

Full Length Research Paper

Characterization of *LeCOP1* gene in *Lycopersicon esculentum* treated with various abiotic and oxidative stresses

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A full-length cDNA of *LeCOP1* was isolated from tomato (*Lycopersicon esculentum*). Phylogenetic analysis based on the deduced amino acid sequence of *LeCOP1* cDNA revealed high sequence similarity to COP1 protein in *Ipomoea nil* (84% identity) and in *Arabidopsis* (77%). *LeCOP1* shared high sequence identity with a hypothetical protein in *Vitis vinifera* and E3 ubiquitin-protein ligase COP1 in *Pisum sativum* (76%). *LeCOP1* gene exists single copy in the tomato genome. Expression of *LeCOP1* gene under abiotic and oxidative stresses was investigated, including exposure to 200 mM NaCl, 200 mM mannitol, cold (4°C), 100 µM abscisic acid (ABA), 10 mM hydrogen peroxide (H₂O₂) and 50 µM methyl viologen (MV). *LeCOP1* was significantly respectively induced at 1, 6, and 24 h after mannitol, NaCl and cold treatment. It was also induced after H₂O₂ treatment at 24 h. However, *LeCOP1* was not induced by MV treatment. These observations suggest that *LeCOP1* gene may be involved in abiotic and oxidative stresses.

Key words: *LeCOP1*, *Lycopersicon esculentum*, abiotic stress, oxidative stress.

INTRODUCTION

Plants exhibit a variety of responses to overcome diverse abiotic environmental stresses, such as salinity, drought and severe temperature changes (Yi et al., 2004). Most of these stresses have been induced by similar mechanisms, most notably, dehydration or water stress (Thomashow, 1998). These stresses damage cellular metabolism, produce the reactive oxygen species (ROS), and inhibit plant photosynthesis (Hasegawa et al., 2000). Plants have developed a diverse and complex set of defense mechanisms to survive under these stresses (Dangl and Jones, 2001). The expression of hundreds of genes is affected by these stresses, and understanding the functions of these genes should help to clarify mechanisms of stress tolerance (Yamaguchi and Shinozaki, 2005; Umezawa et al., 2006). A number of genes that respond to drought, salt and cold stresses at the transcriptional level have been described (Ingram and

Bartels, 1996). Constitutively, photomorphogenic1 (COP1) acts as a central switch in light signal transduction due to majority of light-controlled genome expression attributed to COP1 activity (Zhao et al., 1998; Ma et al., 2002). COP1 contains three functional domains involved in protein-protein interactions: an N-terminal RING-finger domain, a coiled-coil for dimerization, and a WD40 repeat domain implicated in substrate recognition (Deng et al., 1992; Yang and Wang, 2006). In plants, COP1 functions as an E3 ubiquitin ligase to repress light signaling by targeting photoreceptors and downstream transcription factors for ubiquitination (Duck et al., 2004; Yi and Deng, 2005). Ubiquitination is a post-translational modification of proteins that plays important roles in several processes in plants such as abiotic stress responses (Lee and Kim, 2011). Plants overcome various abiotic stresses through ubiquitination and the resulting degradation of components specific to these stress signaling (Lee and Kim, 2011). In mammalian cells, COP1 plays a role in the degradation of p53 protein in cancers and attenuates the tumor suppressor function of p53 (Dornan et al., 2004).

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Abscisic acid (ABA) is an important hormone involved in the adaptive responses of plants to various environmental conditions (Shinozaki et al., 2003). Both ABA-independent and ABA-dependent signal transduction cascades existed during the signaling pathways in response to abiotic stress (Bray, 1997). In *Arabidopsis*, the RING finger E3 ligase, Salt- and drought-induced ring finger1 (SDIR1) has been found to be involved in ABA-related stress signaling and works as a positive regulator in ABA response (Zhang et al., 2008). SDIR1 belongs to the ABA-independent pathway since SDIR1 is induced by drought and salt stress, and not by ABA, however, transgenic *Arabidopsis* plants over expressing SDIR1 display enhanced drought tolerance (Zhang et al., 2007). Over expression of *Arabidopsis* SDIR1 in tobacco and rice also confers improved drought tolerance, suggesting the SDIR1 can function as a drought-tolerance gene in both dicotyledons and monocotyledons (Zhang et al., 2008). COP1 protein in pepper also plays a role in abiotic stress and belongs to ABA-independent regulation system (Guo et al., 2007).

