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Cloning and expression analysis of the *Ccrboh* gene encoding respiratory burst oxidase in *Citrullus colocynthis* and grafting onto *Citrullus lanatus* (watermelon)

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Abstract

A full-length drought-responsive gene *Ccrboh*, encoding the respiratory burst oxidase homologue (rboh), was cloned in *Citrullus colocynthis*, a very drought-tolerant cucurbit species. The robh protein, also named NADPH oxidase, is conserved in plants and animals, and functions in the production of reactive oxygen species (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 homologous *rboh* 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 the rootstock or scion. Results showed that the rootstock significantly affects gene expression in the scion, and some signals might be transported from the root to the shoot. *Ccrboh* in *C. colocynthis* was found to function early during plant development, reaching high mRNA transcript levels 3 d after germination. The subcellular location of Ccrboh was investigated by transient expression of the 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 during the acclimation of plants to stress and also in plant development. It holds great promise for improving drought tolerance of other cucurbit species.

Key words: *Citrullus colocynthis*, *C. lanatus*, drought stress, grafting, NAPDH oxidase, RBOH, watermelon.

Introduction

Water deficit is considered to be the main environmental stress of plants and a major constraint of plant growth and productivity. Tolerance to drought stress is a complex phenomenon, comprising a number of physio-biochemical processes 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 defence response. ROS can function as signalling molecules that mediate responses to various processes in plant cells such as development, pathogen defence, programmed cell death, and stomatal behaviour. Plants have evolved mechanisms of ROS generation, signalling, and removal during development, and biotic and abiotic stress (Apel and Hirt, 2004; Miller *et al.*, 2009).

The respiratory burst oxidase homologue (rboh), also named NADPH oxidase, in mammalian neutrophils has two components located in the plasma membrane (gp91^{-phox} and p22^{-phox}), which interact with several cytosolic proteins (p47^{-phox}, p67^{-phox}, and the small G protein Rac) to become active (Wientjes and Segal, 1995). RBO homologues in the 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 ten members (Atrboh A–J) 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} homologues *AtrbohD* and *AtrbohF* from *Arabidopsis*, *NtrbohD* from *Nicotiana tabacum*, and tomato *rboh* were shown to be required for ROS accumulation in plant defence responses (Simon-Plas *et al.*, 2002; Torres *et al.*, 2002; Sagi *et al.*, 2004; Miller *et al.*, 2009; Pogany *et al.*, 2009). 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 signalling for stomatal closure (Kwak *et al.*, 2003). Ethylene also functions in regulating stomatal aperture, inducing stomatal closure dependent on H_2O_2 production in guard cells generated by AtrbohF (Desikan *et al.*, 2006). These data suggest a complex signalling network with interaction between rbohs and other signalling molecules. In maize, a cross-talk between Ca^{2+} and ROS generated by NADPH oxidase is involved in the ABA signalling 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 the antioxidant defence system against oxidative damage. Ca^{2+} functions upstream and downstream of ROS production in signal transduction in plants. Ogasawara *et al.* (2008) showed that Ca^{2+} binding and phosphorylation synergistically activate the ROS-producing enzyme activity of AtrbohD.

The family Cucurbitaceae includes several economically important cultivated species such as watermelon (*Citrullus lanatus* var. *lanatus*), melon (*Cucumis melo* L.), cucumber (*C. sativus* L.), squashes, pumpkins, and gourds (*Cucurbita* species). Watermelons are often grafted onto *Cucurbita moschata*, *C. maxima*, *Benincasa hispida*, or *Lagenaria siceraria* to impart levels of resistance to soil-borne pathogens (such as *Fusarium oxysporum*), low soil temperatures, salinity, and water stress (Chouka and Jebari, 1999; Lee, 1994; Yetisir *et al.*, 2003, 2006; Yetisir and Sari, 2003). One species, *C. colocynthis*, in the genus *Citrullus* is widely distributed in the Sahara–Arabian desert areas and well adapted to drought stress (Dane *et al.*, 2006). It is a potential rootstock to improve the drought tolerance of watermelon.

Since rboh proteins are important components of signalling pathways, the aim of this study was to isolate and identify the *rboh* gene from drought-tolerant *C. colocynthis*, and to gain information on its 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 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 °C to 30 °C and ambient relative humidity. A half-strength Hoagland's nutrient solution (PhytoTechnology Laboratories, Shawnee Mission, KS) was used to irrigate plants daily after germination.

