneration of the output response of the signaling pathway. SLN1 is a two-component histidine kinase, which senses cellular turgor pressure in prokaryotes. Cytokinin response 1 (Cre1) is a plant (Arabidopsis) hybrid histidine kinase, which can substitute the Sln1 osmosensing function, and its kinase activity is similarly regulated by turgor pressure (Reiser et al. 2003). Another plant HK, AtHK (Urao et al. 1999), was identified as SLN1 homologue. It functions as osmosensor in yeast and complements the yeast double mutant to transmit the signal to a downstream MAPK cascade.

The genome of Arabidopsis encodes about 25 “candidate” G protein-coupled receptors (GPCR)-plasma membrane-localized proteins with a seven-transmembrane topology, while the human genome encodes more than 800 GPCRs (Grill and Christmann 2007). Cell division, ion channel regulation, and disease response are processes regulated by G proteins in both plants and animals (Assmann 2005). GCR1 and GCR2 in Arabidopsis are good candidates for plant GPCRs because they physically interact with GPA1 (G protein) in planta (Pandey and Assmann 2004; Liu et al. 2007). Plants are sessile and incapable of escaping unfavorable environmental conditions such as drought and cold. They rely heavily on ABA to survive these conditions. Therefore, how plants

perceive and transduce the ABA signal is a fundamental question. The *gcr2* mutants show that GCR2 is a major ABA receptor. GCR2 binds ABA with a high affinity and reasonable dissociation constants, and the binding is stereospecific and abides by receptor kinetics. The binding of ABA to GCR2 disrupts GCR-GPA1 interaction (Liu et al. 2007).

MAP-KINASE CASCADES MEDIATE STRESS SIGNALING CROSSTALK

The MAP kinase pathways (reviewed by Zhang et al. 2006; Xiong and Zhu 2001) are intracellular signal modules that mediate signal transduction from upstream receptors to downstream targets in various ways. MAPK cascades consist of three kinase modules (MAPKKK, MAPKK and MAPK) that are activated sequentially by an upstream kinase. In yeast, the HOG1 MAPK pathway is activated upon perception of water loss (Maeda et al. 1995).

There are 20 MAPKs, 10 MAPKKs and 60 putative MAPKKKs in Arabidopsis, which implies crosstalk between various signal transduction pathways because of the imbalance in numbers (Zhang et al. 2006). In plants, it has been observed that drought stress leads to increased gene expression of all three components of a typical MAPK signaling cascade. Several MAPK cascade components are activated by more than one type of abiotic and biotic stress which suggests that MAPK cascades act as points of convergence in stress signaling (Bartels and Sunkar 2005). How a limited set of similar or even identical components is assembled in different ways in distinct cell types to control completely different biological responses is still a remaining question (Ray et al. 2004).

AtMPK3 in Arabidopsis was induced dramatically in response to cold, touch and salinity stress. *AtMPK4* and *AtMPK6* are enhanced by low temperature, low humidity, osmotic stress, touch and wounding. However, MPK4 and MPK6 are involved in distinct signal transduction pathways responding to these environmental stresses (Ichimura et al. 2000). The expression of rice OsMSRMK2 (Agrawal et al. 2002) mRNA was potently enhanced within 15 min by signaling molecules, protein phosphatase inhibitors, ultraviolet irradiation, fungal elicitor, heavy metals, high salt and sucrose, and drought. OsMSRMK2 expression was further modulated by co-application of jasmonic acid (JA), salicylic acid (SA), and ethylene and required de novo synthesized protein factor(s) in its transient regulation. OsEDR1 (Kim et al. 2003) showed a constitutive expression in seedling leaves and is further up-regulated within 15 min upon wounding by cutting, treatment with JA, SA, ethylene, ABA, and hydrogen peroxide. In addition, protein phosphatase inhibitors, fungal elicitor chitosan, drought, high salt and sugar, and heavy metals also dramatically induce its expression. A time course (30-120 min) experiment using a variety of elicitors and stresses revealed that the OsSIPK (Lee et al. 2008) mRNA is strongly induced by JA, SA, ethephon, ABA, cycloheximide (CHX), JA/SA + CHX, cantharidin, okadaic acid, hydrogen peroxide, chitosan, sodium chloride, and cold stress (12 ℃), but not by wounding via cutting, gaseous pollutants, ozone, and sulfur dioxide, high temperature, ultraviolet C irradiation, sucrose, and drought. A MAP kinase homolog from *Poncirus trifoliata* (Meng et al. 2008) was highly induced during cold treatment (4 ℃), but did not show much of an increase during cold acclimation.

For these stress activated MAPKs, it is vital to identify the input and output of the kinases and of the pathways. The input signal could be osmotic stress (e.g. turgor changes)

or derived from osmotic stress injury. The output could be osmolyte accumulation that helps establish osmotic homeostasis, stress damage protection, or repair mechanisms (e.g., induction of LEA/dehydrin-type stress genes) (Zhu 2002).

TRANSCRIPTIONAL REGULATION

Stress signaling primarily includes transcriptional regulation of gene expression and this depends on the interaction of transcription factors with *cis*-regulatory sequences. Phosphorylation of regulatory proteins is a major event in controlling the gene expression in eukaryotes. Therefore, multiple protein-protein and /or protein-DNA interactions frequently determine the rate of transcription by activation/repression of a promoter under given environmental conditions. Up to 1500 transcription factors are present in the *Arabidopsis* genome (Riechmann et al. 2000).

DREB1/CBF and DREB2 regulons

The dehydration-responsive element (DRE) binding protein 1 (DREB1)/ C-repeat binding factor (CBF) and DREB2 function in ABA-independent gene expression. DREBs contain APETALA2 (AP2)/ethylene-responsive element binding factor (ERF) motifs. Therefore, it belongs to ERF family of transcription factors. The AP2/ERF motif is specific to plants and functions as a DNA-binding domain. DREB1 and DREB2, first isolated from Arabidopsis, are involved in two separate signal pathways under cold and dehydration, respectively (Liu et al. 1998). Since then DREB genes with regard to different abiotic stresses were identified in various plants (reviewed by Agarwal et al. 2006). *AtDREB1A* was induced to express within 1 h after exposure to 4 ℃, while

AtDREB2A significantly accumulated 10 min after dehydration and high salt treatment (Liu et al. 1998). A DREB-like factor TINY gene from Arabidopsis was greatly activated by drought, cold, ethylene, and slightly by JA (Sun et al. 2008). Five DREB homologs were isolated from rice (Dubouzet et al. 2003). Expression of *OsDREB1A* was induced by cold and high-salt stress and transiently induced by wounding but not by exogenous ABA or drought. The OsDREB2A gene was induced by dehydration and high-salt stress but not ABA. Maize ZmDREB1A was induced by cold stress and slightly increased by high-salinity stress (Qin et al. 2004). Overexpression of several DREB genes in several plants such as Arabidopsis, rice and wheat showed tolerance to drought, high sanility, or cold stress, and also induced expression of some downstream genes such as *rd29A*, *cor15a*, and *rd17* that have GCCGAC as the DRE core motif in their promoter regions (Nakashima et al. 2009; Liu et al. 1998; Sun et al. 2008; Maruyama et al. 2004). All these studies can be summarized in that DREB transcription factors regulate abiotic stress-related genes and play a critical role in imparting stress endurance to plants. Despite the physiological similarity between the cold and dehydration stresses, it is interesting to note that DREBs can distinguish cold and drought signal transduction pathways (Agarwal et al. 2006).

AREB/ABF regulon

Many ABA-inducible genes respond to drought and high salinity in plants. There is more crosstalk between drought and ABA responses than between ABA and cold responses (Seki et al. 2002). Most ABA-inducible genes contain a conserved ABA-responsive element (ABRE) in their promoter region. ABRE binding factors

(ABFs)/ABA-responsive element binding (AREBs) proteins belong to a distinct subfamily of bZIP proteins. Although ABFs are ABA inducible and can bind to the same ABREs, each ABF might function in different ABA-dependent stress-signaling pathways. They are differentially regulated by various environmental stresses. Arabidopsis *ABF1-4* (Choi et al. 2000), all induced by ABA, showed different expression under abiotic stress. *ABF1* was induced only by cold; *ABF2* and *ABF3* were induced only by salinity. *ABF4* was induced by cold, drought and salinity stress. Overexpression of *ABF2* altered the expression of ABA/stress-regulated genes, and enhanced tolerance to drought, high salt, heat, and oxidative stress, indicating that ABF2 plays a role in the adaptation to abiotic stresses (Kim et al. 2004). *AREB1* and *AREB2* which are induced by drought, NaCl, and ABA in vegetative tissues require ABA for full activation, because their activities were repressed in the ABA-deficient mutant *aba2* and ABA-insensitive mutant *abi1* and enhanced in the ABA-hypersensitive *era1* mutant, probably due to ABA-dependent phosphorylation (Uno et al. 2000). Many abiotic stress-inducible genes contain two *cis*-acting elements, DRE and ABRE in their promoter region, such as *Arabidopsis rd29A* gene (Yamaguchi-Shinozaki and Shinozaki 1994). The DRE may function as a coupling element of ABRE in response to ABA, suggesting synergy between the DREB regulons and the ABRE regulons (Narusaka et al. 2003).

NAC regulon

NAC (NAM, ATAF, CUC2) proteins form a large family of plant-specific transcription factors. 105 predicted NAC genes and 75 predicted NAC genes are present in *Arabidopsis* and rice, respectively (Ooka et al. 2003). NAC family functions in diverse

processes, including developmental programs, defense and abiotic stresses. The complex regulation of NACs consists of microRNA-mediated cleavage of mRNAs and ubiquitin-dependent proteolysis (reviewed by Olsen et al. 2005). The *Arabidopsis ANACs* and rice *OsNAC6*, *SNAC2* genes are induced by cold, drought and high salinity, ABA, wounding and pathogens (Tran et al. 2004; Nakashima et al. 2007; Hu et al. 2008). Microarray analysis of transgenic plants overexpressing *ANAC019, ANAC055,* or *ANAC072* in Arabidopsis, and *OsNAC6, SNAC2* or *ONAC045* in rice (Tran et al. 2004; Nakashima et al. 2007; Zheng et al. 2009; Hu et al. 2008) revealed that several stress-inducible genes were upregulated, resulting in significantly increased stress tolerance to drought, cold or high salinity. These studies suggest that NAC proteins can be important for crosstalk between different pathways. In addition, NACs negatively regulate the stress responsive genes under stresses. *ATAF1* was strongly induced by dehydration and ABA and the knock-out mutant *ataf1* showed drought tolerance with the enhanced expression of stress responsive genes such as *COR47, ERD10, KIN1, RD22* and *RD29A* (Lu et al. 2007).

Transcription factors, including DREB/CBF, AREB/ ABF, NAC, can be used to improve stress tolerance to abiotic stresses in crops. An effective expression system such as suitable promoters will be required for genetic engineering of crops, because constitutive expression promoters are not always functional or can be negative for plant growth and development. Stress-specific promoters are needed for the generation of stress-tolerant crops.

SMALL RNAs IN ABIOTIC STRESS: POST-TRANSCRIPTIONAL REGULATION

Plant growth, development and stress responses depend on the precise regulation of gene expression. Although stress responsive reprogramming of gene expression largely occurs at the level of transcription, post-transcriptional gene expression also play a crucial role in gene expression regulation. The small RNAs, including microRNA (miRNA) and small interfering RNAs (siRNA), are ubiquitous mode of post-transcriptional regulation. These small RNAs are known to silence genes post-transcriptionally by guiding target mRNAs for degradation or by repressing translation. Stress can affect small RNA levels, and stress-responsive genes as small RNA targets have been identified. These provide clues about the role of small RNA in stress response (Sunkar et al. 2007; Shukla et al. 2008).

Arabidopsis miRNA417 was expressed constantly throughout all growth stages and organs. Its expression was regulated by dehydration, salt stress, or ABA. Overexpression of miRNA417 showed that seed germination was retarded in high salt or ABA treatment, implying a role as a negative regulator of seed germination under salt stress (Jung and Kang 2007). Rice miR169g and miR393 were up-regulated under drought stress (Zhao et al. 2007). miR169g promoter region contains two dehydration-responsive elements which is consistent with the drought responsiveness of this precursor. miR393 up-regulation could attenuate growth and development during stress condition, because an increase in miR393 decreases the TIR1 levels, a positive regulator of growth and development. miR393 was strongly upregulated by cold, dehydration, salt and ABA, but miR397b and miR402 were slightly upregulated by all the treatments. miR319 was only upregulated by cold, and miR389a was downregulated by all these stress treatments (Sunkar and Zhu

2004). miR159 was induced by ABA in Arabidopsis. Overexpression of miR159 and miR159-resistant MYB33 and MYB101 resulted in ABA hypersensitivity (Reyes et al. 2007). The predicted targets of miR159 are the MYB transcription factors MYB33, MYB65, MYB101 and MYB104 (Rhoades et al. 2002). Upregulation of miR159 by ABA, gibberellic acid (GA) and drought implies that miR159 may play an important role in hormonal and abiotic stress signaling networks (Reyes et al. 2007). Exposure of plants to abiotic stresses cause generation of excess reactive oxygen species (ROS) which cause oxidative damages. Superoxide dismutase (SOD) acts as superoxide detoxification. Abiotic stress-down-regulated miR398 targets two SOD genes *CSD1* and *CSD2* (Sunkar et al. 2006). Decrease in miR398 level under oxidative stress induced the level of *CSD1* and *CSD2*, and miR398 cleaves CSD1 and CSD2 mRNAs under normal conditions. Transgenic plants carrying miR398-resistant mutations in the CSD2 mRNA showed high tolerance improvement to various abiotic stress conditions.

Salt stress-induced SRO5 mRNA (a gene of unknown function) complements the P5CDH (pyrroline-5-carboxylate dehydrogenase) mRNA to produce a 24-nt nat-siRNA (natural antisense transcript-derived siRNA), and the 24-nt nat-siRNA guides the cleavage of the P5CDH transcript to further produce 21-nt nat-siRNAs. These nat-siRNAs all degrade P5CDH mRNAs to suppress proline degradation. Downregulation of P5CDH also results in P5C-mediated ROS accumulation, and SRO5 also mediates ROS detoxification. Therefore, the SRO5-P5CDH nat-siRNAs together with the P5CDH and SRO5 proteins control proline accumulation, ROS production and stress tolerance (Borsani et al. 2005).