Tomato (*Lycopersicon esculentum*) is one of the most common vegetable crops, however, little research has been carried out on the mechanism of tomato plants in response to environmental stresses. In this study, we characterized the LeCOP1 proteins in tomato plants to further characterize a potential mechanism through which this organism regulates defense responses. The expression of the *LeCOP1* gene was investigated under abiotic and oxidative stresses, including exposure to H₂O₂, 200 mM NaCl, 200 mM mannitol, cold temperature (4°C), 100 µM ABA, 10 mM H₂O₂, and 50 µM MV.

MATERIALS AND METHODS

Plant material and treatment

Tomato (*Solanum lycopersicum* L.) seeds were cultured in Murashige and Skoog (MS) medium (including 3% sucrose, 0.8% agar, pH 5.8). The germinated plants were transferred to pots and kept in a growth chamber at 24°C for four weeks. For cold treatment, the leaves were placed in distilled water and kept in a 4°C cold chamber under dim light for 24 h. The chemical treatments involved incubation under dim light in 200 mM NaCl, 200 mM mannitol, 10 mM H₂O₂, or 50 µM MV for various durations. The ABA stock solution was prepared by dissolving ABA in small aliquots of 1 N NaOH. For the ABA treatment, the tomato plants were removed from the soil and the roots were carefully washed and soaked in a 100 µM ABA.

Multiple amino acid sequence alignment

LeCOP1 cDNA (AF029984) was isolated from tomato, and a BLASTP search was conducted against those of characterized homologous COP1 proteins (Lee et al., 2004). Multiple sequence alignments of tomato LeCOP1 to COP1 proteins from other plants sequences were generated using <http://us.expasy.org/tools>. The accession numbers are AAG31173 (*Ipomoea nil*); NP_180854 (*Arabidopsis thaliana*); CAN71084 (*Vitis vinifera*); and CAA70768 (*Pisum sativum*). After removing the gaps from initial alignments, a

phylogenetic tree was generated using the neighbor-joining (NJ) method (Saitou and Nei, 1987).

RNA isolation and amplification of the full length *LeCOP1* cDNA

Forward (5'-ATGGTGGAAAGTTCAGTTGG-3') and reverse (5'-TCAA GCTGCAAGGACTAACAC-3') primers were designed to amplify the complete, full-length *LeCOP1* gene from tomato cDNA by RT-PCR. Total RNA was isolated from young tomato leaves using TRI-reagent[®] according to the manufacturer's instructions (MRC, USA). From the DNase-treated total RNA (1 µg), first-strand cDNA was synthesized using the AccuPower[®] PCR PreMix (Bioneer, South Korea), containing oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, USA). The PCR reaction was carried out as follows: an initial 5 min of denaturation at 94°C; 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; and a final 7 min incubation at 72°C. The reaction products (12 µl each) were separated on 1% agarose gels and visualized after staining with ethidium bromide. All experiments were performed in triplicate.

DNA isolation and genomic DNA gel blot analysis

Genomic DNA was isolated from mature tomato leaves. Genomic DNA samples (20 µg) were completely digested with *Hind* III and *Xba* I. Digested genomic DNA was separated by electrophoresis on a 1% agarose gel, was denatured, and blotted onto a nylon membrane (Amersham Pharmacia, Uppsala). Membranes were then hybridized with the full-length of *LeCOP1* cDNA probe labeled with [α -³²P] dCTP. Hybridization was performed overnight at 65°C in 5% dextran sulfate, 0.25 M disodium phosphate (pH 7.2), 7% (w/v) SDS, and 1 mM EDTA. After hybridization, the blot was washed twice with 2 × SSC and 0.1% SDS for 10 min each at room temperature and twice with 0.1 × SSC and 0.1% SDS for 5 min each at 65°C. The blots were then dried and developed on X-ray film incubated at -80°C for 1 week.

