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Molecular Characterization, 3D Model Analysis, and Expression Pattern of the *CmUBC* Gene Encoding the Melon Ubiquitin-Conjugating Enzyme Under Drought and Salt Stress Conditions

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Abstract Ubiquitin-conjugating (UBC) enzyme is a key enzyme in ubiquitination. Here, we describe the cloning, characterization, and expression pattern of a novel gene, *CmUBC*, from a melon. Comparison of the deduced amino acid sequences allowed the identification of highly conserved motifs. Synteny analysis between *Cucumis sativus* L. and *Arabidopsis* demonstrated that homologs of several *Cucumis* UBC genes were found in corresponding syntenic blocks of *Arabidopsis.* The homology structure model of the CmUBC protein was constructed. UBCs from melon, yeast, and *Arabidopsis* were highly conserved in their three-dimensional folding. *CmUBC* was ubiquitously expressed in all melon tissues. Increased transcript levels of *CmUBC* were observed during drought and salinity stresses, which suggested that the expression of the *CmUBC* gene in melon plants is responsive to physiological water stress. These results suggested that the *CmUBC* gene might play an important role in the modulation of the ubiquitination pathway.

Keywords *Cucumis melo* L. · Ubiquitin-conjugating enzyme (UBC) · 3D model analysis · Gene expression · Water-stress responses

GenBank Accession No. The *CmUBC* gene was deposited in GenBank under the accession number EU853458.

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Introduction

Plant growth, development, and productivity are adversely affected by abiotic stresses, resulting in the activation of a series of morphological, physiological, biochemical, and molecular changes (Bhatnagar-Mathur et al. 2008). Drought and high salinity are the most frequently encountered abiotic stresses for plants. Although a large number of genes that are regulated by salt and drought stress have been identified (Bray 1997; Zhu 2002), the biological functions of many genes that are related to abiotic stress are still largely unknown in higher plants (Zhou et al. 2010). Therefore, it is important to determine the functions of stress-related genes in order to improve crop tolerance to salt and drought.

Because of its ability to degrade intracellular proteins, ubiquitination is essential for cell growth, regulation of diverse signal transduction, photomorphogenesis, hormone regulation, floral homeosis, senescence, and pathogen defense (Pickart 2001; Suzuki et al. 2002; Xie et al. 2002; Hellmann and Estelle 2002; Devoto et al. 2003). Previous studies have suggested that ubiquitin pathway genes may have a role in plant tolerance against various abiotic stresses. The *Arabidopsis* gene *AtCHIP* that encodes an E3 ubiquitin ligase is upregulated by several stress conditions, such as low and high temperatures, and its overexpression in *Arabidopsis* plays a critical role in temperature stress tolerance (Yan et al. 2003). *HOS1* (Lee et al. 2001) and *SDIR1* (Zhang et al. 2007) encode a RING-finger-protein E3 ubiquitin ligase, which regulates cold-responsive gene expression and enhances drought tolerance through ABA-induced stomatal closure in *Arabidopsis*. Like E3, E2 also plays an important role in stress responses in plants. Many genes that encode the ubiquitin-conjugating (UBC) enzyme have been identified in eukaryotic organisms. There are approximately 11 UBCs in the yeast (*Saccharomyces cerevisiae*), 37 in the *Arabidopsis*, and 50 in the human genomes (Bachmair et al. 2001; Jiang and Beaudet 2004; Kraft et al. 2005). The *RAD6* gene encoding E2 (UBC2) has been shown to provide postreplication repair of ultraviolet (UV)-damaged DNA and induce mutagenesis and sporulation in *S. cerevisiae* (Reynolds et al. 1985). *AtUBC2* from *Arabidopsis thaliana,* which is a structural homolog of *RAD6*, can partially rescue the UV sensitivity in *S. cerevisiae* rad6 mutants (Zwirn et al. 1997). Xu et al. (2009) have reported that Atubc1-1 and Atubc2-1 double mutants show a dramatically reduced number of rosette leaves, which is an early flowering phenotype, and reduced transcript levels of a set of floral repressor genes. Zhou et al. (2010) have cloned the UBC gene *GmUBC2* from soybean, and they have shown that transgenic *Arabidopsis* plants overexpressing *GmUBC2* are more tolerant of salinity and drought stresses compared with control plants. The constitutive expression of the peanut *AhUBC2* gene in wild-type *Arabidopsis* has been shown to confer improved tolerance of water stress that is induced by sorbitol or soil drought in 35S:AhUBC2 transgenic plants (Wan et al. 2011).

UBCs have been shown to play an important role in regulating various aspects of plant growth and development, but the role of plant UBCs in abiotic stress responses needs to be examined further. In the present study, we identified, molecularly characterized, conducted three-dimensional (3D) model analyses, and performed expression profiling of a melon *CmUBC* gene. Transcript level changes of *CmUBC*

during drought and salinity stresses suggested that *CmUBC* may be involved in responses to oxidative stress.