An understanding of post-transcriptional gene regulation by small RNA under abiotic stress is important for understanding and improving stress tolerance in crops. miRNAs can serve as master regulators, because an altered miRNA in response to stress can silence more than one gene simultaneously. Identification of stress-regulated small RNA will help in the design of new strategies for improving stress tolerance (Shukla et al. 2008).

PROTECTIVE PROTEINS

Late embryogenesis-abundant (LEA) proteins are a diverse group of stress-protection proteins which are classified into six groups. Lea proteins comprise the vast majority of stress-responsive proteins. The expression profiles strongly supported a role for LEA proteins as protective molecules which enable the cells to survive protoplasmic water deficit (Ingram and Bartels 1996). Five wheat *lea* genes showed different accumulation patterns in response to drought, cold, salinity or ABA. *Td 29b, Td16, Td27e* genes were highly induced by drought while *Td25a* gene is a better candidate for cold or salt tolerance (Ali-Benali et al. 2005). Several *lea* genes or proteins, belonging to different groups, were induced during water-deficit stress in *Arabidopsis* (Bray 2002) and maize (Boudet et al. 2006), and played distinct roles in cells subjected to the stress. Group 2 LEA proteins or dehydrins are highly hydrophilic, glycine-rich and boiling-stable proteins which are the most frequently described so far (Close 1997; Rorat 2006). The dehydrins are a class of drought-induced proteins that lack a fixed three-dimensional structure. The dehydrin sequence is highly evolved and adapted to remain disordered under conditions of severe dehydration (Mouillon et al. 2008). Drought, cold, freezing

and ABA treatment resulted in accumulation of the thermostable dehydrins in plants (Borovskii et al. 2002). Transcriptional analysis of a sunflower (*Helianthus annuus* L.) dehydrin gene *HaDhn1a* in ABA-deficit mutants indicated the existence of ABA-dependent and ABA-independent pathways for *HaDhn1a* gene accumulation (Giordani et al. 1999). Lea proteins as protective molecules are able to increase stress tolerance as evidenced from overexpression studies in plants. Watermelon plants overexpressing the yeast salt-related gene HAL1 always performed better than non-transformed plants under salt-stress conditions (Ellul et al. 2003).

The heat shock proteins (HSPs) encompass many chaperones, which have an important role in the folding and assembly of proteins during synthesis, and in the removal and disposal of nonfunctional and degraded proteins (Bartels and Sunker 2005). HSPs and HSFs (transcription factors) are induced not only by high temperature but also by drought, cold or high salt stress, suggesting that *Hsp* genes represent an interaction point between multiple stress response pathways (Swindell et al. 2007). In a combination of heat and drought stress, the induction of HSP90, HSP70, HSP100, and small HSP was higher in drought and heat shock as compared to heat shock or drought (Rozhsky et al. 2002). This also implies that HSPs play a role in multiple stress reponses. Overexpression of AtHSP17.6A in Arabidopsis (Sun et al. 2001), NtHSP70 in tobacco (Cho and Hong 2006) and HSP17.7 in rice (Sato and Yokoya 2008) could increase salt and/or drought tolerance.

THE ROLE OF ROS

ROS are versatile molecules mediating a variety of cellular responses in plant cells, including programmed cell death (PCD), development, gravitropism, and hormone signaling (Kwak et al. 2006). ROS are superoxide, hydrogen peroxide, and hydroxyl radicals, which serve as secondary messengers in plant stress responses and in several hormone responses. Drought, salt, heat and oxidative stress are accompanied by the formation of ROS (Wang et al. 2003). Plants possess a sophisticated ROS network, comprising of antioxidative enzymes, antioxidants and ROS-producing enzymes, which allow them to keep ROS levels under tight control. MAPK cascades are major players in ROS signaling pathways including not only induction by ROS but also regulation of ROS production. MAPK pathways and ROS signaling play a key role in controlling normal development and dynamic processes such as flower development, stomatal patterning and stomatal aperture (Pitzschke and Hirt 2009; Bergmann et al. 2004).

ROS and respiratory burst oxidase homolog (RBOH)

The gp91phox homologs *AtrbohD* and *AtrbohF* from *Arabidopsis*, *NtrbohD* from *Nicotiana tabacum*, and *NbrbohA* and *NbrbohB* from *N. benthamiana* were shown to be required for ROS accumulation in plant defense responses (Sagi and Fluhr 2006). The *atrbohd* and *atrbohf* 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 some defense responses in *Arabidopsis* (Torres et al. 2002; Simon-Plas et al. 2002). Interestingly, the *atrbohd* and *atrbohf* double

mutants showed reduced cell death in response to a bacterial pathogen, but enhanced cell death in response to a fungal pathogen. These opposite responses may derive from interaction with SA. ROS produced by RBOHs antagonize SA and suppress cell death in cells that are more distantly located from the cells at the site of infection (Torres et al. 2005). The results indicate that ROS play dual roles in both driving and suppressing PCD in different contexts in the pathogen response.

ROS and ABA signaling

ABA signal transduction is located upstream and downstream of ROS production. ROS is synthesized in response to exogenous ABA, and ROS mediates, at least in part, ABA responses including stomatal closure and gene expression (Pei et al. 2000; Desikan et al. 2001). Analysis of stomatal movement showed that ABA-induced stomatal closure was partially impaired in *atrbohD/atrbohF* double mutant. Cellular events were impaired in the *atrbohD/atrbohF* double mutant guard cells, including ABA-induced ROS increases, ABA activation of I_{ca} channels, and ABA-induced cytosolic Ca^{2+} increase (Kwak et al. 2003). Exogenous application of ROS recovered I_{ca} channel activation and stomatal closure in the *atrbohD/atrbohF* double mutant guard cells. Furthermore, ABA enhances cellular ROS levels in *Arabidopsis* and *Vicia faba* guard cells (Pei et al. 2000; Zhang et al. 2001). ABA-induced ROS production and ABA activation of I_{ca} channels was impaired in the *abi1-1* protein phosphatase 2C mutant. The *abi2-1* mutant resulted in disruption of H_2O_2-induced stomatal closures (Murata et al. 2001).

ROS scavengers

ROS scavengers which detoxify the cytotoxic effects of ROS under various stress conditions include enzymes such as SOD, glutathione peroxidase (GPX) and ascorbate peroxidase (APX) as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins. Additional compounds such as osmolytes can also function as ROS scavengers (Wang et al. 2003). Large-scale transcriptome analysis of plants that were subjected to various abiotic stress and biotic stress revealed the induction of a large set of genes that encodes ROS-scavenging enzymes (Seki et al. 2002; Mittler et al. 2004). Scavenging enzymes have been utilized to engineer plants. Overexpression of ROS scavengers or mutants with higher ROS scavenging ability showed increased tolerance to environmental stresses (Bartels and Sunkar 2005). Overexpression of Mn-SOD reduced drought injury in transgenic alfalfa (Mckersie et al. 1996). In tobacco, overexpressing genes encoding GPX and glutathione *S*-transferase (GST) showed significant increases in growth following chilling or salt treatment (Roxas et al. 1997). Overexpression of cucumber ascorbate oxidase (AO) in tobacco increased the capacity to detoxify the reactive oxidative burden created by a variety of ROS-inducing agents (Fotopoulos et al. 2006). Accumulation of compatible solutes may also protect plants by scavenging of ROS, and by their chaperone-like activities in maintaining protein structures and functions. Wild watermelon (*Citrullus lanatus* sp.) in response to drought/high light stress conditions showed massive accumulation of a novel compatible solute, citrulline (Kawasaki et al. 2000), which was found to be one of the most potent hydroxyl radical scavengers (Akashi et al. 2001). In tomato and watermelon, temperature stress (heat and cold) could induce the accumulation of phenolics in the plant by

activating their biosynthesis as well as inhibiting their oxidation (Rivero et al. 2001). Engineered overproduction of these compatible solutes provides an opportunity to generate more tolerant plants (reviewed by Wang et al. 2003).

ROS present a significant point of convergence between abiotic and biotic stress pathways. Plants are equipped with tight regulation mechanisms to balance ROS production and scavenging. Dissecting the genetic network that regulates ROS signaling (for example, ROS generation, ROS targets, and the interaction between ROS producer and ROS scavenger) in response to stresses will be important for future study (Kwak et al. 2006; Fujita et al. 2006).

STRESS COMBINATION

Comparing the effects of different stresses is an important step toward understanding plant behavior under realistic field conditions where stresses rarely occur alone. In the field, crops and other plants are routinely subjected to a combination of different abiotic stresses. For example, in drought-stricken areas, many crops encounter a combination of drought and other stresses, such as heat or salinity (Mittler 2006).

Physiological characters are different between the acclimation responses of plants to different stresses. When they are combined, conflicting or antagonistic responses might occur. During heat stress, plants open stomata to cool their leaves by transpiration. However, if heat stress is combined with drought, plants would not be able to open the stomata and the leaf temperature would be high. If salinity or heavy metal stress is combined with heat stress, the increase in transpiration would cause the increase in

uptake of salt or heavy metals. Temperature stress combined with high light increases ROS production by the photosynthetic apparatus (reviewed by Mittler 2006).

A transcriptome analysis identified several hundred Arabidopsis transcripts that accumulated in Arabidopsis exposed to heat and drought simultaneously (Rizhsky et al. 2004). Fewer than 10% of the regulated genes in this dual-stress treatment overlapped with the genes of heat or drought, but certain drought- or heat-response-specific transcripts were elevated by the combined stress (Rizhsky et al. 2004). Similar changes in metabolite accumulation were also observed. Sucrose and other sugars such as maltose and glucose accumulated during a combination of drought and heat. Proline that accumulated in drought was strongly suppressed during a combination of drought and heat (Rizhsky et al. 2004). In sunflower, 105, 55 and 129 transcripts were significantly changed in response to high light, high temperature and a combination of high light and high temperature, respectively. A significant number of these transcripts were specific to each stress, and only 7 genes responded to all three treatments (Hewezi et al. 2008). This indicates that multiple stresses control largely separate gene networks that cannot be predicted from studying the individual stresses alone.

Tolerance to a combination of different stresses is a complex trait involving multiple pathways and cross-talk between different sensors and signal transduction pathways. Reproduction of natural conditions in experimental studies by incorporating multiple stresses will increase our understanding of the diversity of stress adaptation mechanisms and provide opportunities to further improve crops in natural conditions (Voesenek and Pierik 2008)

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II GENE EXPRESSION CHANGES IN RESPONSE TO DROUGHT STRESS IN *CITRULLUS COLOCYNTHIS*

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 relative quantitative (RQ) real-time RT-PCR to be differentially regulated. RQ real-time PCR was used to quantify the expression of these genes in root and shoot tissues at five time points (4, 8, 12, 24, and 48 h, respectively) following 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 h and 12 h, in shoots following 12 h and 48 h of drought. Some genes showed tissue specific expression patterns. Seedlings were treated with salicylic acid (SA), jasmonic acid (JA) or abscisic acid (ABA) and analyzed using real-time PCR. A complex interplay between hormone signaling pathways regulate plant gene expression during adaptive responses to abiotic stress.

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 level 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 (Rodríguez et al. 2006): (1) 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) function in signaling cascades and transcriptional control, such as kinases, phospholipases and transcriptional factors.

Plant hormones ABA, JA, 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 deeply 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 were

induced after drought, high salinity and cold stress treatments (Seki et al. 2002). ABA affects JA biosynthesis, suggesting that it 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 mutant showed that SA plays an important role to modulate redox balance and protect plants from oxidative stress (Yang er al. 2004). SA-treatment induces a sharp accumulation of ABA, which in turn is an inducer of various antistress reactions in plants (Sakhabutdinova et al. 2003). There is the 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 the 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 Saharo-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 high 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 homologue 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. Bitter apple 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. The objective of this study is to characterize the expression patterns of the drought induced genes and to identify potential pathways in response to drought stress.

MATERIALS AND METHODS

Plant material and treatments

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

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. Leaves and roots were harvested 8 h following ABA treatment, 4 h following SA, or 4 h following JA treatment. Leaves 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) * 100] (Smart and Bingham 1974).

RNA isolation and cDNA-AFLP

RNA was extracted from root or shoot 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% formadehyde agarose gel electrophoresis.

RNAs from root at 0 h and 8 h PEG treatment were used in cDNA-AFLP analysis. cDNA was synthesized using RETROscript™ (Ambion, Austin, TX) 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, 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

Differential 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) 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, USA). 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 15s 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, a start 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 were 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 $2^{-\Delta\Delta C}{}_T$, 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). When drought stress started, leaf RWC decreased dramatically (ca. 15%) until 12 hours and then gradually adjusted its leaf RWC to 82% and maintained a RWC of 80% during treatment. This indicates that *C. colocynthis* has mechanism to cope with drought stress.

Identification of drought-related transcripts

cDNA-AFLP was used to study the response of *C. colocynthis* to drought stress. A total of 32 different primer combinations were used. Over 100 putative differentially expressed DNA fragments from root were cloned and sequenced. 34 cDNA fragments show significant homology to known genes in the GenBank database using the blastx search utility on NCBI. 18 cDNA fragments were confirmed to be differentially expressed in drought treated plants (Table 1).

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 probably function in stress tolerance such as HSP70 (CC4), HSP22 (CC85), grpE like protein (CC47), PR-protein (CC61), synaptobrevin-related protein (CC48), TOC34-1 (CC24), ABC transporter (CC16), RBOHD (CC37), pyruvate kinase (CC32), beta-amylase (CC64), alpha7 proteasome subunit (CC36), and TIP1 (CC75). The second group is involved in signaling cascades and in transcriptional control, such as protein kinases (CC23 and CC19), 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

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 have been 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 cochaperons and therefore control the Hsp70 chaperone cycle (Groemping and Reinstein 2001). HSP22 induction by extreme temperature and oxidative stress in different species are 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, CC4 and CC47 were slightly up-regulated during drought, while CC85 was highly up-regulated over 100 fold (Fig 2). This has been also observed in maize under heat stress (Lund et al. 1998). Maize HSP70 did not change to any extent 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 appeared to be expressed only during stress (Lund et al. 1998).