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

Seedlings at the 5–6 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 –80 °C. Relative water content (*RWC*) was measured during PEG treatment according to the method by Smart and Bingham (1974). For other treatments, seedlings at the 5–6 leaf stage were treated with 100 μM ABA, 1 mM SA, 50 μM JA or 150 mM NaCl by spraying and/or irrigation. Shoots and roots were harvested at 8 h for ABA, 4 h for SA, 4 h for JA, and 24 h for NaCl treatment. Shoots 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, and 60 d after germination.

RNA isolation and cDNA synthesis

RNA was extracted from roots or shoots according to the 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. cDNA was synthesized using RETROscript™ (Ambion).

Cloning of full-length Ccrboh cDNA using 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 sativum*, *Lycopersicon esculentum*, and *Nicotiana tabacum*, deposited in GenBank. PCR analysis was initiated with the hot start method

Table 1. Oligonucleotide primer sequences for Ccrboh cDNA cloning and relative quantitative real-time RT PCR

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

using a 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* were chosen from the NCBI database. Multiple sequence alignment was carried out with CLUSTAL W at the default setting. Treeview software was used for displaying the phylogenetic trees, and pSORT was used to predict protein localization.

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 the reference gene, was amplified in parallel with the target gene allowing normalization of gene expression and providing quantification. Primers were designed based on conserved regions, and primer sequences of the *Ccrboh* (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 PCR Master mix kit (Applied Biosystems) following the manufacturer's recommendations. Quantification of the relative transcript levels was performed using the comparative C_T method. The induction ratio (*IR*) was calculated as recommended by the manufacturer and corresponds to $2^{-\Delta\Delta CT}$, where

$$\Delta\Delta C_T = \left(C_{T,\,\text{Target gene}} - C_{T,\,\text{actin}}\right)_{\text{stressed}} - \left(C_{T,\,\text{Target}} - C_{T,\,\text{actin}}\right)_{\text{control}}$$

Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in the target sample versus the reference sample (Pfaffl, 2001).

Southern blot analysis

Genomic DNA isolated from *C. colocynthis* seeds (20 µg per sample) was digested with different restriction enzymes (*Hind*III, *Eco*RV, or *Xba*I 20 unit g^{-1} DNA) for 16 h at 37 °C, followed by separation on a 0.8% agarose gel. After electrophoresis, gels were washed with water and 10× SSC and blotted with Hybond N^+ (Amersham Pharmacia Biotech, Piscataway, NJ) prewetted with 10× 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-labelled probe. Full-length cDNA of the *Ccrboh* gene as a probe was obtained by PCR using the following gene-specific primers: CcrbohFW3 and CcrbohRV4 (Table 1).

Green fluorescent protein (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 cut pENTR vector and gene were ligated using T4 DNA ligase (Invitrogen). Entry clones containing the *Ccrboh* gene lacking a stop codon were transferred from the entry clone vector to the destination clone vector pEarleyGate 103 with GFP on C-terminal using the LR reaction (Earley *et al.*, 2006).

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 leaf mesophyll 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 previously (Sheen, 1991; Rashotte *et al.*, 2006). Electroporation was typically carried out with $1–2\times10^5$ protoplasts in 300 µl of wash solution and about 40–50 µg of plasmid DNA, and treated for electroporation at 300 V in a 0.1 mm electroporation cuvette using an Eppendorf Electroporator 2510. After overnight incubation in the dark, protoplasts were examined under 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 dye initially to observe cells and to identify nuclei in intact cells as a measure of the cells' viability. A GFP filter that blocks both chlorophyll fluorescence and Hoechst 33342 fluorescence was used to examine the localization of GFP fusion proteins. All photographs were taken with a Qimaging Fast 1394 digital camera and composed as a composite image in Adobe Photoshop CS3.