Real time RT-PCR analysis

To determine whether the *LeCOP1* gene in tomato mediates plant responses to different physical stress conditions, its expression was investigated via real time RT-PCR after exposure to abiotic stresses, including treatment with 200 mM NaCl, 200 mM mannitol, cold (48°C), and 100 mM ABA treatment. Total RNA was isolated from young tomato leaves using Trizolreagent[®] according to the manufacturer's instructions (Invitrogen, Carlsbad, California USA) and was digested with DNaseI (Promega, Madison, WI, USA) to remove DNA contamination. 5 µg of total RNA from each pool was reverse transcribed in the presence of Oligo(dT) and 9-mer random primer in a volume of 20 µL. The synthesized cDNA was diluted with 180 µL of water and used as template in real-time RT-PCR. PCR primers were designed to amplify ORF of *LeCOP1* as follows, forward: 5'- ATGGTGGAAAGTTCAGTTGG-3'; reverse: 5'- TCAA GCTGCAAGGACTAACAC -3'. The Actin (accession no. U60480) gene was used as an internal reference to normalize the amount of total RNA present in each reaction. The primers for amplification of Actin gene were as follows, forward: 5'- TGGCATCATACTTTCTACAATG-3'; reverse: CTAATATCCTCGTCACATTTTCAT. The reactions were performed on MJ ResearchOptic onTM2 machine (Bio-Rad/MJ Research, Hercules, CA). The reactions were conducted in triplicate in a total volume of 20 µL containing 10 µL 2 × SYBR green premix ExTaq (TaKaRa Biotech, Otsu, Shiga, Japan), 0.25 µM each primer and 2 µL of template (equivalent to 0.05 µg of total RNA). The

(A)



Figure 1. Multiple alignments of *Lecop1* with other COP1 proteins. (A) Comparison of the derived amino acid sequences of *Lecop1* with other COP1 proteins in *I. nil* (AAG31173), *A. thaliana* (NP_180854), *V. vinifera* (CAN71084), and *P. sativum* (CAA70768). The RING finger domain was underlined by “—”; the WD40 repeats were underlined by “---”. (B) Phylogenetic tree of *Lecop1* homologs in these plants.

(B)

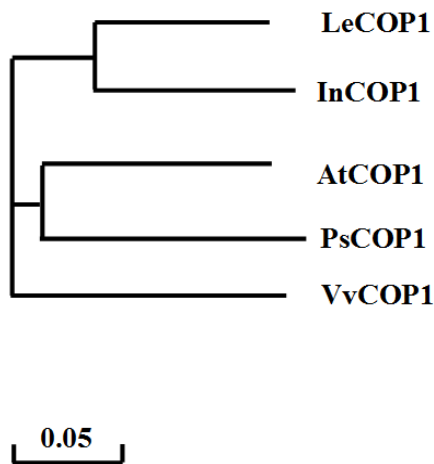


Figure 1. Continued.

amplification was carried out with the following cycling parameters: 94°C for 30 s followed by 45 cycles of 94°C for 12 s, 60°C for 30 s, 72°C for 45 s, and 1 s at 81°C for plate reading. A melting curve

was generated for each sample at the end of each run to serve as an assessment of the purity of the amplified products. The expression levels were calculated from the threshold cycle according to delta-delta Ct method (Livak and Schmittgen, 2001). The experiments were performed at least four times.

RESULTS

Sequence analysis of *LeCOP1*

A full-length of 2034 bp cDNA was obtained (GenBank accession no. AF029984). Phylogenetic analysis revealed high sequence similarity of the deduced amino acid sequence of *LeCOP1* cDNA to other COP1 proteins (Figure 1A). *LeCOP1* shared 84% AAID with *I. nil* (AAG31173), 77% AAID with *A. thaliana* (NP_180854), 76% AAID with *V. vinifera* (CAN71084), and 76% AAID with COP1 protein in *P. sativum* (CAA70768). The RING finger domain exists in the N-terminal and is conserved among *LeCOP1* and COP1 proteins determined from other plants, suggesting that this domain might have a significant biological function. Seven copies of the repeat WD40 domain were present in COP1 proteins. The evolutionary relationship among these COP1 proteins is shown in Figure 1B. *LeCOP1* protein showed the most similarity to COP1 proteins in *I. nil*.