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 may also be involved in abiotic stress, since CC61 was highly induced over 50 fold in root under drought (Fig 2).

Transcript CC26, which corresponds to APC11 (anaphase promoting complex subunit 11), is homologous to a RING-H2 finger protein that 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. 2003). Most Arabidopsis APC/C protein subunits are a single-copy gene in Arabidopsis (Capron et al. 2003). 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 would execute distinct functions required for growth and environmental adaptation in specific tissues and /or cellular compartments (Capron et al. 2003). This conclusion is supported by our study since CC26 did not change in root, but was upregulated over 8 folds in shoot after 24 h 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, α6and 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. CC36, putative α7 subunit, was induced in shoot after 8 hours of drought condition. The results indicate 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 the new cellular identity. Currently, a number of developmental stages and environmental responses have been linked with or shown to depend on significant changes in proteasome abundance or activity (Kurepa and Smalle 2007).

The inferred amino acid sequence of 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 *atrbohf* 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 some defense responses in *Arabidopsis* (Torres et al. 2002; Simon-Plas et al. 2002). ROS as secondary messenger triggers 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 at al. 2006). Transcript level of CC37 was induced in both root and shoot following drought treatment. This may result in the increase of ROS production and then drought tolerance in the plants. 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 serve to restrict

ROS burst, to limit cell death, and help retain responsiveness to pathogen invasion (Lequeu et al. 2005).

Transcript CC48 shows homology to the synaptobrevin-related protein, a small integral membrane protein, which belongs to 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). CC48 was highly induced in shoot following drought, while no change occurred in root. These results indicate that some SNAREs may be involved in stress-related signaling pathways.

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. So 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 (Frelet and Klein 2006; Schulza and Kolukisaoglub 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 at al. 2003). CC16 was significantly up-regulated under drought conditions and ABA, JA and SA as well (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 homologues 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. 2006). 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. CC24 showed tissue specific expression pattern which was induced only in shoot following drought, ABA, JA and SA treatment.

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 spanning across different functional groups. 2157 genes had more transcript abundance in *Atvip2* compared with Col-0, whereas 2084 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 quite 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. CC38 was induced in shoot after 4 h drought condition, and also responded to ABA, SA and JA treatment in shoot.

Transcript CC64 has high homology to a beta-amylase (BMY), 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 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). CC64 clone was strongly induced both in root and shoot upon drought and hormones. The results indicate that β-amylase is stress-related protein that can function in osmotic protection.

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* c*ytosolic pyruvate kinase 1* (*CaPKc1*) gene was induced in the 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. CC32 was up-regulated in root, but not in shoot during drought, which also indicated that PK plays a role in abiotic stress in plant.

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). 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). CC75 was induced after 8 h of PEG treatment in shoot, but suppressed in root.

Transcript CC27 shows high homology to *HAIRY MERISTEM (HAM)* in *Petunia*

(Stuurman et al. 2002). *HAM* encodes a putative transcription factor of the GRAS ((GAT, RGA and SCR) 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 At SCL6 (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). CC27 was significantly down-regulated in root, while slightly induced in shoot under drought (Fig 2).

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 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 the 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). In our study, CC23 was significantly suppressed in root, and only slightly induced in shoot.

Transcript CC76 shows high homology to GmNAC2 (NAM, ATAF and CUC2) 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. 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 either ANAC019, ANAC055, or ANAC072 in Arabidopsis, and OsNAC6 in rice (Tran et al. 2004; Nakashima et al. 2007) revealed that many genes that are inducible by abiotic and biotic stresses were upregulated. CC76 was highly induced in shoot under drought and ABA, JA and SA. 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. RQ 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 slightly induced or without change in shoots. Other TDFs such as CC26, CC36, CC38 CC47, CC48 CC75 and CC76 with homology to APC11, α 7 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 h and 12 h in roots, whereas at 8 h and 24 h in shoots. This might imply that roots sense soil water content 4 hours earlier than shoot, and some signals transport from root to shoot, such as ABA which acts 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 (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). Among these drought responsive genes, six genes in root and twelve genes in shoot were changed under drought, ABA, JA and SA treatments. Three drought-responsive genes CC85 (HSP22), CC61 (PR-protein) and CC16 (ABC-transporter) were induced by all treatments in shoot and root, which are likely to be regulated by the same or overlapping defense signaling pathways. Most drought-responsive genes are stress-related genes

under tissue specific regulation, because they were induced under treatments in shoot, but without change or suppressed in root. CC47 (grpE) was upregulated in shoot by all treatments and downregulated in root by ABA, SA and JA treatments. CC4 (HSP70) was induced by SA and JA in shoot. CC37 (RBOHD) and CC64 (β-amylase) were induced by ABA and SA in shoot. Protein kinases CC19 and CC23 were induced by both ABA and JA treatments, but suppressed in root by both signals. These results showed that the differences and crosstalk of gene expression among these signals. A coordinated range of hormones is necessary to achieve the proper response in plant environment interaction (Robert-Seilaniantz et al. 2007).

CONCLUSIONS

In this study, we identified drought-inducible genes in *C. colocynthis*, and examined their expression in root and shoot during drought stress over time and following different hormone treatments. Some stress gene induction occurs primarily at the level of transcription. 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 the 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 that roots respond to drought earlier than shoots. Overall, the study showed that *C. colocynthis* undergoes a complex adaptive process during drought stress. A complex interplay between ABA, JA and SA signaling pathways regulates plant gene expression during adaptive responses to abiotic stress.

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.

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

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

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

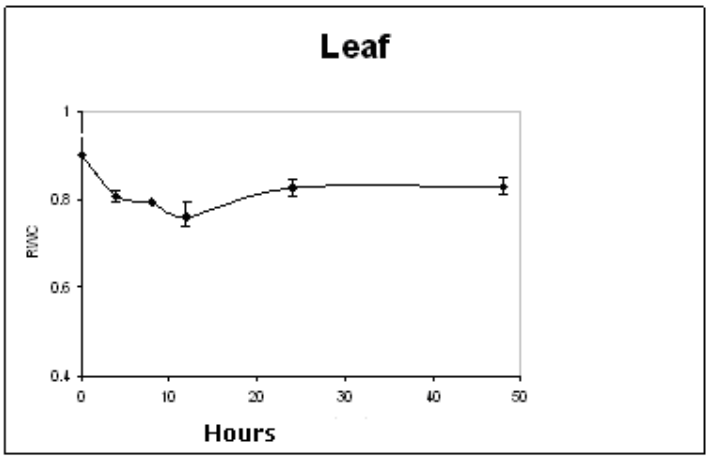
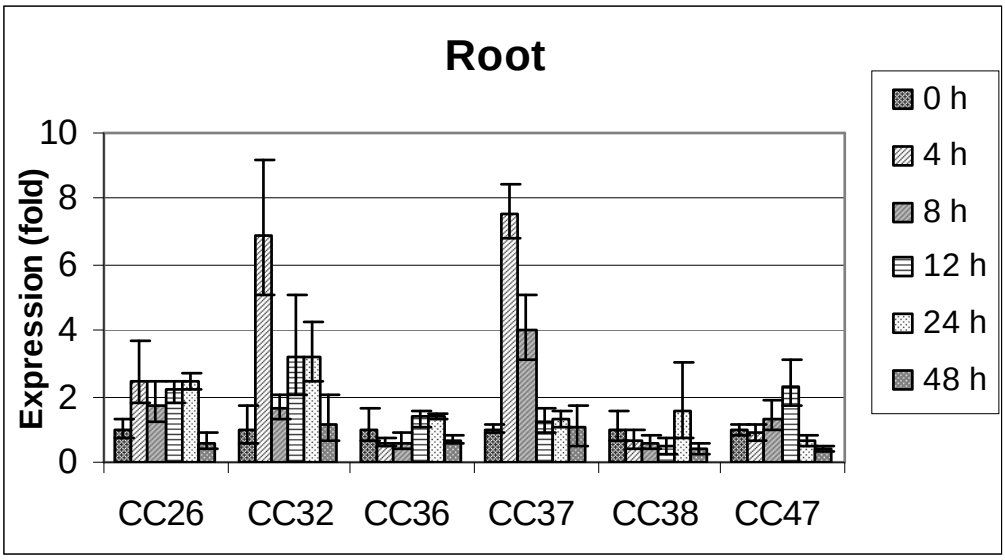


Fig 2A. The relative expression level of genes in shoot and root during 0, 4, 8, 12, 24 and 48 h of drought (PEG) treatment; Gene expression was normalized by comparing ΔΔCt to control (0 h)



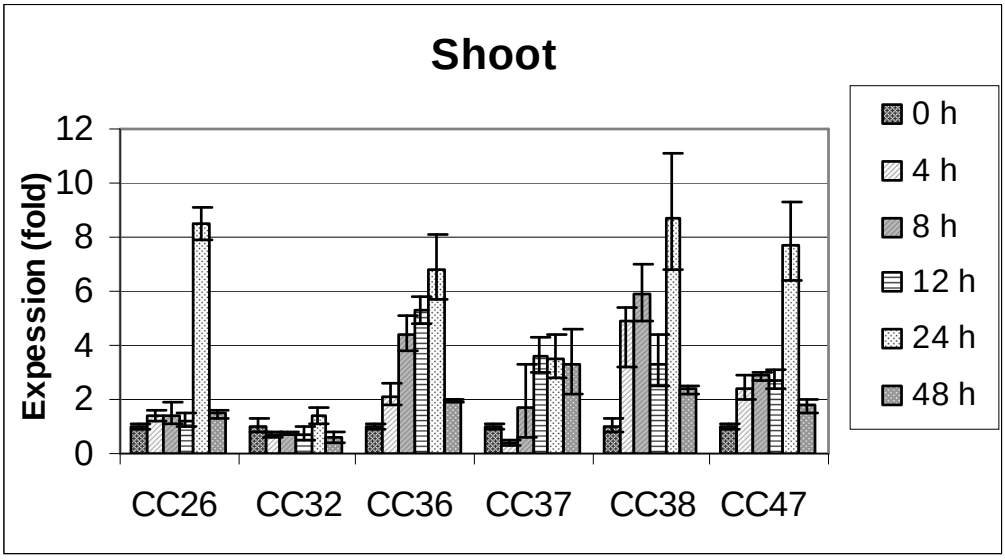
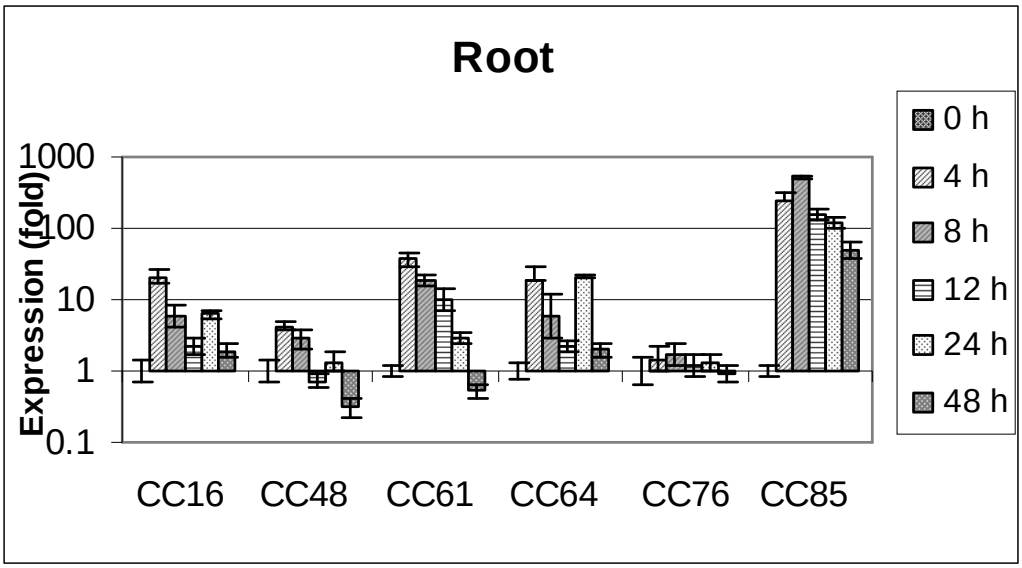


Fig 2B. The relative expression level of genes in shoot and root during 0, 4, 8, 12, 24 and 48 h of drought (PEG) treatment; Gene expression was normalized by comparing ΔΔCt to control (0 h)