Materials and Methods

Plant Culturing and Stress Applications

The Galia type of Turkish melon was used for the gene expression studies with quantitative polymerase chain reactions (qPCR). The seeds were kindly provided by Batı Akdeniz Agricultural Research Institute (Antalya, Turkey). The seed coats were removed, and the seeds were washed with distilled water three times. Then, they were transferred to plastic containers and grown in hydroponic culture containing half-strength Hoagland's solution (Hoagland and Arnon 1950) for 14 days in a plant growth chamber at 24 ± 2°C with a 16-h light and 8-h dark photoperiod at a light intensity of 400 μmol m^{-2} s^{-1}. All stress treatments were initiated on day 14 of normal growth. For drought stress, 10% polyethylene glycol 6000 (PEG-6000) was added to the half-strength Hoagland's solution; for salt stress, 200 mM sodium chloride was added. Both treated (stress) and nontreated (control) plants were kept in the growth chamber with the same growth conditions. Leaf samples from the treated and control plants were harvested after 1, 3, 6, 24, and 48 h of stress application. Time point zero (0 h) was used as a control. The leaf tissues from three biological replicates were collected and immediately frozen in liquid nitrogen.

Total RNA Isolation and Cloning of *CmUBC*

Total RNA extraction was performed with the Trizol reagent (Life Technologies Corp., Grand Island, NY, USA) according to the method of Chomczynski (1993). DNA contamination in samples was removed with DNase I (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. A Quant-iT RiboGreen RNA assay kit (Life Technologies Corp.), which employs a sensitive fluorescent dye, was used for the quantification of RNA samples. The fluorescence was recorded with a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific Inc.). The RNA concentrations of the samples were determined according to the standard curve generated with at least five different dilutions (1–10 μg/mL) of ribosomal RNA standard. The quality and integrity of the total RNA was checked with agarose gel electrophoresis and the Agilent 2100 Bioanalyzer.

The full-length *CmUBC* gene was cloned with the rapid amplification of cDNA ends (RACE) method, with the 5′-GAATCTACGCTCGAAGATCGACATACC-3′ oligonucleotide primer for the 5′ Race reaction and the 5′-TTTTCCCCACCCG AAGTCCACATTT-3′ oligonucleotide primer for the 3′ Race reaction. These primers were designed according to the highly conserved amino acid sequences in previously identified genes encoding the UBC enzyme (E2) from various plant species. The GeneRacer 5′ and 3′ primers were used for the 3′ Race and 5′ Race

reactions, respectively. First-strand cDNA synthesis was conducted with 10 μg of total RNA. One microliter of cDNA was used for PCR with specific E2 primers. A high-fidelity PCR system (Life Technologies Corp., Paisley, UK) was used with the following PCR parameters: 2 min of template denaturation at 95°C for 1 cycle, followed by 5 cycles at 95°C (30 s), 72°C (90 s), and 72°C (90 s), and then 20 cycles at 94°C (30 s), 57°C (30 s), and 68°C (90 s), with a final 10-min extension step at 68°C. The amplified gene fragment was cloned into a pCR8/GW/TOPO vector (Life Technologies Corp.) and validated by colony PCR. The inserted cDNA was sequenced with an ABI 310 DNA sequencing system.

Nucleotide Sequence and Bioinformatics Analyses

Primer finding and open-reading-frame determinations for the novel melon *E2* cDNA sequence were performed with Mega5 (Tamura et al. 2011). The protein sequence was deduced and analyzed with Sequence Annotated by Structure (SAS), which was developed by EMBL-EBI (Cambridgeshire, UK). Blastn and Blastp programs were utilized for the identification of the homologous sequences of the melon *CmUBC* cDNA sequence and the protein sequence in GenBank, respectively.

The gene sequences of the melon UBC enzyme (*CmUBC*) were phylogenetically compared to those of other species found in GenBank. Multiple alignments were conducted with the ClustalW program in Mega5 (Thompson et al. 1994). In order to illustrate the phylogenetic relationships among the sequences, a phylogenetic neighbor-joining tree was constructed based on the uncorrected p distance method. The reliability of the tree was evaluated by bootstrapping 1,000 replicates.

Tandem duplications of UBC genes in the *Cucucmis sativus* genome were predicted by determining their physical locations on individual chromosomes with the Cucumber Genome DataBase (Han et al. 2008; Ren et al. 2009). Tandemly duplicated genes were defined as adjacent homologous genes on a single chromosome, with no more than one intervening gene. For the synteny analysis, syntenic blocks within the *C. sativus* genome, as well as between the cucumber and *Arabidopsis* genomes, were downloaded from the Plant Genome Duplication Database (Tang et al. 2008), and those containing cucumber and *Arabidopsis UBC* genes were identified.

Homology Modeling of *CmUBC*

Homology models were constructed with Molecular Operating Environment software (MOE 2006.08; Chemical Computing Group, Montreal, Quebec, Canada). The CmUBC amino acid sequence was aligned with *S. cerevisiae* UBC4 [Protein Data Bank (PDB) code, 1QCQ] and *A. thaliana* UBC1 (PDB code, 2AAK) with MOE's multiple sequence and structural alignment algorithm with the structural alignment tool and the BLOSUM62 substitution matrix. The alignment of the CmUBC was based on both the sequence and structural homology with the experimentally derived structure of *S. cerevisiae* UBC4 and *A. thaliana* UBC1. The 3D structure models were formed with the MOE homology program (Azad et al. 2011) and were based on a segment matching procedure. The stereochemical quality

of the models was assessed by structural analyses with the Protein Report Function of the MOE Protein Structure Evaluation. In order to evaluate how much the structural models deviated from the template, the root-mean-square deviation value between the template and the superposed model was determined by SuperPose Version 1.0 (Maiti et al. 2004).