Results

Cloning and sequence analysis of the Ccrboh gene

The cDNAs *Ccrboh* (EU580727) encoding respiratory burst oxidase protein was cloned from *C. colocynthis* and sequenced. Sequence analysis indicated that the full-length cDNA contains the 5′-UTR, the complete open reading frame (ORF), 3′-UTR and the Poly (A) tail. *Ccrboh* has a 2781 bp ORF encoding a protein of 926 amino acids. The 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 the N-terminal. Six tentative transmembrane segments were predicted by pSORT. In *Arabidopsis*, the EF-hands are present in the third small exon of *rbo* 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 rbohs 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 (Sagi 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, the FAD-binding domain, and the NAD-binding domain which have greater overall sequence similarity to gp91[phox] in animals (Torres *et al.*, 1998; Torres and Dangl, 2005). Phylogenetic analysis of Ccrboh and 10 rboh proteins from *Arabidopsis* showed that Ccrboh has high homology to the AtrbohD (Fig. 1A). Plant rbohs are structurally related to human NOX and are part of a large superfamily that arose early in evolution before the animal/plant divergence (Fluhr, 2009).
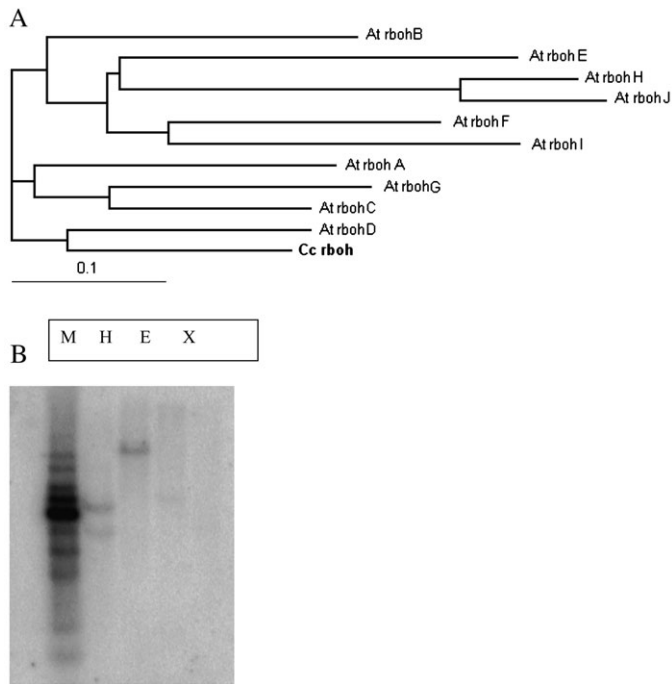
Fig. 1. (A) Phylogenetic tree of Ccrboh and 10 Arabidopsis rbohs. Ccrboh (EACF05505), AtrbohJ (Q9LZU9), Atrbohl (Q9SUT8), AtrbohC (O81210), AtrbohD (Q9FIJ0), AtrbohA (O81209), AtrbohG (Q9SW17), AtrbohF (O48538), AtrbohE (O81211), AtrbohB (Q9SBl0), and AtrbohH (Q9FJD6). (B) Southern blot analysis of *Ccrboh*. Genomic DNA digested with *Hin*dIII (H), *Eco*RV (E), or *Xba*I (X), respectively, followed by hybridization using full-length gene as probe. M=1 kb DNA marker.

To investigate the genomic organization of the *Ccrboh* gene in *C. colocynthis*, genomic DNA was digested with *Hin*dIII, *Eco*RV, or *Xba*I, respectively, and hybridized with a probe, which was a full-length cDNA of the *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 *Hin*dIII), and one band in the second (digested with *Eco*RV) and third lane (digested with *Xba*I) under high stringency conditions (Fig. 1B), indicating that *Ccrboh* potentially exists as one or two copies in the genome.

Expression profiles of the Ccrboh *gene in* C. colocynthis

Leaf *RWC* of *C. colocynthis* was measured to define the induction of the drought condition following PEG treatment. The leaf *RWC* in control *C. colocynthis* and watermelon was 90% and 95%, respectively. Upon treatment with PEG, the leaf *RWC* of both species decreased rapidly to 75% within 12 h. However, *C. colocynthis* gradually adjusted its leaf *RWC* to maintain an 80% level during treatment (Si *et al.*, 2009), while the *RWC* of watermelon continually decreased to 68% resulting in the wilting of leaves. This indicates that *C. colocynthis* has some mechanism to cope with drought stress.