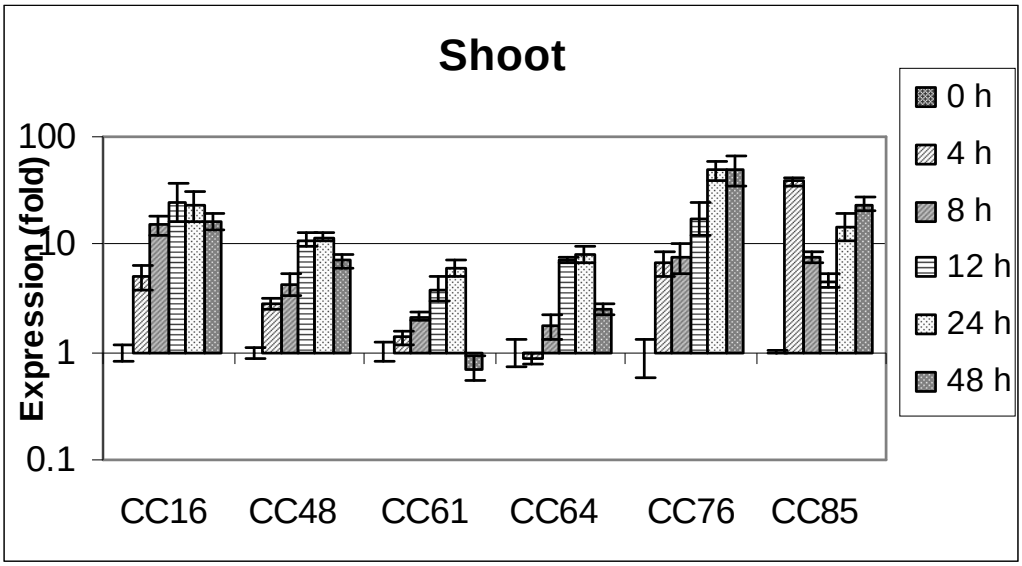
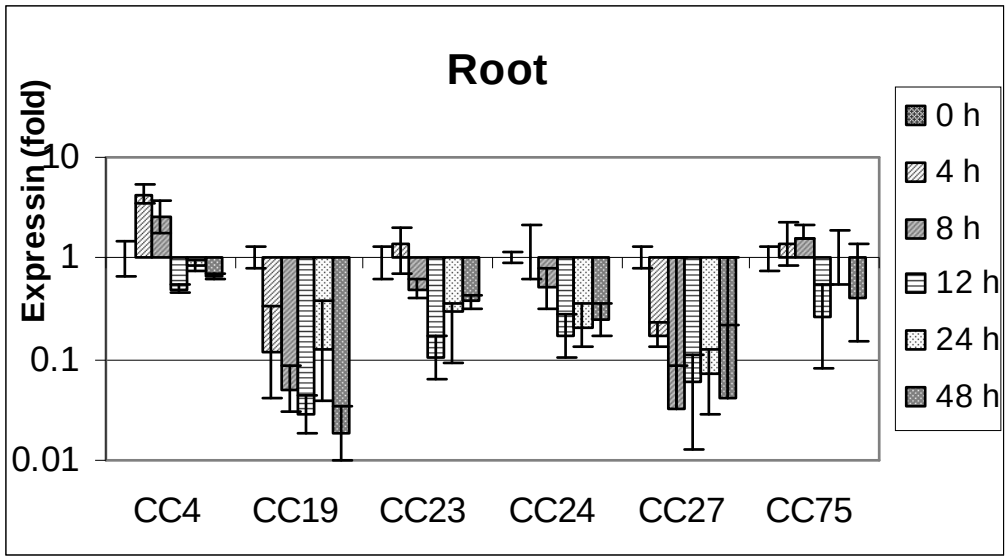


Fig 2C. The relative expression level of genes in shoot and root during 0, 4, 8, 12, 24 and 48 h of drought (PEG) treatment; Gene expression was normalized by comparing ΔΔCt to control (0 h)



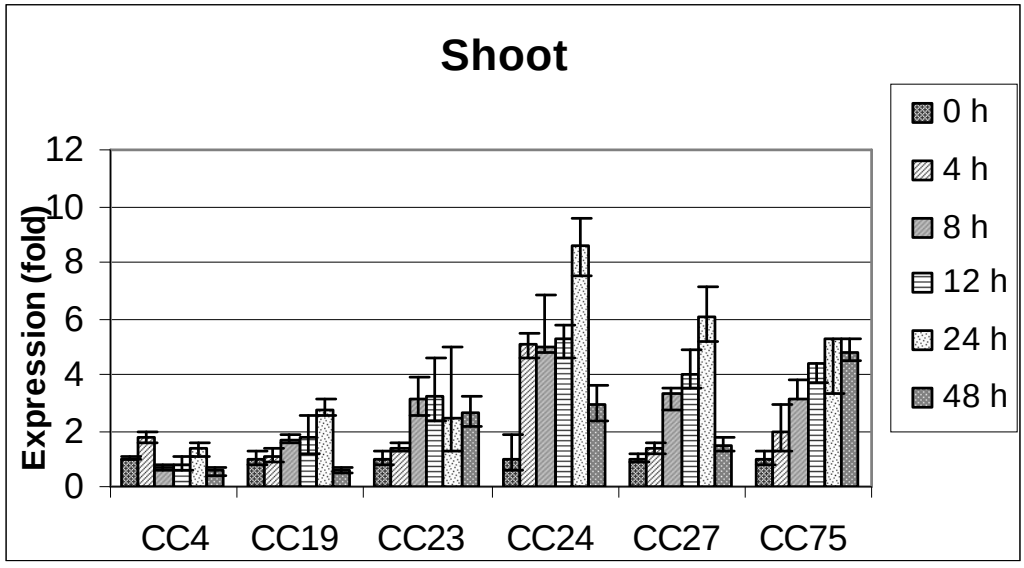
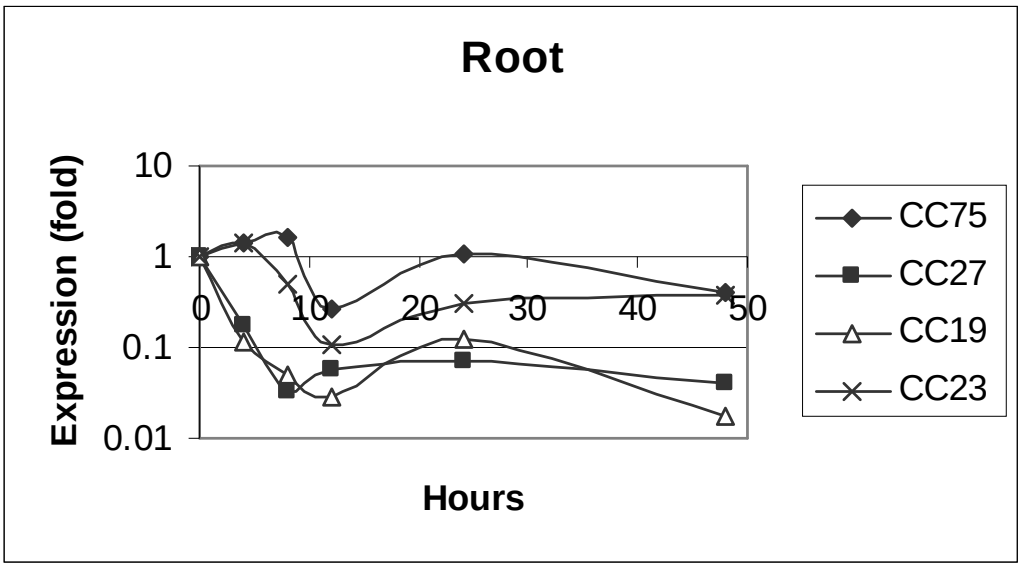


Fig. 3A Comparison of expression profiles of some genes in root and shoot during drought (PEG) treatments. Gene expression was normalized by comparing ΔΔCt to control (0 h).



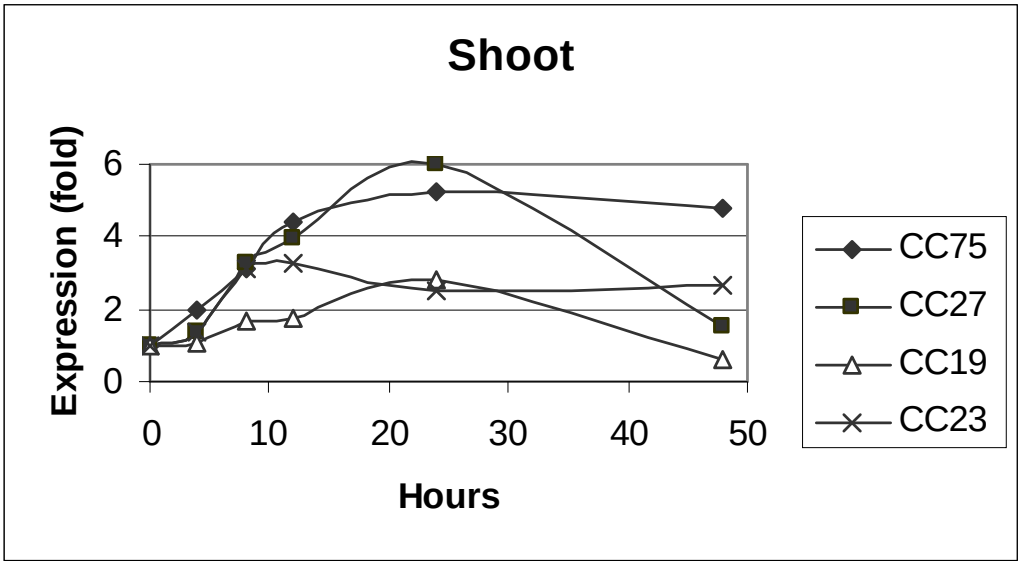
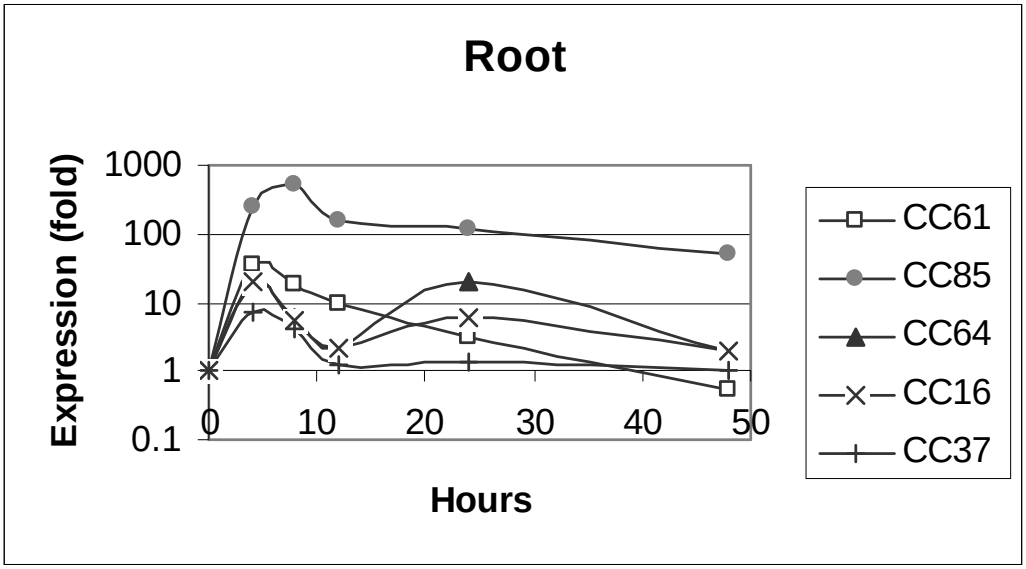
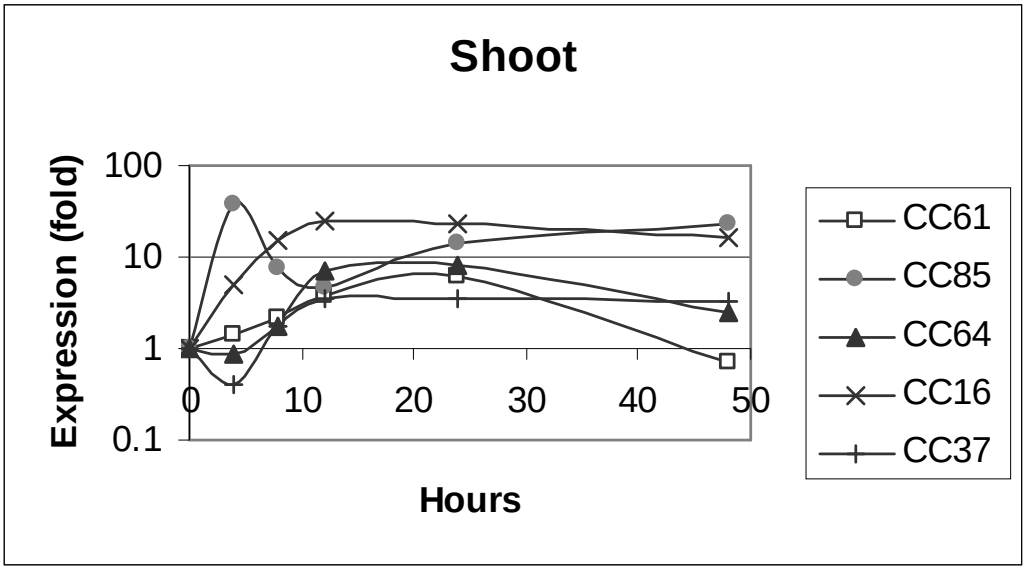


Fig. 3B Comparison of expression profiles of some genes in root and shoot during drought (PEG) treatments. Gene expression was normalized by comparing ΔΔCt to control (0 h).





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III EXPRESION ANALYSIS OF *Ccrboh* GENE ENCODING RESPIRATORY BURST OXIDASE IN *CITRULLUS COLOCYNTHIS*

ABSTRACT

A full length drought-responsive gene *Ccrboh*, encoding respiratory burst oxidase homolog (RBOH), was cloned in *Citrullus colocynthis*, a very drought tolerant cucurbit species. This protein also named NADPH oxidase is conserved in plants and animals, and functions in the production of ROS. The *Ccrboh* gene accumulated in a tissue specific pattern when *C. colocynthis* was treated with PEG, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) or NaCl, while the *Ccrboh* gene did not show any change in *C. lanatus* var. *lanatus*, cultivated watermelon, during drought. Grafting experiments were conducted using *C. colocynthis* or *C. lanatus* as rootstock or scion. Results showed that *C. colocynthis* rootstock significantly affects gene expression in *C. lanatus* scion, and some signals might be transported from root to shoot. *Ccrboh* in *C. colocynthis* was found to function early during root and vegetative development, reaching high mRNA levels 3-7 days after germination. The subcellular location of Ccrboh was investigated by transient expression of *35S :: Ccrboh :: GFP* fusion construct in protoplasts. The result confirmed that Ccrboh is a transmembrane protein. Our data suggest that *Ccrboh* might be functionally important in acclimation of plant to stress and also in plant development. It holds great promise for improving drought tolerance of other cucurbit species.