Expression Analysis of *CmUBC* in Tissues

Evaluation of *CmUBC* gene expression levels in different organs of melon was achieved by reverse transcription (RT)-qPCR. An aliquot of 1 μg of total RNA was pretreated with DNase I and used as a template for cDNA synthesis in 20-μL reactions with random hexamers (Qiagen, Hilden, Germany). Forward and reverse primers were designed according to the melon *CmUBC* cDNA sequence and tested to ensure amplification of single discrete bands with no primer-dimers. The forward primer was 5′-TCGAGGAATTAATGGCTTCAAAGC-3′. The reverse primer was 5′-GCTGTAGTCCACTATCCCATAG-3′. It has been previously shown that the expression of the *Cucumis melo 18SrRNA* gene was stable across all tissues and conditions tested (Baloglu et al. 2011). The *Cm18SrRNA* gene was used for normalization. The *Arabidopsis 18SrRNA* forward primer 5′-AAACGGCTACCAC ATCCAAG-3′ and the *Arabidopsis 18SrRNA* reverse primer 5′-CCCATC CCAAGGTTCAACTA-3′ were used for *18SrRNA* amplification. RT-qPCR was performed in triple technical replicates for each sample. The target cDNA was amplified according to that previously described in the section on cloning. The amplification products that belonged to different plant parts were visualized on a 1% agarose gel.

RT-qPCR Analysis of *CmUBC* During Water Stress

The evaluation of *CmUBC* gene expression levels during salt and drought stress conditions was performed with a one-step RT-PCR method (Qiagen), which provided cDNA synthesis and PCR reactions in a single tube. The reactions were performed with the SYBR Green PCR Master Mix (Qiagen) and analyzed in the Corbett Rotor-Gene 6000. RT-qPCR reactions were conducted in a total volume of 20 μL containing 0.5 μM of each forward and reverse primer, 1× QuantiTect SYBR Green RT-PCR Master Mix, 0.2 μL QuantiTect RT Mix, and 100 ng total RNA from the leaf tissues of control and stressed melon seedlings. The *CmUBC* forward primer 5′-CTGTCTAGACTCCATATTCAGGAGGTG-3′ and the *CmUBC* reverse primer 5′-TACATCGATGCAATTTCAGGAACGAGA-3′ were designed to ensure amplification of single discrete bands with no primer-dimers. These primer pairs amplified fragments of 250 bp. The following thermal cycle conditions were used: 50°C for 30 min and 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 54°C for 30 s, and 72°C for 30 s.

In order to analyze the *CmUBC* gene expression level changes with RT-qPCR, an absolute quantification method (Baloglu et al. 2012) was performed with external standards of plasmid DNA containing fragments of *CmUBC*. *CmUBC* gene fragments were amplified with conventional PCR with primers that were also used

in real-time PCR analyses and cloned into pCR8/GW/TOPO cloning vectors. The sequences were confirmed with universal M13 forward (−20) and M13 reverse primers. The threshold cycle (C_T value) was plotted versus the $\log_{10}$ of the dilution series of seven different concentrations (10^7 and 10^1 copies/μL) of the standard to generate a standard curve. No-template control reactions that contained all qPCR components except the template were performed in each run of RT-qPCR. The specificity of the primer pairs was confirmed with melting curve analyses performed after each run. RT-qPCR was performed in triple technical replicates for each RNA sample from three biological replicates. The dilution series and *CmUBC* gene fragments were amplified in separate wells. The C_T values of the standards were determined. The C_T values of the RNA samples that were obtained from control and stressed melon plants were compared with the standard curve in order to determine the copy number of transcripts of *CmUBC* gene fragments in the samples.

Results

Isolation and Sequence Characterization of *CmUBC*

The cDNA was synthesized from total RNA for the cloning of the full length of the *CmUBC* gene with the RACE method with degenerate primers that were designed from the highly conserved amino acid sequences of previously identified *UBC*s. The *CmUBC* gene was deposited in GenBank under the accession number EU853458.

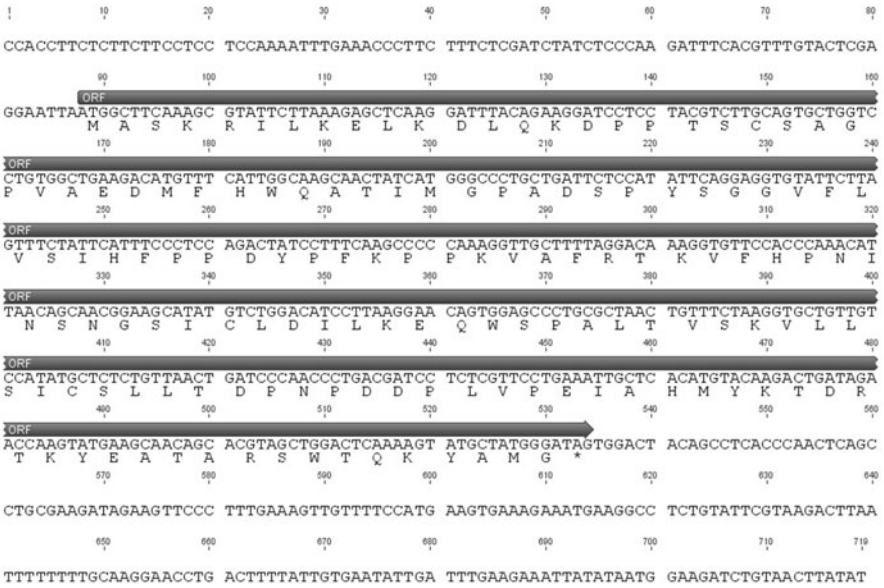

Fig. 1 Nucleotide and deduced amino acid sequences of the *CmUBC* gene. The amino acid sequence is shown in a single-letter code below each nucleotide codon. Nucleotide numbers are indicated on the ORF *box*. The *asterisk* below the nucleotide sequence indicates a stop codon