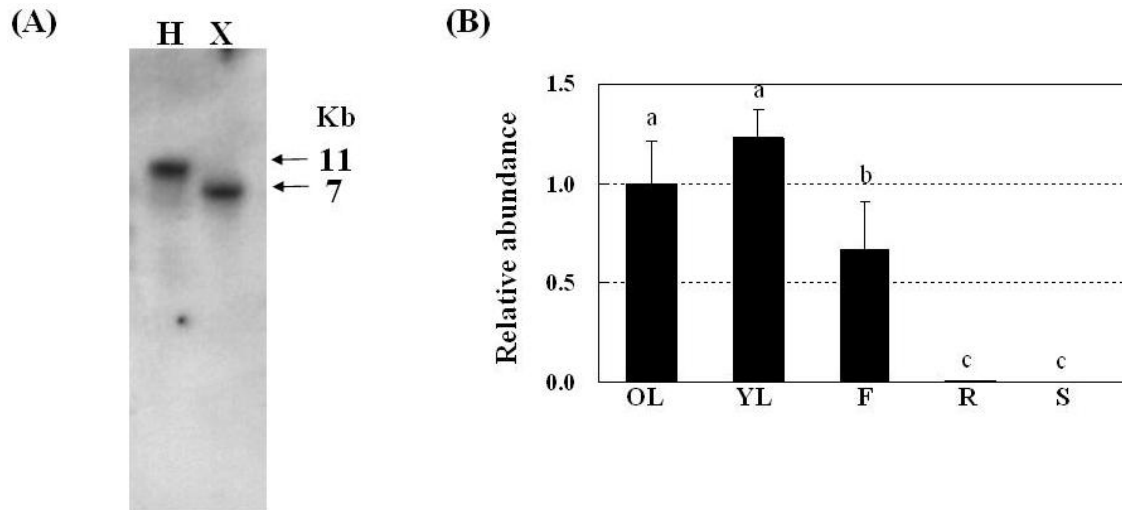


Figure 2. Characterization of the *LeCOP1* gene. (A) Southern blot analysis: genomic DNA was digested with *Hind* III (H) and *Xba* I (X), loaded on an agarose gel, and hybridized with a ^{32}P -labeled probe corresponding to the full-length *LeCOP1* cDNA. (B) Tissue-specific expression of the *Lecop1* gene: *Lecop1* RNA (1 μg) levels were monitored by real time RT-PCR in mature tomato leaves (ML), young tomato leaves (YL), flowers (F), stems (S), and roots (R). Values are expressed as mean ($n = 4$). Errors bars show the SD for each experiment. Bars with different letter differ ($P < 0.05$).

Southern blotting analysis of *LeCOP1*

To assess the copy number of the *LeCOP1* gene in tomato, DNA gel blot analysis was performed on tomato genomic DNA digested by *Hind* III and *Xba* I using ^{32}P -labeled *LeCOP1* full-length cDNA. Hybridization of the genomic DNA blot resulted in a single band in DNA samples digested by *Hind* III and *Xba* I (Figure 2A). These results indicate the presence of the other *LeCOP1*-related genes. *LeCOP1* gene exists as single copy in the tomato genome.

Analysis of *LeCOP1* expression level in various tissues

The expression level of the *LeCOP1* gene in various tomato tissues was determined utilizing RT-PCR (Figure 2B). The *LeCOP1* transcript was strongly expressed in tomato leaves and flowers. No signal was detected in the stems and roots. *cop1* transcripts differentially accumulated in various organs of the mature tomato plant, and the developmental regulation of the *cop1* gene expression is likely to vary among different organs.

Expression of *LeCOP1* mRNA in response to various stresses

To determine whether the *LeCOP1* gene in tomato mediates plant responses to different physical stress conditions, its expression was investigated via real time RT-PCR after exposure to abiotic stresses, including

treatment with 200 mM NaCl, 200 mM mannitol, cold (4°C), and 100 μM ABA treatment (Figure 3). Treatment with distilled water was used as a control condition for abiotic stress (Figure 3A). The *LeCOP1* transcript increased significantly to 40 times compared to untreated samples after 6 h of exposure to NaCl and reached about 60 times until 24 h (Figure 3B). In cold-treated tomato leaves, *LeCOP1* expression was induced significantly after 24 h of chilling treatment, approximately 40-fold higher (Figure 3C). In mannitol-treated tomato leaves, *LeCOP1* expression began to accumulate within 1 h of mannitol treatment and reached the highest level until 12 h, approximately 200-fold higher, and declined after then (Figure 3D). However, *LeCOP1* transcript level was not induced by ABA treatment (Figure 3E). These results suggest that *LeCOP1* gene is not involved in ABA stresses signal pathway but is related to cold mediated signal transduction at the transcriptional level.