INTRODUCTION

Water deficit is considered to be the main environmental stress of plants and a major constraint of plant productivity. Tolerance to drought stress is a complex phenomenon, comprising a number of physio-biochemical processes at both the cellular and whole organismal level which are activated during different stages of plant development (Ramanjulu and Bartels 2002; Wang et al. 2003). Production of ROS at the cell surface is one of the earliest events detected in the plant defense response. ROS can function as signaling molecules that mediate responses to various processes in both plant and animal cells such as development, pathogen defense, programmed cell death, and stomatal behavior. Plants have evolved mechanisms of ROS generation and removal during development and under biotic and abiotic stress (Apel and Hirt 2004).

The respiratory burst oxidase homolog (RBOH) also named NADPH oxidase, in mammalian neutrophils has two components located in the plasma membrane (gp91^{-phox} and p22^{-phox}), which become active when several cytosolic proteins (p47^{-phox}, p67-^{phox} and the small G protein Rac) join these membrane components (Wientjes and Segal 1995). RBO homologs in plant and animal kingdoms contain cytosolic FAD- and NADPH-binding domains and six conserved transmembrane helices. In addition, some include calcium-binding elongation factor (EF) hands (Sagi and Fluhr 2006; Torres et al. 1998). The Arabidopsis genome contains 10 members (Atrboh A-J) of basically similar structures with two EF hands at the N terminus (Sagi and Fluhr 2006). Function overlap between different RBOH proteins has been observed (Torres et al. 2002; Kwak et al. 2003).

The gp91-^{phox} homologs *AtrbohD* and *AtrbohF* from *Arabidopsis*, *NtrbohD* from

Nicotiana tabacum, and tomato *rboh* were shown to be required for ROS accumulation in plant defense responses (Simon-Plas et al. 2002; Torres et al. 2002; Sagi et al. 2004). ABA signal transduction is located upstream and downstream of ROS production. ROS is synthesized in response to exogenous ABA, and ROS mediates, at least in part, ABA responses including stomatal closure and gene expression (Pei et al. 2000; Desikan et al. 2001). The *Arabidopsis* genes (*AtrbohD* and *AtrbohF*) function in ROS-dependent ABA signaling for stomatal closure (Kwak et al. 2003). Analysis of stomatal movement showed that ABA-induced stomatal closure was partially impaired in *atrbohD/atrbohF* double mutant. Cellular events were impaired in *atrbohD/atrbohF* double mutant guard cells, including ABA-induced ROS increase, ABA activation of I_{ca} channels, and ABA-induced cytosolic Ca^{2+} increase. Ethylene may also function in regulating stomatal aperture. Ethylene induces stomatal closure that is dependent on H_2O_2 production in guard cells generated by AtrbohF (Desikan et al. 2006). The Arabidopsis ethylene receptor mutants *etr1-1* and *etr1-3* were insensitive to ethylene, resulting in the failure to induce stomatal closure and to generate H_2O_2. These data suggest a complex signaling network with interaction between RBOHs and other signaling molecules.

In maize, a cross-talk between Ca^{2+} and ROS generated by NADPH oxidase is involved in the ABA signal pathway leading to the induction of antioxidant enzyme activity and antioxidant metabolism (Jiang and Zhang 2002; 2003). Water stress-induced ABA accumulation triggered the generation of ROS by NADPH oxidase, resulting in the induction of antioxidant defense system against oxidative damage. Ca^{2+} functions in upstream and downstream of ROS production in signal transduction in plants.

The Cucurbitaceae is a large and diverse family containing several domesticated

species such as watermelon (*Citrullus lanatus* var. *lanatus*), melon (*Cucumis melo* L.), cucumber (*C. sativus* L.), squashes, pumpkins and gourds (*Cucurbita* species). One species (*C. colocynthis*) in the genus *Citrullus* is a source of genetic improvement for drought resistance, since this species is widely distributed in the Sahara-Arabian desert areas and well adapted to drought stress (Dane et al. 2006). Watermelons are often grafted onto *Cucurbita moschata, C. maxima, Benincasa hispida* and *Lagenaria siceraria* to impart levels of resistance to soil borne pathogens (such as *Fusarium oxysporum*), and low soil temperatures, salinity and water stress tolerance, as well as increases in yield by enhancing water and nutrients uptake (Chouka and Jebari 1999; Lee 1994; Yetisir et al. 2003; 2006; Yetisir and Sari 2003). Therefore, *C. colocynthis* is a potential rootstock to increase drought tolerance for watermelon.

Since RBOH proteins are important components of signaling pathways, the aim of this study was to isolate and identify *rboh* gene from drought tolerant *C. colocynthis*, and gain information on how *Ccrboh* gene functions under stress conditions and during plant development. To our knowledge, this is the first report on the cloning of a full length *rboh* gene and the analysis of the transcriptional profiles of this gene in *Citrullus* species.

MATERIALS AND METHODS

Plant material and treatments

C. colocynthis seeds (No. 34,256) from Israel and *C. lanatus* var. *lanatus* seeds ('AU Producer') were sown in turface or soil in the greenhouse with a 14-h photoperiod at temperatures ranging from about 22℃ to 30 ℃, with ambient relative humidity. A 1/2

strength Hoagland's nutrient solution (PhytoTechnology Laboratories, Shawnee Mission, KS) was used to daily irrigate plants after germination.

The seedlings with at least one true leaf were grafted using one cotyledon or slant graft method (Davis et al. 2008). To facilitate rootstock and scion union, seedlings were placed in a shaded plastic tunnel with a herrmidifier (Fedders, Sanford, NC) to maintain 100% humidity and temperatures around 28 ℃ for a period of 7 to 10 days, followed by acclimation for 7 days to the natural conditions of the greenhouse by slowly decreasing the humidity.

Seedlings at 5 to 6 leaf stage were placed in 20% PEG 8000 solution to induce drought. Leaf and root samples were collected at 0, 4, 8, 12, 24 and 48 h and immediately stored at –80ºC. For other treatments seedlings at 5 to 6 leaf stage were treated with 100 μM abscisic acid (ABA), 1 mM salicylic acid (SA), 50 μM jasmonic acid (JA) or 150mM NaCl by spraying and/or irrigation. Leaves and roots were harvested at 8 h for ABA, 4 h for SA, 4 h for JA and 24 h for NaCl treatment. Leaves and roots from untreated seedlings were harvested as controls. For gene expression during vegetative growth, samples were collected at 1, 3, 7, 14, 21, 30, 60 days after germination.

RNA isolation and cDNA synthesis

RNA was extracted from root or shoot 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% formadehyde agarose gel electrophoresis.

cDNA was synthesized using RETROscript™ (Ambion) according to the manufacturer's instructions.

Cloning of *Ccrboh* core cDNA fragment and rapid amplification of cDNA ends (RACE)

The primers CcrbohFW1 and CcrbohRV1 (Table 1) used for the cloning of *Ccrboh* core cDNA fragment were designed and synthesized according to the conserved regions of the *rboh* gene sequences of *Arabidopsis thaliana, Oryza sativa, Triticum sativa, Lycopersicon esculentum,* and *Nicotiana tabacum,* deposited in GenBank. PCR analysis was initiated with hot start method using single strand cDNA template and Taq DNA polymerase (New England BioLabs, Ipswich, MA). The PCR product was subcloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced.

RACE was performed according to the manual of the 5'-RACE System Version 2.0 and 3'-RACE System (Invitrogen, Carlsbad, CA). Gene specific primers CcrbohRV1 and CcrbohRV2 for 5'-RACE, and CcrbohFW2 for 3'-RACE (Table 1) were generated based on the cloned conserved core cDNA sequences.

Sequences analysis

Amino acid sequences encoding rboh genes from Arabidopsis, rice, tomato, maize, potato, tobacco, and alfalfa were chosen from the NCBI database. Multiple sequence alignment was carried out with CLUSTAL W at default setting. Treeview software was used for displaying the phylogenetic trees.

Relative quantitative (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 or ABI, Foster City, CA). The *C. colocynthis* specific actin gene (GH626171) used as reference gene was amplified in parallel with the target gene allowing normalization of gene expression and providing quantification. The primer sequences of the gene (CcrbohFW4 and CcrbohRV2) and actin (ACTFW and ACTRV) are listed in Table 1. Detection of RQ real-time RT-PCR products was done using the SYBR® Green Universal Master mix kit (ABI) following manufacturer's recommendations. Ten microliters of each sample were analyzed using 2% agarose gel electrophoresis and visualized with ethidium bromide.

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 (threshold cycle) 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: $E = [(10^{-1/y}) - 1] \times 100$. Quantification of the relative transcript levels was performed using the comparative C_T method. Transcript levels of target genes were normalized against the β-actin gene transcript levels as described in the ABI PRISM 7500 Sequence Detection System user bulletin #2 (ABI). The induction ratio (IR) was calculated as recommended by the manufacturer and corresponds to $2^{-\Delta\Delta C}{}_T$, 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 sample (Pfaffl 2001).

Southern blot analysis

Genomic DNA isolated from *C. colocynthis* seeds (20ug/sample) was digested with different restriction enzymes (*Hind*III, *EcoR*V, or *Xba*I 20 unit/g DNA) for 16 h at 37 ℃, followed by separation on a 0.8% agarose gel. After electrophoresis, gels were washed with water and 10X SSC and blotted with Hybond N+ (Amersham Pharmacia Biotech, Piscataway, NJ) prewetted with 10X SSC. Hybridization was performed at 65°C with Church buffer (1% BSA, 200 μM EDTA, 0.5 M sodium phosphate, 7% SDS) containing a ^{32}P-labeled probe. Full length cDNA of *Ccrboh* gene as a probe was obtained by PCR using the following gene-specific primers: CcrbohFW3 and CcrbohRV4 (Table 1).

GFP conjugated plasmid construction

The plasmid for protoplast transformation was generated using the Invitrogen Gateway system according to the manufacturer's instructions. Ccrboh DNA lacking a stop codon was amplified by PCR using CcrbohFW3 and CcrbohRV5 (Table 1), and subcloned into a TOPO vector (Invitrogen, Carlsbad, CA). The TOPO vector with the gene and pENTR 1A dual selection vector were cut by *Kpn*I and *Not*I, and the cutted pENTR vector and gene were ligated using T4 DNA ligase (Invitrogen). Entry clones containing *Ccrboh* gene lacking a stop codon were transferred from entry clone vector to the destination clone vector pEarleyGate 103 with GFP on C-terminal (A gift from Dr. Aaron Rashotte, Auburn University) using the LR reaction (Invitrogen).

Protoplast isolation and transformation

C. colocynthis cotyledons from soil-grown plants were excised, cut into 1 mm strips and immediately placed into an enzyme solution for overnight digestion in the dark. The enzyme solution which contained 2% cellulose R10, 0.5% macerozyme R10, 0.5% driselase, 2.5% KCl, 0.2% $CaCl_2$, pH 5.7 was filter sterilized. After overnight incubation, leaf tissue was gently shaken for 30 min at 40 rpm to release protoplasts, followed by filtration through a 40 µm cell sifter to remove debris and centrifugation at 150 g to pellet the protoplasts. Protoplasts were washed twice with a washing solution (0.5 M mannitol, 4 mM MES pH 5.7 and 20 mM KCl) and re-centrifuged at 150 g. The protoplasts were suspended in washing solution on ice for electroporation.

Protoplasts were transformed in a manner essentially as described (Sheen 1991; Rashotte et al. 2006). Electroporation was typically carried out with 1 - 2 x 10^5 protoplasts in 300 uL of wash solution and about 40 ug to 50 ug of plasmid DNA, and treated for electroporation at 300V in a 0.1 mm electroporation cuvette using an Eppendorf Electroporator 2510. Protoplasts were immediately placed on ice and left in the dark at 22°C for 18 h before examination.

Microscopy

Microscopy for subcellular localization was conducted using a Nikon Eclipse 80*i* epifluorescence microscope with a UV source. A standard UV filter was used in addition to 1 ng·ml^{-1} of Hoechst 33342 stain to initially observe cells and identify nuclei. A GFP filter that blocks chlorophyll fluorescence and Hoechst 33342 fluorescence was used to

examine localization of GFP fusion proteins. All photos were taken with a Qimagine Fast 1394.

Overexpression and RNAi vector construction

To generate the RNAi construct for gene knock-out, a fragment of *Ccrboh* gene (Product a, 132 bp) was amplified by PCR with *C. colocynthis* cDNA as a template using forward primer P1 and reverse primer P2 (Table 1). The *GUS* fragment (Product b, ca. 1 kb) was amplified using pBI 121 as a template with forward primer P3 and reverse primer P4 (Table 1). The antisense and sense fragment (Product c and d, respectively) were amplified by PCR with Product a as a template using primers P5 and P7, and P6 and P8 (Table 1), respectively. P5 and P6 primers contain the *Ccrboh* gene sequence (in small letters) and *Gus* gene sequence (in capital letters). P7 and P8 primers contain *Kpn*I-*Xba*I and *Sca*I–*Ava*I restriction sites (in capital letters), respectively. The RNAi construct was amplified using P7 and P8 primers with Product b, c and d as templates. The construct was subcloned into the pGEM-T-Easy vector (Promega, Madison, WI), and sequenced to check for correct orientation and sequence. Binary vector pE1803 and RNAi construct clone were digested with *Kpn*I and *Sac*I, and ligated with T4 DNA ligase. The pE1803 vector with the RNAi construct (*pMSP*::*RNAi*), which carries kanamycin- and hygromycin-resistance markers under the control of an enhanced mannopine synthase promoter (MSP), was developed for *Agrobacterium*-mediated transformation of *C. colocynthis*.

To generate the over-expression (OE) construct, the full length *Ccrboh* gene was synthesized by PCR using *C. colocynthis* cDNA as a template and CcrbohFW3 and

CcrbohRV4 as primers (Table 1), and subcloned into the TOPO vector (Invitrogen). The TOPO vector with the gene and pE1803 vector were cut with *Kpn*I and *Xba*I, and ligated using T4 DNA ligase (Invitrogen). The *pMSP*::*Ccrboh* vector was used for *Agrobacterium*-mediated transformation.