The 719-bp cDNA, which encoded the *CmUBC* gene, comprised an 88-bp 5′-untranslated region, a 444-bp open reading frame, and a 187-bp 3′-untranslated region. No introns interrupted the coding sequence. The deduced number of amino acids was 148, and the predicted polypeptide sequence of *CmUBC* was a basic protein with an isoelectric point of 7.72 and a molecular mass of 16.5 kDa (Fig. 1).

Multiple sequence alignment of the deduced amino acids of CmUBC showed that the CmUBC proteins shared high sequence similarity and sequence conservation of cotton, legume, spruce, rice, tomato, sorghum, and grape UBCs (Fig. 2). CmUBC contained a conserved active-site cysteine that was required for the catalytic activity of E2 enzymes and that was surrounded by the consensus active site motif HPN(I/V)(X)3-GX(I/V/L)C(I/L)X(I/V)(I/L), which is present in almost all plant, animal, and yeast E2 enzymes that have been characterized (Zhou et al. 2010). CmUBC is predicted to not have any signal peptides by the iPSORT prediction, which was consistent with observations in soybean GmUBC2 (Zhou et al. 2010) and peanut AhUBC2 (Wan et al. 2011).

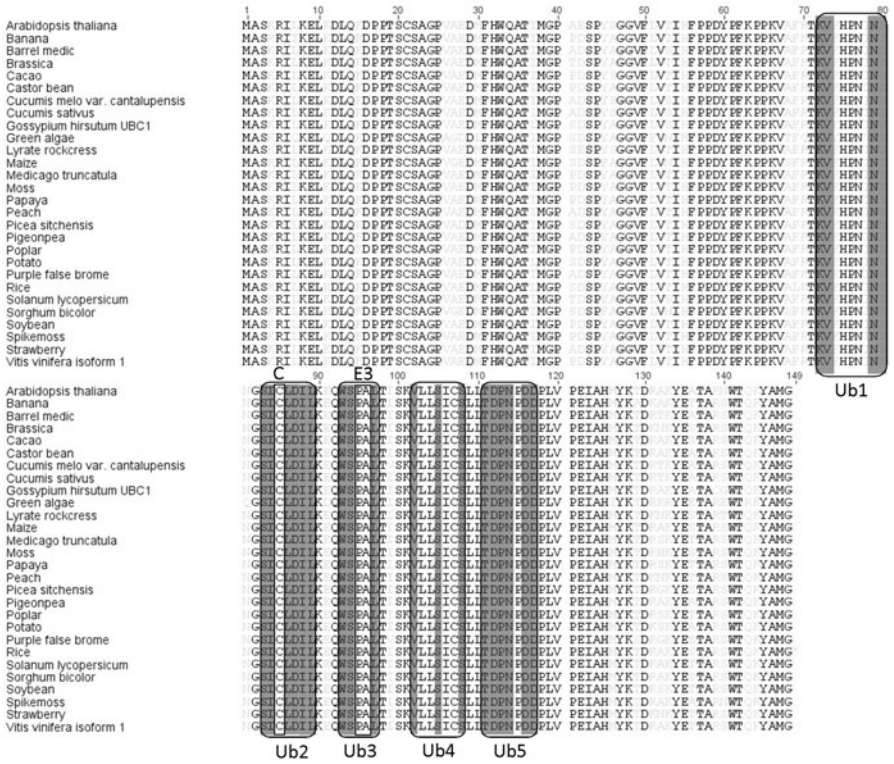

Fig. 2 Alignment of the deduced amino acid sequences of melon CmUBC with cotton, legume, spruce, yeast, tomato, sorghum, and grape. Identical amino acid residues are shaded in *gray*. Above the alignment, *C* indicates the active-site cysteine of UBCs and *E3* indicates the E3 interaction residues. Ubiquitin thioester intermediate interaction residues are bordered and shown as Ub1-5

Phylogenetic Analysis and Evolutionary Relationship Between the *UBC* Genes of Melon and *Arabidopsis*

UBC sequences from 37 different organisms, including fungi, animals, and plants, were selected for the construction of a phylogenetic tree that was used to determine the evolutionary relationship among E2s in eukaryotes and prokaryotes (Fig. 3). The resulting unrooted phylogenetic tree suggested that the plant UBC proteins are evolutionarily diverse, with the 37 UBC tested sequences classified into six subgroups. As expected, UBC protein from melon generally exhibited closer