Ccrboh was induced in both roots and shoots (Fig. 2) following drought (PEG) treatment, but the induction in
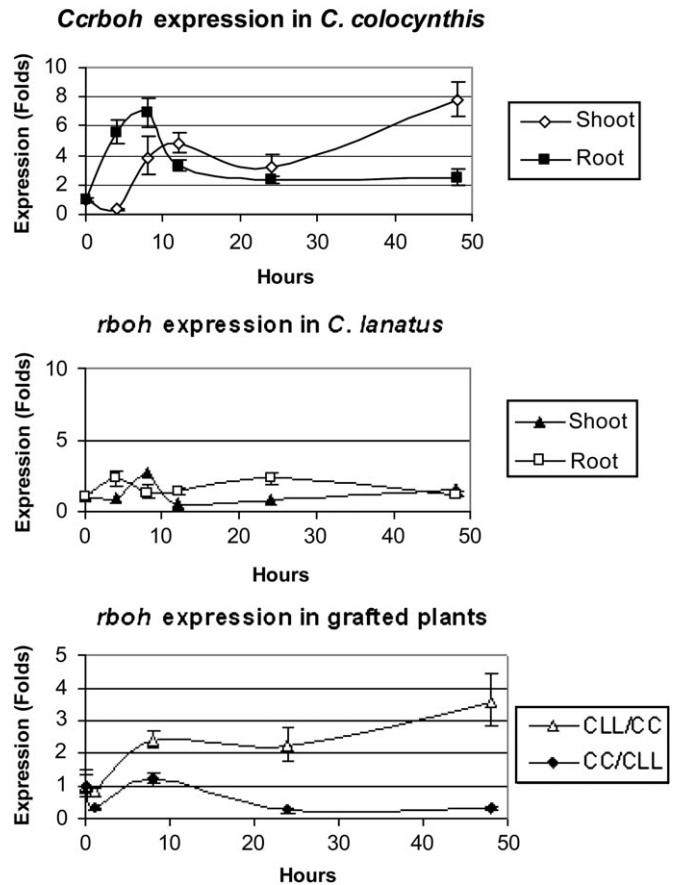
Fig. 2. Comparison of expression profiles of the *rboh* gene in the root and shoot of *C. colocynthis* and *C. lanatus* var. *lanatus* during drought (PEG) treatments and grafting. CLL/CC: *C. lanatus* var. *lanatus* grafted onto the *C. colocynthis* rootstock; CC/CLL: *C. colocynthis* grafted onto the *C. lanatus* var. *lanatus* rootstock. Gene expression was normalized by comparing ΔΔCt to control (0 h) (*n*=3).

roots was 4 h earlier than in shoots. The highest induction level in roots was between 4 h and 8 h and decreased to control levels after 12 h, while it was highly induced in shoots after 12 h of treatment 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 programmes 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; Fluhr, 2009).

Ccrboh was also induced by ABA, JA, SA, but not NaCl in a tissue-specific pattern (Fig. 3). The results are similar to other reports, showing that application of ABA, IAA, or BA leads to the 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). SA and JA act as local and systemic signal molecules in plant defence against pathogen attack and a complex interplay between SA and rboh occurs in
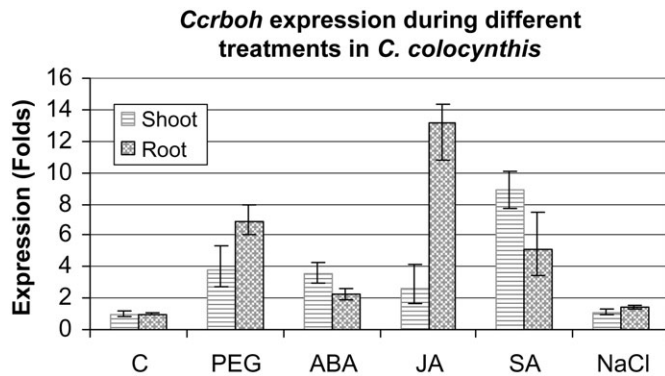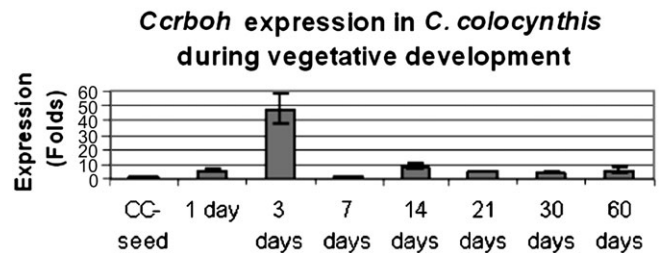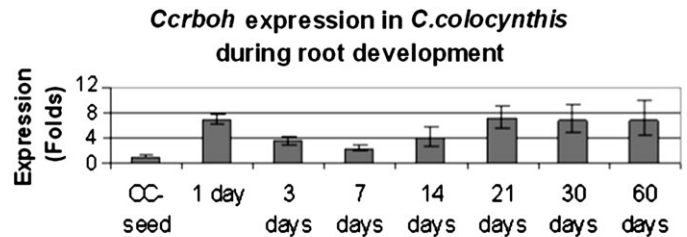
Fig. 3. Comparison of expression profiles of the *Ccrboh* gene in the root and shoot during different treatments in *C. colocynthis*. Gene expression was normalized by comparing ΔΔCt to control (0 h) (*n*=3).