Plant materials and *Agrobacterium*-mediated transformation

Seeds of watermelon were surface sterilized in 70% ethanol for 30s, and 1% hypochloride solution for 30 min, and rinsed three times with sterile distilled water. The sterilized seeds were germinated on one-half strength MS medium (Murashige and Skoog 1962) in dark at 28 °C for 2 days, followed by a transfer to a tissue culture room at 26±2°C under a photoperiod of 16 h light/8 h dark and light intensity of 90 umol $m^{-2}s^{-1}$. Cotyledons from 3 (-5) -day-old seedlings were excised into eight segments and used as explants for regeneration and transformation.

Cotyledon explants were transferred to a pre-culture medium consisting of MS medium supplemented with 0.1 mg l^{-1} 6-benzylaminopurine (BA) and 1 mg l^{-1} indoleacetic acid (IAA), and placed in the culture room at 25 °C for 3-5 days until they expanded to approximately four fold their initial size. *A. tumefaciens* C58C1 strain carrying a binary vector pE1803 which contained OE or RNAi construct was used to transform cotyledon segments. The control plasmid pBI121 contains the marker gene *npt*II under control of a *nos* promoter near the right border and reporter gene *gus* under the control of the *CaMV*35S promoter close to the left border (Jefferson et al. 1987). Plasmid pE1803 contains enhanced mannopine synthase promoter, and *hpt* gene for hygromycin selection (Ni et al. 1995). Transformed *Agrobacteria* grown to log phase in

LB liquid medium (OD_{600}: 0.7-0.9) were centrifuged at 3,500 rpm for 10 min. Pellets were resuspended in MS liquid medium containing 200 uM acetosyringone. Explants were infected by immersing them in the above mentioned *Agrobacterium* inoculum for 30 min, and cocultivation on MS medium supplemented with 0.1 mg l^{-1} BA, 1 mg l^{-1} IAA, and 0.2 uM acetosyringone at 28 °C for 3 days in the dark. Explants were briefly washed with MS basic liquid medium containing 500 mg l-1 carbenicillin, dried on sterile filter paper and placed on selection and shooting medium (Table 2) supplemented with 0.1 mg l^{-1} BA, 1 mg l^{-1} IAA, 300 mg l^{-1} carbenicillin, and 100 mg l^{-1} kanamycin or 15 mg l^{-1} hygromycin for 4-5 weeks at 25°C. Green shoots were transferred to rooting medium (basic medium containing 1 mg l^{-1} indole-3-butyric acid (IBA) and 100 mg l^{-1} carbenicillin) for 5-6 weeks at 25°C.

PCR

Genomic DNA was isolated from young leaves using DNeasy Plant Minikit (Qiagen, Valencia, CA). PCR detection of *gus* gene was performed using primers P3 and P4 (Table 1) and standard PCR methodologies.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *Ccrboh* gene

The cDNAs *Ccrboh* encoding respiratory burst oxidase protein was cloned from *C. colocynthis* and sequenced. Sequence analysis indicated that the full-length cDNA

contained the 5'-UTR, the complete open reading frame (ORF), 3'-UTR and the Poly (A) tail. *Ccrboh* has a 2,781 bp ORF encoding a protein of 926 amino acids. BLASTp search utility identified the ATG translation start in *Ccrboh* as well as a Ca^{2+}-binding motif of the EF-hand loop type occurring at N-terminal (Fig 1). In Arabidopsis, the EF-hands are present in the third small exon of rboh homologues (Torres et al. 1998). The presence of the highly conserved motif in rboh proteins suggests a possible direct effect of Ca^{2+} on the function of NADPH oxidase in plants. It has been shown that the EF-hand motif in plant rbohs bind $^{45}Ca^{2+}$ (Keller et al. 1998), and Ca^{2+} stimulates rboh to produce ROS in plasma membranes (Sigi and Fluhr 2001; Heyno et al. 2008). The C- terminal of the Ccrboh protein contains other functional motifs such as the ferric reductase like transmembrane component domain, FAD-binding domain and NAD-binding domain (Fig 1). The C-termini of the plant rboh proteins show greater overall sequence similarity to $gp91^{phox}$ in animals especially in the last intracellular domain containing FAD and NADPH binding site, and have some amino acids known to be absolutely required for $gp91^{phox}$ (Torres et al. 1998; Torres and Dangl 2005). Phylogenetic analysis between Ccrboh and 10 rboh proteins in Arabidopsis showed that Ccrboh has high homology to the AtrbohD protein (Fig 2). Phylogenetic tree was constructed based on rboh protein sequences to investigate the evolutionary relationship among monocot and dicot species. The results showed that rboh proteins are conserved in both monocot and dicot plants, suggesting its evolution before monocot and dicot split (Fig 3, 4).

Expression analysis of *Ccrboh* gene in *C. colocynthis*

Ccrboh was induced in both roots and shoots (Fig.5) following drought (PEG) treatment, but the induction in roots was 4 h earlier than in shoots. The highest induction level in roots was between 4 and 8 h and decreased to control levels after 12 h, while it was highly induced in shoot after 12 h and continually increased up to 48 h. 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. The tissue-specific division of transcript distribution falls into three basic classes in Arabidopsis: expression throughout the plant (AtrbohD and F), in the roots (Atrboh A-G, I), or in a pollen-specific manner (Sagi and Fluhr 2006).

Ccrboh was induced in all other treatments such as ABA, JA, SA, but not in NaCl in a tissue specific pattern (Fig.6). The results correspond with other reports, showing that application of ABA, IAA, or BA leads to accumulation of rboh transcript (Kwak et al. 2003; Sagi et al. 2004). It is likely that rboh could function as a signal transponder for hormone action (Sagi et al. 2004). Hormones might regulate rboh in two ways. Hormones might affect rboh by generating ROS burst that may be mediated by functions in N-terminal regulatory regions. A more lasting and long-term effect of hormones may be achieved by the upregulation of rboh levels. Also, the regulation of *Ccrboh* is tissue specific as shown earlier. Regulation of the temporal and spatial expression patterns is an important part of the plant stress response. Tissue-dependent variations in the expression pattern of transcripts under different abiotic stress between roots and shoots have been documented in many plant species (Kreps et al. 2002, Baisakh et al. 2006; Kawasaki et al. 2001; Liu and Baird 2003). Arabidopsis *rboh* genes are differentiated by their expression

sensitivity to environmental inputs (Sagi and Fluhr 2006). The most common abiotic inducers are nitrogen stress and conditions of anoxia/hypoxia, and AtrbohC to F are also induced by various biotic stresses. AtrbohD is identified as the major constitutively active form (Torres et al. 2002). The diverse transcription patterns of rboh genes suggest that Rboh proteins function in a broad range of growth, biotic and abiotic stress responses (Torres and Dangl 2005).

To understand how Ccrboh functions in plant development, we analyzed the expression of *Ccrboh* gene during root and vegetative growth (Fig. 7) using *C. colocynthis* seeds as a control. In roots, *Ccrboh* was increased to 7 folds in 1- day roots, and then decreased to 3-4 folds in 3-14-days roots. After 14 days after germination, *Ccrboh* expression level gent back to 7 folds. In shoots, *Ccrboh* expression level increased 1 day after germination, and then dramatically increased in the 3 day-old-seedlings, followed by a leveling off to control level. However, the gene maintained constitutive expression levels up to 5 to 10 fold in 7-60 day-old-seedlings. The results indicate that *Ccrboh* gene also functions during plant development, such as root growth and leaf morphogenesis. A role for AtrbohC in root hair growth and in mediating the tip-focused Ca^{2+} gradient was discovered in Arabidopsis root hair cells (Foreman et al. 2003). *AtrbohC* transcript is present in the epidermis in the proximal regions of the meristem, in the elongation zone, in the differentiation zone and in elongating root hairs. The *AtrbohC* mutant *RHD2* (*ROOT HAIR DEFECTIVE2*) have short root hairs and stunted roots. Lowered rboh levels in the antisense lines of tomato were shown to have a profound influence on plant growth. The curled and inverted leaves, and abnormal flowers and fruits were observed in the mutants. Rboh induction responded to application of ABA,

IAA, BA and ACC (Sagi et al. 2004). All these observations indicate the rboh function in a plethora of developmental effects in many plant organs, and imply the involvement of a range of hormones. This is not surprising, because ROS are required for cell expansion during morphogenesis of organs such as roots, leaves and pollen (reviewed by Carol and Dolan 2006). A number of histone 3.3 variants were modulated in tomato rboh antisense mutants, implying that attenuation of rboh activity may influence chromosome structure, and eventually impinge on fundamental cellular processes (Sagi et al. 2004).

Expression analysis of *Ccrboh* in *C. lanatus* var. *lanatus* (watermelon) and grafted plants under drought

Watermelon plants, treated with 20% PEG 8000 to induce drought stress, were examined at 0, 4, 8, 12 and 48 h. The mRNA transcript level of *Ccrhoh* gene did not change in root and shoot during treatment (Fig. 5). As expected, the analysis confirmed significant transcript accumulation differences of *Ccrboh* gene in the two species. Interestingly, when watermelon was grafted onto *C. colocynthis* rootstock, *Ccrboh* was induced following 8 h of treatment and continually increased up to 24 h in watermelon scions (Fig. 8). The expression pattern in grafted plants (watermelon scion / *C. colocynthis* rootstock (CLL/CC)) was the same as that in *C. colocynthis*, but the induction level was lower than in *C. colocynthis* (Fig. 8 and Fig. 5). The *Ccrboh* gene expression level did not change in *C. colocynthis* grafted onto watermelon rootstock (CC/CLL) (Fig. 8) during drought. The grafting experiment indicates that the rootstock is important for regulation of genes in the scion, and there might be long distance signaling of ABA

(Thompson et al. 2007), microRNA (Ruiz-Medrano et al. 1999), or some small proteins (Corbesier et al. 2007).

Watermelons are often grafted onto *Cucurbita moschata*, *C. maxima*, *Benincasa hispida* and *Lagenaria siceraria*. Several studies showed that grafted watermelons are protected from Fusarium wilt, and increased low soil temperature, salinity and water stress tolerance, as well as show increases in yield by enhancing water and nutrients uptake (Chouka and Jebari 1999; Lee 1994; Yetisir et al. 2003; 2006; Yetisir and Sari 2003). Root characteristics are of primary importance in determining stress tolerance in other plant species, such as tomato (Fernández-García et al. 2002), apple trees (Jensen et al. 2003), and tobacco (Ruiz et al. 2006). Therefore, *C. colocynthis* is a potential rootstock to increase drought tolerance for watermelon. However, how the rootstock affects the fruit quality and how the grafted plants perform under stress conditions in the field environment needs to be further investigated.

Southern blot analysis

To investigate the genomic organization of *Ccrboh* gene in *C. colocynthis*, genomic DNA was digested with *Hind*III, *Eco*RV, or *Xba*I, respectively and hybridized with the probe, which was full length cDNA of *Ccrboh* gene generated by PCR. The result showed that at least two hybridizing bands ranging from 2 kb to 10 kb were present in the first lane (digested with *Hind*III), and one band in the second (digested with *Eco*RV) and third lane (digested with *Xba*I) under high stringency conditions (Fig. 9), indicating that *Ccrboh* potentially exists as one or two copies in the genome.

Subcellular localization of the Ccrboh protein

To address the subcellular localization of Ccrboh in living cells, a construct containing Ccrboh fused in-frame with the GFP (Ccrboh::GFP) driven by the *CaMV*35S promoter was transiently expressed in leaf protoplasts. Hoechst 33342 stain was used to initially observe if protoplasts were intact and nuclei could be identified (Fig.10 right). As shown in Fig. 10 (top), Epifluorescent microscope examination revealed that cells transferred with the unconjugated GFP (control) exhibited a diffused distribution of green fluorescence throughout the cell. By contrast, when GFP was fused with Ccrboh, the GFP signal was confined to the plasma membrane (Fig. 10 bottom), confirming that Ccrboh protein is localized on the plasma membrane. The result is consistent with previous studies conducted using cellular fractionation of plant tissues in tobacco (Sagi and Fluhr 2001; Simon-Plas et al. 2002).

Overexpression and RNAi transformation

All explants inoculated with *A. tumefaciens* containing Ccrboh::OE vector and a Ccrboh::RNAi vector turned brown and died within 2-3 weeks on shooting medium with 50 mg l-1 hygromycin and 300 mg l^{-1} carbenicillin. In a follow-up experiment, a lower concentration of hygromycin (15 mg l^{-1}) was used to select transformants. However, all explants turned brown and died after 2-3 weeks.

Explants inoculated with *A. tumefaciens* containing pBI121 vector as a control stayed green on shooting medium with 100 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin. Most of these explants produced green kanamycin-resistant callus, but only 3 explants induced shoots. These 3 explants were transferred into rooting medium, and two

produced roots. However, PCR analysis of the *GUS* gene indicated that these plants were not transformed.

Watermelon is recognized as one of the most recalcitrant plants regarding *Agrobacterium* mediated transformation. It has been difficult to establish a regeneration system. These results indicate that 1. The transformation efficiency is very low for watermelon; 2. Watermelon is difficult to regenerate after transformation; 3. *Ccrboh* gene might be toxic to explants; 4. Hygromycin might not be an effective selectable marker (personal communication with Dr. Nong). The growth patterns of transformed watermelon selected using kanamycin or hygromycin were nearly identical, but the recovery of plantlets following transformation was more difficult for hygromycin-selected plants, and the hygromycin-selected seedlings have been known not to recover easily (Park et al. 2005).

CONCLUSIONS

In summary, a full-length cDNA clone, *Ccrboh*, encoding respiratory burst oxidase has been identified from *C. colocynthis*. Sequence analysis showed that Ccrboh is highly homologous to AtrbohD, and rboh proteins are conserved in monocot and dicot plants. *Ccrboh* transcript was induced to express in a tissue-specific pattern following drought stress, ABA, JA and SA treatment in *C. colocynthis* seedlings. No change in *C. lanatus* var. *lanatus* (watermelon) under drought stress was observed, but *Ccrboh* gene was induced in watermelon scion grafted onto *C. colocynthis* rootstock. *Ccrboh* also functions in leaf morphogenesis because of changes in expression detected during vegetative growth. Transient expression of Ccrboh::GFP fusion protein in protoplast confirmed that

Ccrboh protein is localized on the plasma membrane. Rboh appears to be a highly regulated, sensitive, and versatile mediator of developmental and environmental signals (Sagi et al. 2004). Depending on the incoming signals from the plant, pathogen, or environment, the redox state might be altered such that it governs a transcriptional response aimed at maximizing plant fitness in a changing environment. All these results provide very useful information for the functional analysis of Ccrboh and its implications in plant genetic improvement.