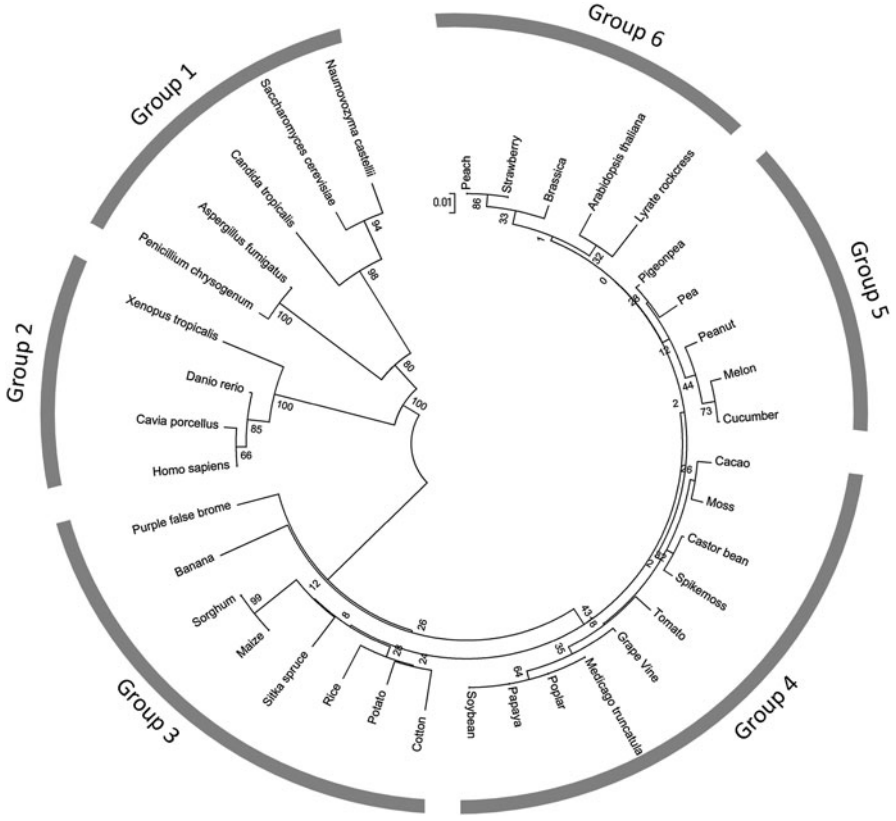

Fig. 3 Phylogenetic analysis of the amino acid sequences of melon CmUBC and homologs from animals, plants, fungi, and bacteria. Accession numbers of E2s used in the phylogenetic tree are: NP 009638 (*Saccharomyces cerevisiae*), XP 003674019 (*Naumovozyma castellii*), XP 002546386 (*Candida tropicalis*), XP 002563316 (*Penicillium chrysogenum*), XP 755003 (*Aspergillus fumigatus*), NP 001072891 (*Xenopus tropicalis*), NP 001082922 (*Danio rerio*), NP 057067 (*Homo sapiens*), XP 003465877 (*Cavia porcellus*), XP 003563818 (purple false brome), GSMUA Achr3G15810 (banana), XP 002438462 (sorghum), ACG31068 (maize), ABR16809 (*Picea sitchensis*), NP 001054191 (rice), ABA81863 (potato), AAL99222 (cotton), XP 003554749 (soybean), XP 002309194 (poplar), XP 003600541 (*Medicago truncatula*), XP 002284203 (grape vine), NP 001234437 (tomato), XP 002983205 (spikemoss), XP 002532653 (castor bean), XP 001764546 (moss), EU853458 (melon), AAV34697 (peanut), XP 002867477 (lyrate rockcress), NP 851114 (*Arabidopsis thaliana*), ACC38297 (brassica), ACB87921 (peach)

relationships to UBC proteins from dicotyledonous angiosperms than to those from monocotyledonous angiosperms. These results suggest that although plant *UBC* genes may be derived from a common ancestor, a number of them may have undergone further differentiation in monocotyledon and dicotyledon lineages.

A chromosomal synteny analysis, which is important for genomic comparison, is a relatively rapid method that is used to reveal the genomic evolution of related species. Shared synteny provides information for cross-species analyses of gene function, including the conservation of gene order, contents, and frequencies with similar traits (Choi et al. 2004). Syntenic genes are orthologs that are located in these syntenic fragments, and thus they share similar functions (Cheng et al. 2012). Because of the good characterization of the functions of *UBC* genes in *Arabidopsis*, we analyzed a comparative synteny map between cucumber and *Arabidopsis* genomes in order to provide further insights into the functions of melon UBC genes. With regard to the correspondence of cucumber and *Arabidopsis* UBC genes, the syntenic fragments included the following ortholog pairs: Csa019968 in Chr 3, AT1G64230.4; Csa011177 in Chr 4, AT2G16740.1; Csa015454 in Chr 5, AT4G27960.2; Csa007655 in Chr 6, AT3G08690.1; Csa014033 in Chr 7, AT2G16740.1; and Csa019968 in Chr 3, AT5G41700.4 (Fig. 4).