Fig. 4. Comparison of expression profiles of the *Ccrboh* gene in the roots and shoots following days after germination in *C. colocynthis* (CC). Gene expression was normalized by comparing ΔΔCt to control (CC seeds) (*n*=3).

the regulation of cell death (Torres *et al.*, 2005; Pogany *et al.*, 2009). Tissue-dependent variations in gene expression patterns under different abiotic stress conditions have been documented in many plant species (Kawasaki *et al.*, 2001; Kreps *et al.*, 2002; Liu and Baird, 2003; Baisakh *et al.*, 2006). Regulation of the temporal and spatial expression patterns is an important part of the plant stress response.

To understand how Ccrboh functions in plant development, the expression of the *Ccrboh* gene was analysed during root and vegetative growth (Fig. 4) using *C. colocynthis* seeds as a control. In roots, *Ccrboh* expression levels showed a 7-fold increase in 1-d-old roots, followed by a 3–4 fold increase in 3–14-d-old roots, and 7-fold induction in older seedlings. In shoots, *Ccrboh* expression levels increased 1 d after germination, and then dramatically increased up to 48-fold in the 3-d-old seedlings, followed by a reduction to 5–10 fold in 7–60-d-old seedlings. The results indicate that the *Ccrboh* gene also functions during root growth and leaf morphogenesis.

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

Watermelon plants, treated with 20% PEG 8000 to induce drought stress, were examined at 0, 4, 8, 12, and 48 h. Several pairs of primers were designed based on the conserved regions of rbohD. After testing, primers CcrbohFW4 and CcrbohRV2 (Table 1) were used to analyse *rboh* gene expression in two species. The *rhoh* mRNA transcript levels in watermelon did not change in roots and shoots during PEG treatment (Fig. 2). Interestingly, when watermelon was grafted onto a *C. colocynthis* rootstock, *rboh* was induced following 8 h of treatment and continually increased up to 48 h in watermelon scions (Fig. 2). The expression pattern in grafted plants [watermelon scion/*C. colocynthis* rootstock (CLL/CC)] was the same as that in non-grafted *C. colocynthis*, but the induction level was lower (Fig. 2). The *Ccrboh* gene expression level did not change in *C. colocynthis* grafted onto watermelon rootstock

(CC/CLL) during drought (Fig. 2). Miller *et al.* (2009) reported that plants can mediate rapid cell to cell communication over a long distance which is dependent on the *rbohD* gene. This grafting experiment indicates that the rootstock is important for the regulation of genes in the scion as a result of long-distance signalling of ROS (Miller *et al.*, 2009), ABA (Thompson *et al.*, 2007), microRNA (Ruiz-Medrano *et al.*, 1999), or some small proteins (Corbesier *et al.*, 2007).