Further studies, including characterizing the regulation of the signal transduction network that controls Ccrboh production and activity, as well as primary downstream targets modulated by ROS bursts, will extend our understanding of the biological role and function of rboh in plant development and growth as well as the responses to various biotic and abiotic stresses.

Table 1. Oligonucleotide primer sequences

Primer name	Sequence (5'—3')
CcrbohFW1	CCTGTTTGTCGAAACACCATCACT
CcrbohRV1	GAATGATCCTTGTTCCCTAGTCAC
CcrbohRV2	AATGGGCGATTGCGTGTAATCCC
CcrbohRV3	AGGAACGATGACGCCTAATT
CcrbohFW2	GGAGGAGCTCCTAATCCTAAGT
CcrbohFW3	ATGAGACCTCACGAACCTTATTCTG
CcrbohRV4	AGTGCGGTATGTGTCAACCTTCACC
CcrbohFW4	AATTAGGCGTCATCGTTCCT
CcrbohRV2	AATGGGCGATTGCGTGTAATCCC
CcrbohFW3	ATGAGACCTCACGAACCTTATTCTG
CcrbohRV5	AGTGGATGTTTTACGAGAGAAAT
ACTFW	CAACATACATAGCAGGCACA
ACTRV	TGACTGAGGCTCCACTCAAC
P1	tcaattacttcagccccagaa
P2	gagaagtccacttttccagc
P3	CCGACGAAAACGGCAAGAAAAAGC
P4	CCAGAAGTTCTTTTTCCAGTACCT
P5	TTTCTTGCCGTTTTCGTCGGTAtcaattacttcagccccagaa
P6	ACTGGAAAAAGAACTTCTGGCCTtcaattacttcagccccagaa
P7	GGTACCACTCTAGGATgagaagtccacttttccagc
P8	GTCATGACCTAGGCGATgagaagtccacttttccagc

Table 2. A protocol for transformation of watermelon

Step	Description	Duration
Germination	Darkness	2-3 days
	Light	2-3 days
	½ MS + 3% sucrose + 0.8% agar, pH 5.8	
Explant	Cotyledon	
Pre-culture	Basic medium (MS + 3% sucrose + 0.8% agar, pH 5.8), 1.0 mg/l BA + 0.1 mg/l IAA	5-7 days
cDNA insert	Ccrboh, RNAi construct, GUS	
Agrobacterium strains	*C58C1*	
Inoculation	MS + 3 % sucrose + 200 uM acetosyringone	30 min
Co-culture	Basic medium 1.0 mg/l BA + 0.1 mg/l IAA + 0.2 uM acetosyringone	3 days in dark
Washing	MS + 3 % sucrose + Carbenicillin 500 mg/l	10 min
Selection and shooting	Basic medium 1.0 mg/l BA + 0.1 mg/l IAA + carbenicillin 300 mg/l + hygromycin 15 mg/l (or kanamycin 100 mg/l)	Shooting 4-5 weeks
Rooting	Basic medium 1 mg/l IBA + carbenicillin 100 mg/l	Root formation 5-6 weeks

Fig.1 Putative conserved domains of Ccrboh protein generated by NCBI Blastp.

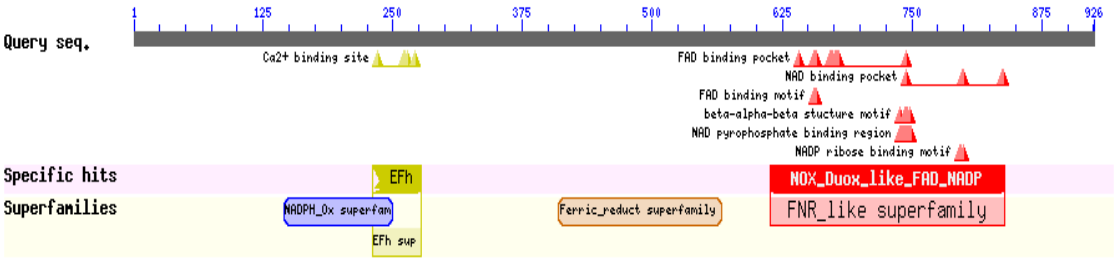


Fig.2 Phylogenetic tree of Ccrboh and 10 Arabidopsis rbohs. Ccrboh, AtrbohJ (Q9LZU9), AtrbohI (Q9SUT8), AtrbohC (O81210), AtrbohD (Q9FIJ0), AtrbohA (O81209), AtrbohG (Q9SW17), AtrbohF (O48538), AtrbohE (O81211), AtrbohB (Q9SBI0), AtrbohH (Q9FJD6).

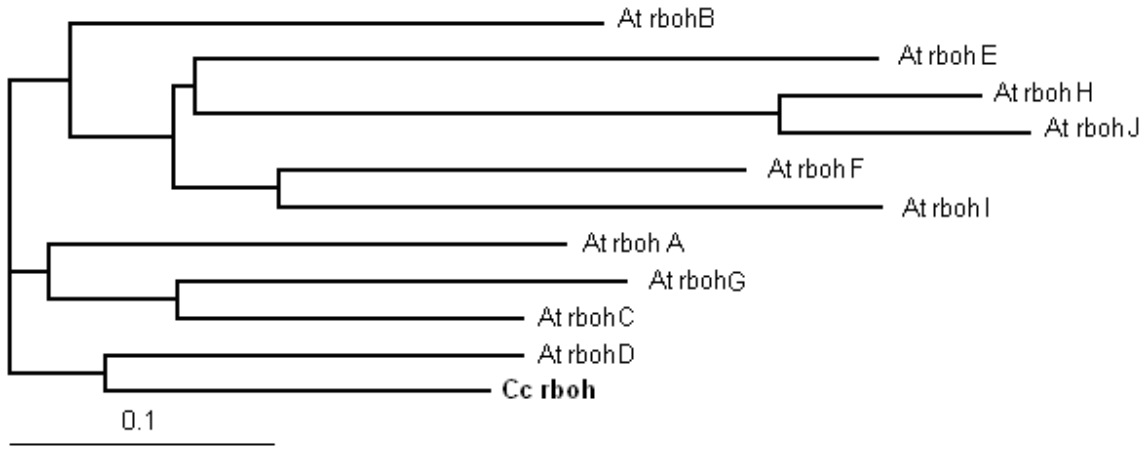


Fig.3 Alignment of the amino acid sequences of rbohD in different species. Ccrboh from *C. colocynthis*; AtrbohD (Q9FIJ0) from Arabidopsis; StrbohD (Q2HXK9) from *Solanum tuberosum* (potato); Ntrboh (CAC84140) from *Nicotiana tabacum* (tobacco); Zmrboh (ABP48737) from *Zea mays* (Maize); OsrbohD (ABA94089) from *Oryza sativa* (rice).

Fig.4 Phylogenetic tree of rboh proteins in different species. Ccrboh from *C. colocynthis*; AtrbohJ (Q9LZU9), AtrbohI (Q9SUT8), AtrbohC (O81210), AtrbohD (Q9FIJ0), AtrbohA (O81209), AtrbohG (Q9SW17), AtrbohF (O48538), AtrbohE (O81211), AtrbohB (Q9SBI0), AtrbohH (Q9FJD6) from Arabidopsis; StrbohC (BAE79344), StrbohF (BAB84124), Strboh (BAC06825), StrbohB (Q948T9), StrbohA (Q948U0), StrbohD (Q2HXK9), StrbohC (Q2HXL0) from *Solanum tuberosum* (potato); Lerboh (AAD25300) from Lycopersicon esculentum (tomato), Ntrboh (CAC84140) from *Nicotiana tabacum*, Mtrboh (CAM35833) from *Medicago truncatula*; Zmrboh (ABP48737) from *Zea mays* (Maize); OsrbohD (ABA94089), OsrbohH (ABA99453) from *Oryza sativa*.

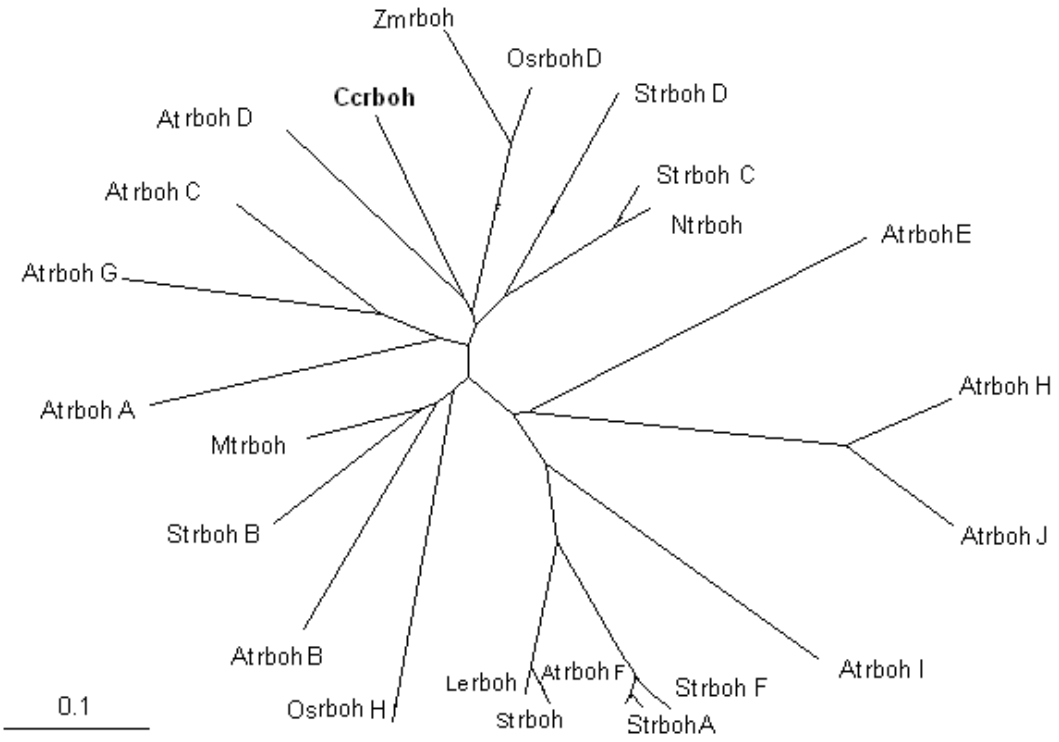
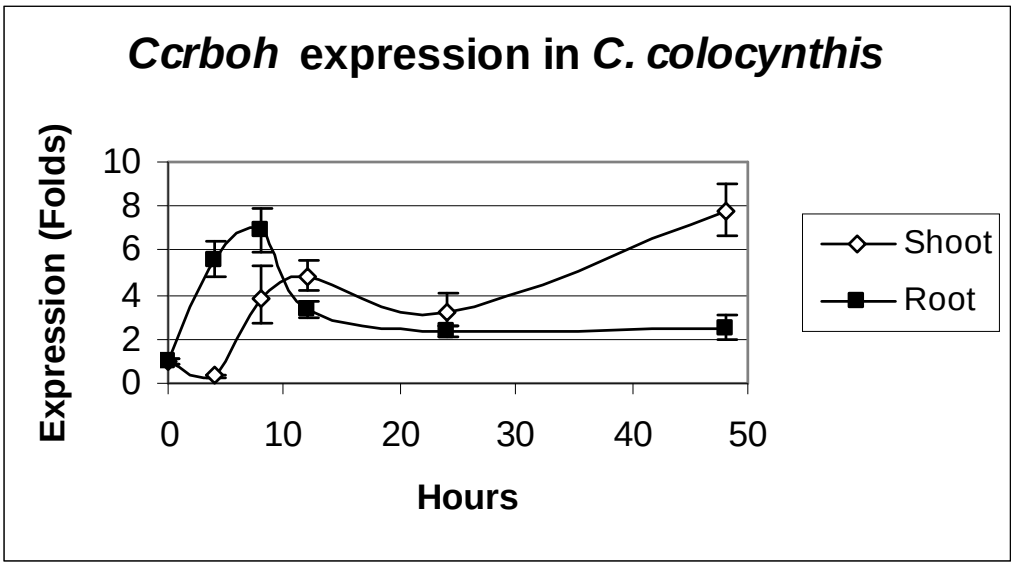


Fig.5 Comparison of expression profiles of *Ccrboh* gene in root and shoot in *C. colocynthis* and *C. lanatus* var. *lanatus* during drought (PEG) treatments. Gene expression was normalized by comparing ΔΔCt to control (0 h).



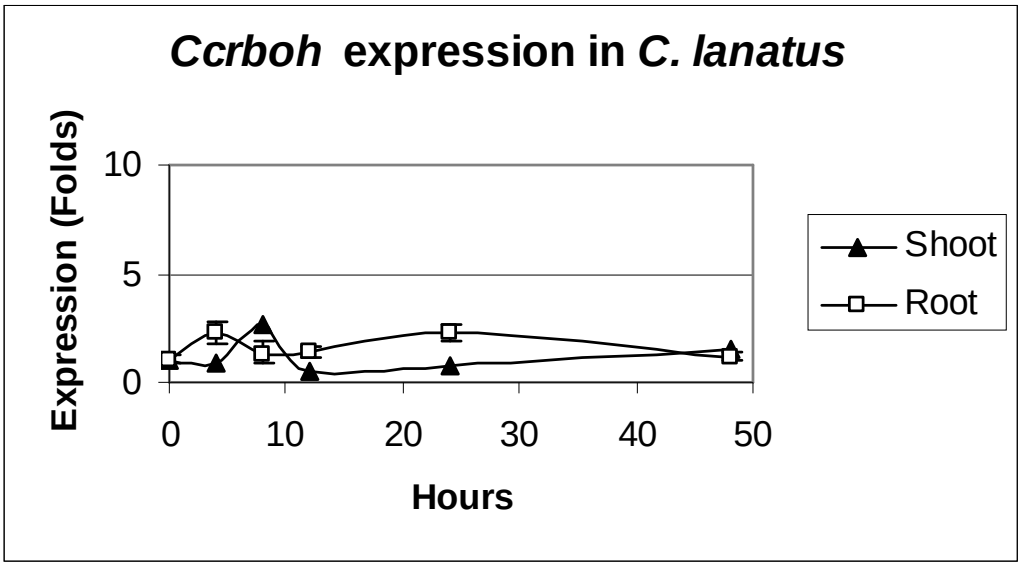


Fig.6 Comparison of expression profiles of *Ccrboh* gene in root and shoot during different treatments in *C. colocynthis*. Gene expression was normalized by comparing ΔΔCt to control (0 h).