Structural Characterization and Comparison of CmUBC with UBC1 and UBC4

Based on the high fraction of sequence conservation, the homology structure model of the CmUBC protein was constructed according to its homolog from *S. cerevisiae* UBC4 (Cook et al. 1993). The 3D structure model of CmUBC showed an alpha/beta protein with four alpha-helices and a four-stranded antiparallel beta-sheet (Fig. 5). CmUBC belongs to the class of E2s that consists entirely of the relatively conserved globular core domain (Cook et al. 1992). An active-site residue (Cys85) is located in the cleft between two loops. Ubiquitin-thioester intermediate interaction residues and E3 interaction residues (E3) were found between the 70 and 120 amino acid residues. The amino and carboxy termini occurred at opposite ends of the long axis

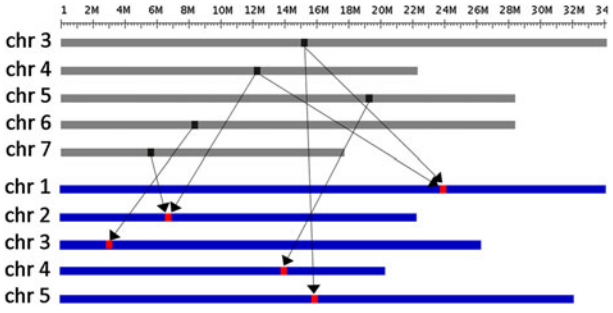

Fig. 4 Synteny analysis of *UBC* genes of cucumber and *Arabidopsis*. Chromosomes are depicted as *horizontal gray* (cucumber) and *blue* (*Arabidopsis*) *bars*. UBC genes are indicated by *vertical black* (cucumber) and *red* (*Arabidopsis*) *lines*. *Arrows* denote syntenic regions between cucumber and *Arabidopsis* chromosomes (Color figure online)

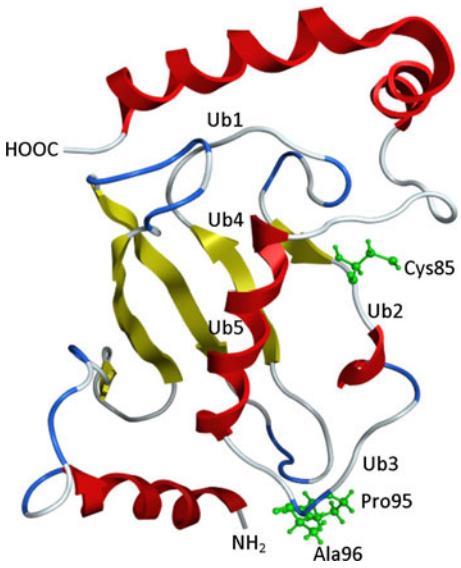

Fig. 5 Homology modeling of CmUBC. The 3D model of CmUBC was constructed based on experimental X-ray crystal structure of UBC4 from *Saccharomyces cerevisiae* (PDB code 1QCQ). The amino acid residues that form active-site cysteine (Cys85) and E3 interaction residues (Pro95, Ala96) are shown as *sticks*. The regions of the ubiquitin thioester intermediate interaction residues are indicated by Ub1–Ub5

of the molecule. Comparisons with the structure of different UBC enzymes suggested that UBC enzymes are highly conserved in their 3D folding.

The 3D model of CmUBC was superposed separately on the structure of UBC4 from *S. cerevisiae* (Cook et al. 1993) and UBC1 from *A. thaliana* (Cook et al. 1992). Superposition of the 3D models of CmUBC on the experimental structures of UBC4 showed that altered residues were found in the conserved sites of Ub1 and Ub5 (ubiquitin-thioester intermediate interaction residues) (Supplementary Fig. 1). Although the Ub1 and Ub5 residues are highly conserved among the different plant species, it was observed that a hydrophobic Val (V73) and Phe (F74) in the Ub1 residue of CmUBC changed to Ile (I74) and Thy (Y75) in the Ub1 residue of UBC4, respectively. In addition, Pro (P113) shuffled to Ala (A114) in the Ub5 residue of *S. cerevisiae* UBC4. There was no great variation found between the surfaces of the two molecules, as most of the identical residues between the two proteins were clustered on the surface that lies adjacent to the residues between 50 and 120. Based on the data reported herein, it can be concluded that the Ub1 and Ub5 residues and their hydrophobic nature did not differ between CmUBC and UBC4, despite some disparities.

Deviations in sequence length and insertions were observed when the CmUBC model was constructed and superposed with the *A. thaliana* UBC1 (data not shown). The 3D model of CmUBC showed that seven residues located in Ub1, Ub4, Ub5, and E3 (E3 interaction residues) were substituted (Supplementary Fig. 2). Despite the existence of some amino acid changes, such as R75 and M76 in Ub1 and I105 in the Ub4 residues of UBC1, the hydrophobic properties of the Ub1 and Ub4 residues did not change in CmUBC. However, because of the conversion of the acidic amino acid Asp (D116-D117) to the uncharged polar amino acids Asn (N119) and Ser (S120), the acidic nature of the Ub5 residues of melon CmUBC changed to uncharged for *A. thaliana* UBC1. The E3 interaction residues consisted of two

highly conserved amino acids, Pro (P) and Ala (A). There was a replacement between Ala (A96) and Ile (I99) in the E3 residue of UBC1, which resulted in no change occurring in the hydrophobic nature of the E3 residue of UBC1 and CmUBC.