Expression of *LeCOP1* mRNA in response to various oxidative stresses

To examine the influence of ROS on *LeCOP1* expression, we treated plants with 10 mM H_2O_2 and 50 μM methyl viologen (MV). H_2O_2 treatment caused the *LeCOP1* transcript level to increase at 1 h, declined after then, and then reached a maximum level at 24 h; about 50-fold higher (Figure 4A). As a response to MV treatment, no significant difference of the *LeCOP1* transcript was observed at all time points during the treatment (Figure 4B).

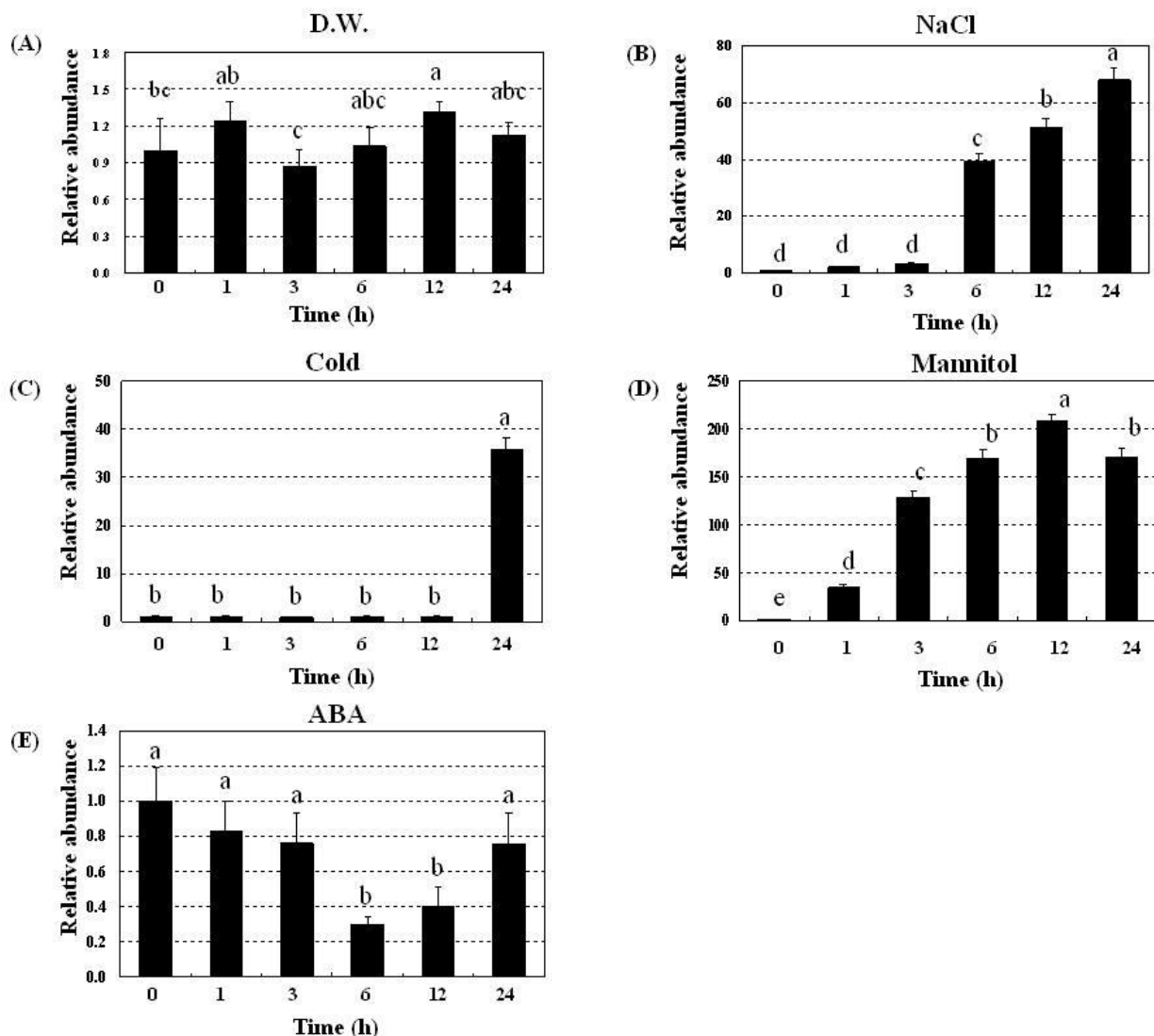


Figure 3. Real time RT-PCR analysis of *LeCOP1* gene expression in tomato leaf tissues after exposure to various abiotic stresses. (A) Buffer treatment used as the control. (B) NaCl (200 mM). (C) Cold treatment at 4°C. (D) Mannitol (200 mM). (E) ABA (100 μM). Relative expression level = gene expression level under stress/gene expression level under control condition. Values are expressed as mean (n = 4). Errors bars show the SD for each experiment. Bars with different letter differ (P < 0.05).

DISCUSSION

COP1 protein contains several functional domains, which may be involved in environmental stress signaling pathways (Figure 1A). RING-finger domain is a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc (Zheng et al., 2000); it is defined by the 'cross-brace' motif C-X₂-C-X(9-39)-C-X(1-3)-H-X(2-3)-(N/C/H)-X₂-C-X(4-48)-C-X₂-C. It is probably involved in mediating protein-protein interactions and is always identified in a protein with a wide range of functions such as viral replication, signal transduction, and development (Gack et al., 2007). WD40 domain plays a wide variety of functions including

adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly (Orlicky et al., 2003).

ABA is involved in the regulation of defense-related signaling in response to abiotic stresses (Mauchi-Mani and Mauch, 2005). Some genes are induced by water stress but are not responsive to exogenous ABA treatment, which suggests the existence of both ABA-independent and ABA-dependent signal transduction cascades between the initial signal of drought or cold stress and the expression of specific genes (Bray, 1997).

It was reported that E3 ligases were involved in ABA-dependent or independent pathways in drought/salt stress responses (Lee et al., 2010).