Subcellular localization of the Ccrboh protein

To address the subcellular localization of Ccrboh in living cells, a construct containing Ccrboh fused in-frame with GFP driven by the *CaMV* 35S promoter (35S::Ccrboh::GFP) was transiently expressed in leaf protoplasts. Hoechst 33342 dye was used to observe if protoplasts were intact and viable initially (Fig. 5, right panels). As a control the unconjugated GFP vector was transformed into protoplasts as shown in Fig. 5 (bottom), revealing cells that exhibit a diffused distribution of green fluorescence throughout the cell. By contrast, when GFP was fused with Ccrboh, the GFP signal was confined to the outermost region of the cell (Fig. 5, top), which could be followed under high magnification by focusing through the *z*-axis of the cell. This result is consistent with the localization of the Ccrboh protein to the plasma membrane, in agreement with results from previous studies using the cellular fractionation of plant tissues in tobacco (Sagi and Fluhr, 2001; Simon-Plas *et al.*, 2002), and localization of NtrbohD on chemically distinct membrane microdomains called lipid rafts (as reviewed by Fluhr, 2009). It also confirmed the prediction by pSORT that the Ccrboh protein has six transmembrane segments, and is localized to plasma membrane with a probability of 52.2%.
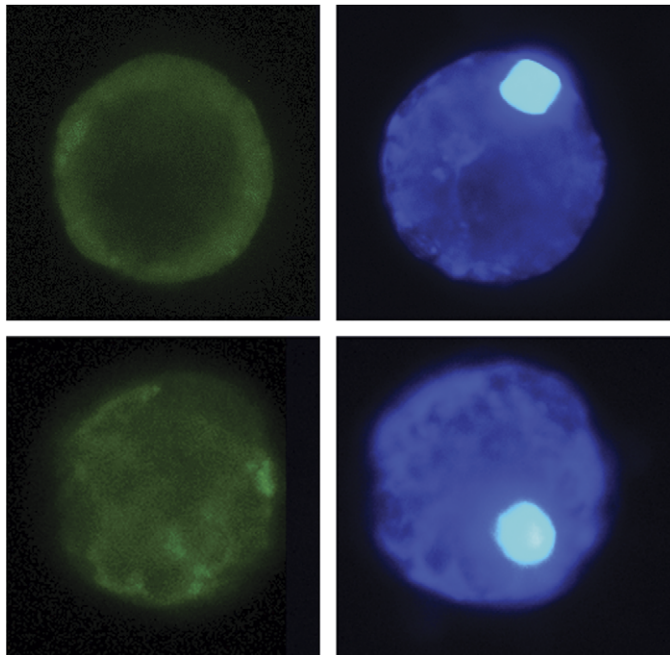
Fig. 5. The subcellular localization of the Ccrboh protein in *C. colocynthis* protoplasts. A representative example of 35S::Ccrboh::GFP fusion in a leaf mesophyll protoplast of *C. colocynthis* (top) compared with a protoplast transformed with the control vector pEarleyGate 103 (bottom). Protoplasts were visualized under UV light in the presence of Hoechst 33342 dye showing the subcellular position of the nucleus (right). Visualization using a GFP wavelength filter (left) blocks general background and Hoechst dye fluorescence thus revealing the location of Ccrboh to the outermost region of the cell (top) in contrast to the control with diffusely distributed fluorescence around the cell (bottom).

Discussion

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–F* are also induced by various biotic stresses. AtrbohD is identified as the major constitutively active form (Torres *et al.*, 2002). *Ccrboh* was induced by hormones such as ABA, JA, and SA (Fig. 3). The results correspond with other reports, showing that application of ABA, IAA, or BA leads to the 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; as reviewed by Mori *et al.*, 2009). 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 up-regulation of rboh levels. 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). It was recently discovered that the initiation and propagation of a systemic signal in *Arabidopsis* requires the function of the *rbohD* gene. Surprisingly, this systemic signal is independent of JA, SA or ethylene, hormones known to be involved in stress and wounding responses and ROS production (Miller *et al.*, 2009). It is possible that ROS act downstream of these hormones.

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*) has 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. 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 that rboh proteins function in a plethora of developmental effects in many plant organs, and imply the involvement of a range of hormones (Harir and Mittler, 2009; Mori *et al.*, 2009). This is not surprising, because ROS are required for cell expansion during the morphogenesis of organs such as roots, leaves, and pollen (as 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).

Domesticated watermelons have been selected for their productivity and quality. Domestication and breeding have contributed to an increased susceptibility to environmental stresses, diseases, and pests. For this reason, watermelons are often grafted onto different rootstocks. Root characteristics are of primary importance in determining stress tolerance in plant species (Fernández-García *et al.*, 2002; Jensen *et al.*, 2003; Ruiz *et al.*, 2006). The identity and relative contribution of signals from the root during water deficit conditions remains controversial (Schachtman and Goodger, 2008), but rboh appears to be a highly regulated, sensitive, and versatile mediator of developmental and environmental signals (Miller *et al.*, 2009). Since *rboh* gene induction was observed in watermelon grafted onto a *C. colocynthis* rootstock during drought, *C. colocynthis* can be considered as a potential rootstock for watermelon production or as a source for improving drought tolerance via gene manipulation. However, how the rootstock affects the fruit quality and how the grafted plants perform under stress conditions in the field environment needs to be investigated further.

Conclusions

In summary, a full-length cDNA clone, *Ccrboh*, encoding respiratory burst oxidase has been identified in *C. colocynthis*. Sequence analysis showed that Ccrboh is highly

homologous to AtrbohD. *Ccrboh* transcript was expressed in a tissue-specific pattern following drought stress, ABA, JA, and SA treatment in *C. colocynthis* seedlings. Although no change in *rboh* transcript level was observed in watermelon under drought stress, the *rboh* gene was induced in the watermelon scion grafted onto a *C. colocynthis* rootstock. *Ccrboh* also functions in leaf morphogenesis because of changes in expression that were detected during vegetative growth. Transient expression of Ccrboh::GFP fusion protein in protoplasts confirmed that the Ccrboh protein is localized on the plasma membrane. 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.

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