Construction of Standard Curves

The standard curve was generated for the *CmUBC* gene expression analysis. The linear range of quantification was determined after serial dilutions of standard plasmid containing the *CmUBC* gene. Serial dilutions from 1.0×10^7 to 1.0×10^1 copies of pCR8/GW/TOPO-CmUBC were tested. The optimal range of RT-qPCR that is commonly accepted (Taylor et al. 2010) was obtained between dilutions of 10^2 and 10^3 for pCR8/GW/TOPO-CmUBC. The efficiency of pCR8/GW/TOPO-CmUBC was 98.47% with $R^2 = 0.99754$ (Supplementary Fig. 3).

Expression Analysis of the *CmUBC* Gene

The transcript levels of the *CmUBC* gene in different organs of melon were investigated by a RT-qPCR. *CmUBC*-gene specific primers were amplified to the expected fragment of about 470 bp. The levels of expression of the *Cm18SrRNA* housekeeping gene (250-bp fragment) were also measured as a control. The *CmUBC* gene was expressed constitutively in all of the tissues. However, compared with other tissues, including the stem and the root, an increase of about twofold in *CmUBC* gene expression was observed in leaf tissues (Fig. 6). RT-qPCR was performed in order to inspect the effects of drought and salt stresses on the transcript levels of the *CmUBC* gene in melon leaf tissues. The expression levels of *CmUBC* peaked at 1 h post-treatment (hpt), with similar patterns over time during both salinity and drought stresses. Although the expression profiles of *CmUBC* were similar for both stresses, the concentrations of the transcripts were different. The transcript concentrations of *CmUBC* were approximately 300 molecules/ng for salt and 200 molecules/ng for drought stress applications (Fig. 7). The *CmUBC* gene expression levels were sharply decreased after 3 hpt for both stresses. Comparing the salt stress samples to the control samples, the transcription levels of *CmUBC* were significantly upregulated twofold at 6 and 24 hpt. However, there was a slight decrease in the levels of expression of *CmUBC* at 48 hpt during salinity and drought stresses.

Discussion

In this study, we cloned and characterized the melon UBC enzyme gene, *CmUBC*. The phylogenetic analyses demonstrated that all UBC genes from land plants were clustered into four groups (Groups 3–6), while the yeast and animal UBC genes fell into their own separate groups (Groups 1 and 2). Although CmUBC had a high sequence similarity to UBC from cotton, grape vine, poplar, and soybean, it phylogenetically clustered together with cucumber and pea. Thus, this suggested

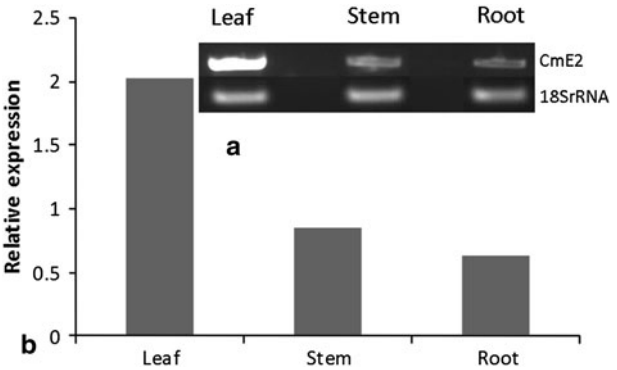

Fig. 6 *CmUBC* gene expression in three organs of melon plants under normal conditions. *a* Semiquantitative RT-PCR analysis of the *CmUBC* gene in leaf, stem, and root tissues of melon plants. *b* Bar graph showing expression of ubiquitin-conjugating enzyme *CmUBC* gene normalized to *Cm18SrRNA* gene in three organs of melon plants. Data are expressed as mean ± SEM of three separate individuals

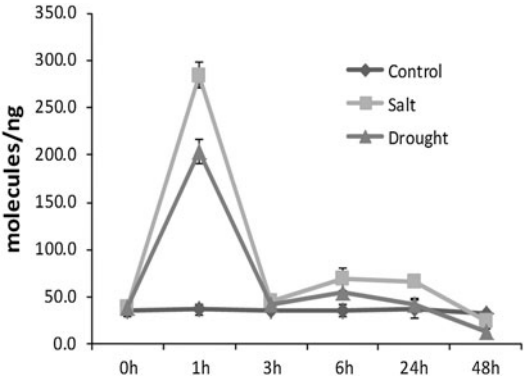

Fig. 7 Expression patterns of the *CmUBC* gene of melon during salt and drought stress conditions at time points from 0 to 48 h

that CmUBC, like soybean UBC, could also be an active UBC enzyme. Further biochemical analyses are needed to confirm whether CmUBC binds ubiquitin through a thiol ester linkage (Zhou et al. 2010).

We screened the Cucumber Genome Database and identified at least five UBC gene family members in the cucumber chromosomes that displayed all of the same characteristics as the melon *CmUBC* gene. Comparative genomic analyses provided the transfer of functional information from different taxa. Thus, well-studied taxa allow for a better understanding of the genome structure, function, and evolution of another less well-studied taxon (Paterson et al. 2012). In this study, we indicated that the cucumber and *Arabidopsis* UBC genes were located in syntenic regions of the two genomes (Fig. 4). To date, several important and divergent biological processes that are regulated by UBC genes have been reported in *Arabidopsis*, including UV sensitivity in *S. cerevisiae* rad6 mutants (Zwirn et al. 1997), the reduction in the number of rosette leaves and transcript levels of a set of floral repressor genes, an early-flowering phenotype (Xu et al. 2009), more tolerance to

salinity and drought stresses (Zhou et al. 2010), and improved plant tolerance to water stress that is induced by sorbitol or soil drought (Wan et al. 2011). Together with our expression data, these results will help to infer the probable functions of the melon *CmUBC* gene.