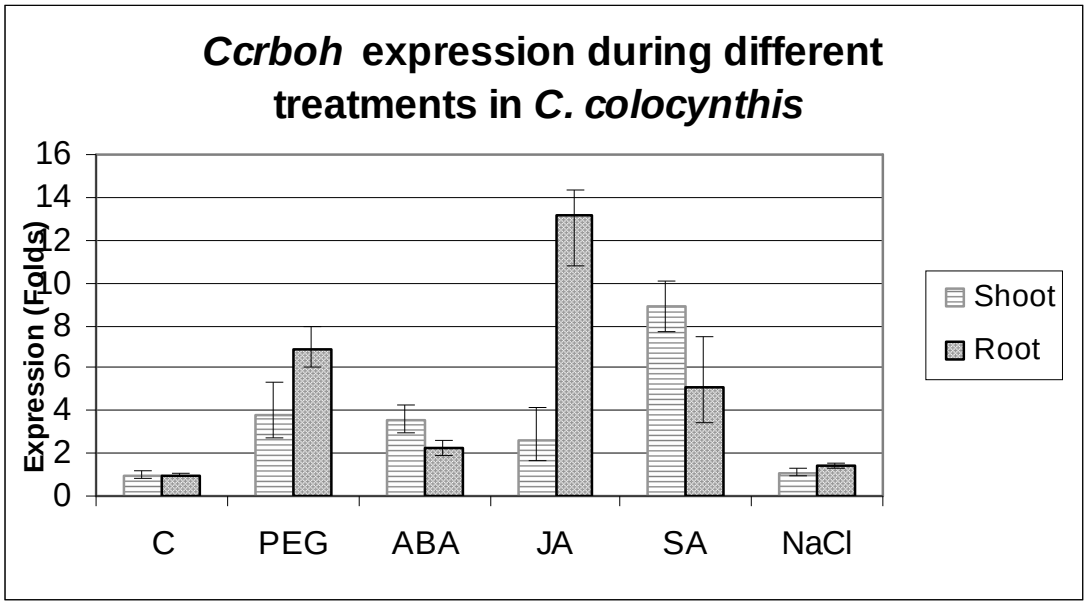
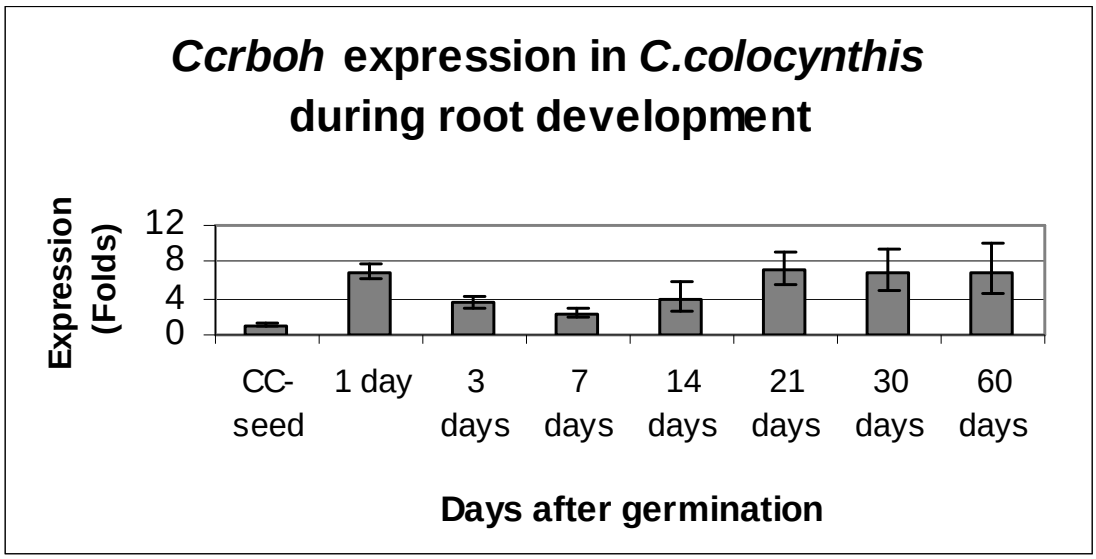


Fig.7 Comparison of expression profiles of *Ccrboh* gene in roots and shoots during days after germination in *C. colocynthis* (CC). Gene expression was normalized by comparing ΔΔCt to control (CC seeds).



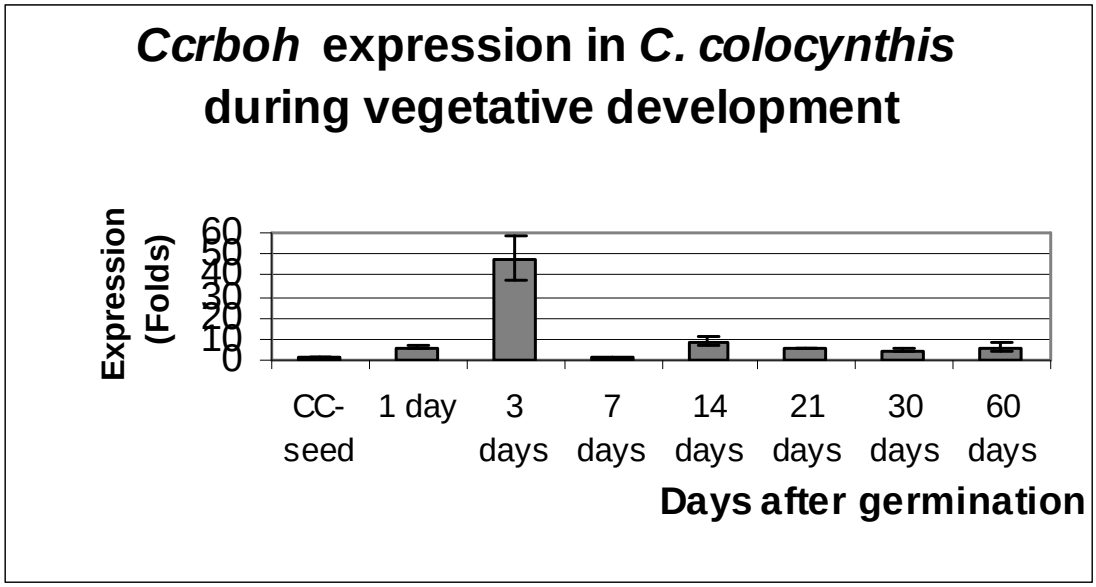


Fig.8 Comparison of expression profiles of *Ccrboh* gene in grafted plants during drought (PEG) treatments. Gene expression was normalized by comparing ΔΔCt to control (0 h). CLL/CC: *C. lanatus* var. *lanatus* grafted onto *C. colocynthis* rootstock; CC/CLL: *C. colocynthis* grafted onto *C. lanatus* var. *lanatus* rootstock.

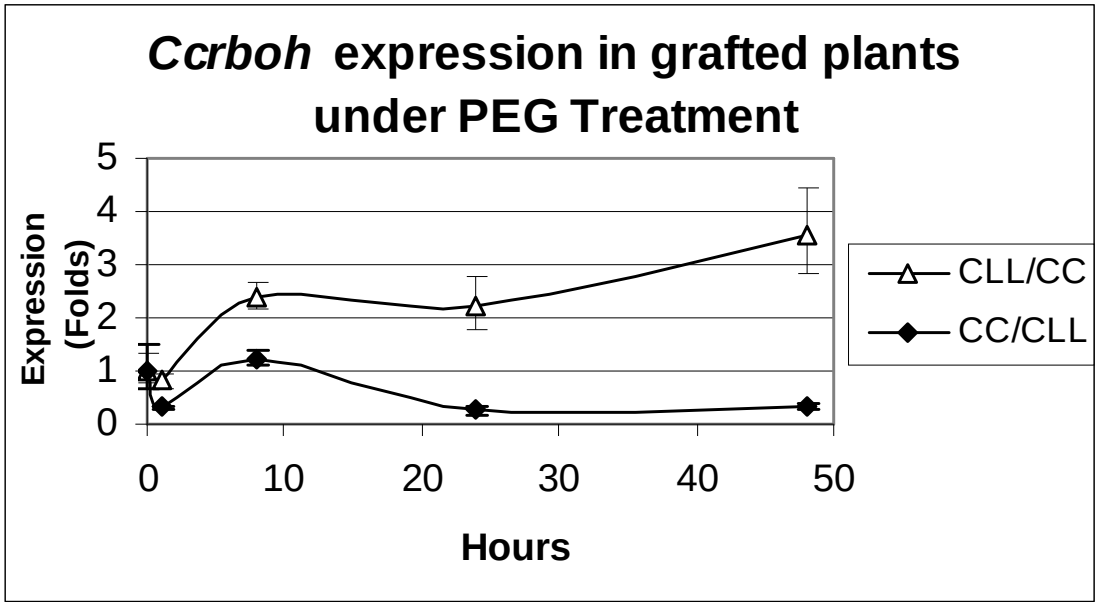
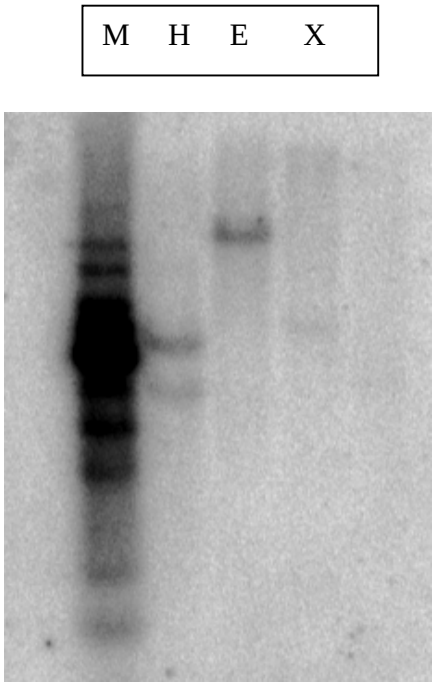
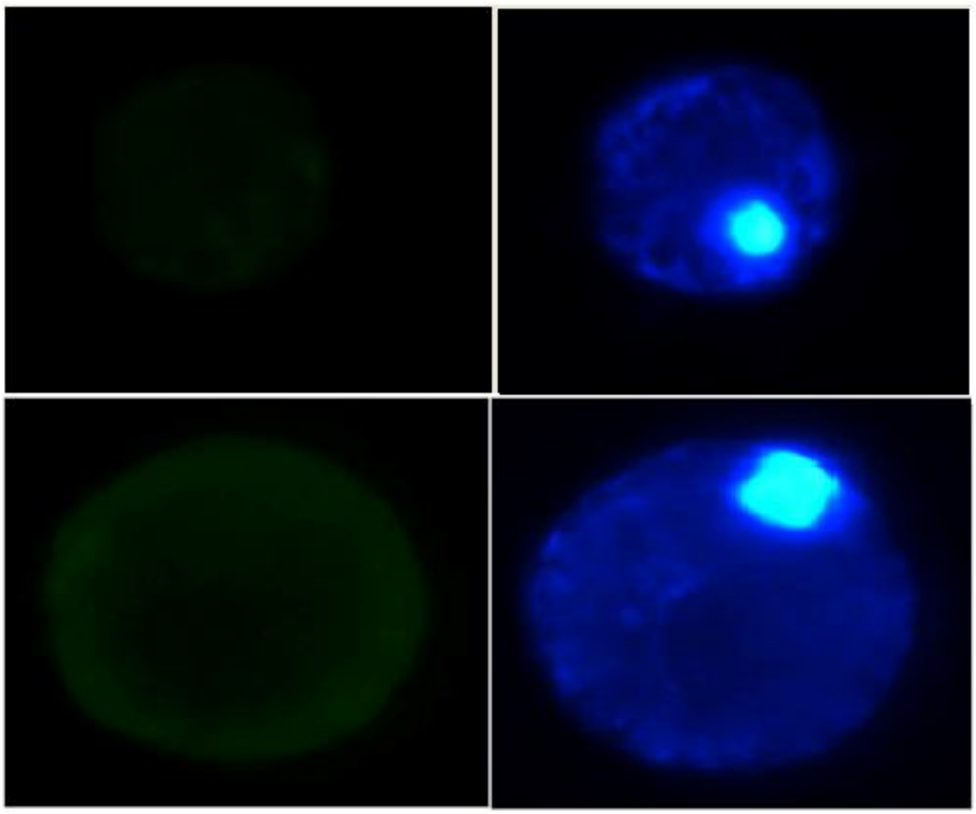


Fig. 9 Southern blot analysis of *Ccrboh*. Genomic DNA digested with *Hind*III, *Eco*RV, or *Xba*I, respectively, followed by hybridization using full length gene as probe.



M: 1 kb DNA marker; H: *Hind*III; E: *Eco*RV; X: *Xba*I

Fig. 10 The subcellular localization of Ccrboh protein. Left: Visualization of Ccrboh::GFP at plasma membrane; Right: Visualization of nuclei using Hoechst 33342 stain under UV light; Top: Control, pEarleyGate 103 vector; Bottom: Ccrboh::GFP fusion vector.



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