Multiple sequence alignment revealed high similarity and sequence conservation in all of the UBCs from the different classes of plant species. The *CmUBC* gene has three conserved sites. The first one is a catalytic domain containing a cysteine that plays an important role in the ubiquitin-mediated protein degradation pathway. A thiol-ester linkage is formed between the conserved cysteine and the C-terminus of ubiquitin and complexes with the E3 ubiquitin protein ligase enzymes (Worthylake et al. 1998; Xiao et al. 1998). In addition, other E2s form thiol-ester linkages without the use of E3s, and several UBC homologs (TSG101, Mms2, Croc-1) lack the active site cysteine that is essential for ubiquitination (Weissman 2001; Xiao et al. 1998). Ubiquitin-thioester intermediate interaction residues (indicated as Ub1-5 in Fig. 2) are the second conserved sites that provide complementary surfaces for ubiquitin and the E2 protein to form an unstable E2-ubiquitin-thiolester intermediate prior to the transfer of ubiquitin to an E3-ligase protein (Hamilton et al. 2001). The last conserved region shown is E3 (Fig. 2), which consists of E3 interaction residues. This motif is recognized by the E3s, and an E2–E3 complex is formed (Weissman 2001; Zheng et al. 2000).

We constructed the homology structural model of CmUBC and its homologs from other eukaryotes. The homology structural model of CmUBC showed highly conserved 3D folding with yeast and *Arabidopsis*. A homology modeling analysis may be informative for the functional characterization of plant UBC enzymes. Although the region around the active-site cysteine, ubiquitins, and the E3 residues is one of the most highly conserved, two striking exceptions were observed. The gene products of yeast CDC34 and wheat UBC7 have a 12-residue segment that corresponds to an insertion between residues 95 and 96 in *Arabidopsis* UBCl (Van Nocker and Vierstra 1991; Goebl et al. 1988). This extra segment forms a large loop that provides the formation of multiubiquitin chains for wheat UBC7 and yeast CDC34, suggesting that this region may be important for transferring ubiquitin to itself (Van Nocker and Vierstra 1991; Haas et al. 1991). However, we did not observe an insertion between the specified residues in the melon CmUBC protein. The characterization and 3D model analyses suggested that CmUBC may act as an active UBC enzyme that regulates protein degradation and water-stress responses in melon. The identification and functional characterization of substrate proteins of CmUBC will be useful for the elucidation of the regulatory mechanisms of the ubiquitination–proteasome pathway in abiotic stress responses in plants.

UBC proteins play roles in diverse cellular processes, including DNA repair, signal transduction and cell differentiation (Xiao et al. 1998; Zwirn et al. 1997), the regulation of ion homeostasis, osmolyte synthesis, oxidative stress, and abiotic stress responses (Zhou et al. 2010). There are some lines of evidence to suggest that ubiquitination may play a vital role in plant responses to abiotic stresses (Wan et al. 2011; Zhou et al. 2010; Mukoko et al. 2010; Zhang et al. 2007; Yan et al. 2003). Wan et al. (2011) have investigated the effects of abiotic stresses on the transcript levels of the *AhUBC2* gene in different organs of peanut. In contrast to our findings,

the *AhUBC2* transcript levels in peanut roots were relatively higher than those in leaves and stems under normal conditions. Our results showed that the *CmUBC* gene was highly expressed in the leaf tissues of melon under PEG-induced dehydration and high salinity stresses, consistent with the RT-qPCR expression analysis of the *AhUBC2* gene in leaves and stems, which was significantly upregulated by PEG-6000-induced dehydration, high salinity, and low temperature (Wan et al. 2011). These results suggest that the expression of the *CmUBC* gene, like the *AhUBC2* gene, was responsive to physiological water stress. The levels of expression of *GmUBC2* were also examined in different tissues of soybean seedlings under abiotic stress conditions (Zhou et al. 2010). The expression analysis of *AhUBC2* from peanuts (Wan et al. 2011), *AtUBC1–AtUBC2* from *Arabidopsis* (Xu et al. 2009), and *CmUBC* from melons were consistent with the *GmUBC2* gene (Zhou et al. 2010), which was detected in leaves, stems, and roots of soybean plants without stress treatments. This implies that ubiquitously expressed plant UBCs have an important role in plant development and physiology. The mRNA levels of *GmUBC2* increased after exposure to salt and drought stress. *GmUBC2* transcripts increased in soybean seedlings that were treated with a solution containing 200 mM NaCl and 20% PEG. After 6 h of treatment with 200 mM NaCl, the transcript levels of *GmUBC2* in stems and leaves increased about twofold over those in the control, a result similar to that of *CmUBC*, in which expression was upregulated twofold at 6 hpt of salt stress. The transcript concentrations of *CmUBC* burst after 1 hpt of salt and drought stress applications. These results suggest that ubiquitination mainly occurs in leaves at very early time periods, triggering the degradation of target proteins that restrain active water-stress responses in plants.

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