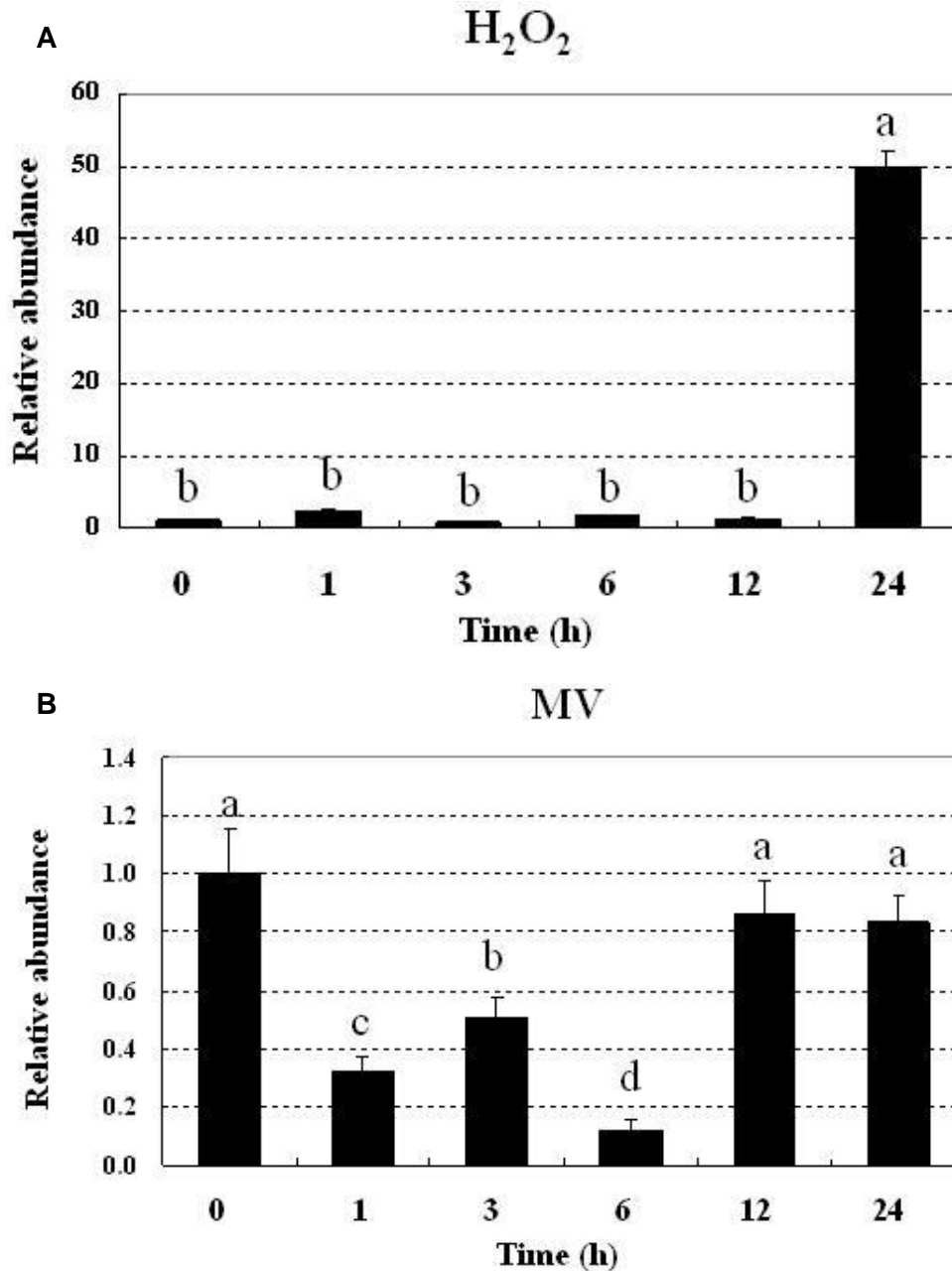


Figure 4. Real time RT-PCR analysis of *LeCOP1* gene in tomato plants treated with chemicals related to oxidative and osmotic stresses. Total RNA was extracted from leaves exposed to 10 mM H_2O_2 (A) or 50 μ M MV (B), at the indicated times after treatment. Relative expression level = gene expression level under stress/gene expression level under control condition. Values are expressed as mean ($n = 4$). Errors bars show the SD for each experiment. Bars with different letter differ ($P < 0.05$).

AtCHIP, an E3 ubiquitin ligase in *Arabidopsis*, is involved in ABA-dependent stress response. Its transcripts are upregulated by several environmental stress conditions such as low and high temperatures (Yan et al., 2003). Although some reports on the connection of E3 ligase and abiotic stress signaling have been published, more elucidation of the biological roles of newly identified E3

ligases should be further carried out. In our study, *LeCOP1* was specifically induced after low temperature and drought treatments but not by ABA (Figure 3), which confirm this result that at least two separate regulatory systems function in gene expression during drought and cold stress.

Studies of abiotic stress signal transduction will play an

important role in agricultural improvement. Transgenic tobacco and *Arabidopsis* plants which overexpressed some cold-induced genes exhibited enhanced cold tolerance properties, which were attributed to the increased expression of cold-regulated genes (Kim et al., 2001; Huang et al., 2005). Our investigation of COP1 proteins will become another target for engineering biotech crops with enhanced tolerance to abiotic stresses.

Reactive oxygen species (ROS) belong to byproducts of normal metabolic process produced by all organisms, including superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2^1) (Foyer et al., 1994). ROS, especially H_2O_2 , are involved in the responses of plants to both abiotic and biotic stresses (Dat et al., 2000). H_2O_2 is a signal molecule in signal transduction pathways triggered during stress response (Willekens et al., 1995). As various environmental stresses lead to the generation of ROS in plants, plant injury caused by environmental stresses may be related to ROS-initiated oxidative damage at the cellular level (Kuzniak, 2002). MV reduces O_2 and generates superoxide anion in the chloroplast (Babbs et al., 1989). We applied MV in our study to be a kind of oxidative stress (Figure 4). Oxidative stress application unregulated the transcripts of encoding proteins involved in antioxidant metabolism (Seong et al., 2007).

In the present study, we investigated the expression of *LeCOP1* gene under abiotic stresses by RT-PCR. *LeCOP1* was induced significantly in cold, drought and high salinity. Furthermore, *LeCOP1* was not induced by plant hormone ABA. These observations collectively provide initial evidence that *LeCOP1* gene was related to abiotic stresses and maybe belong to ABA-independent regulation system. The mechanisms underlying the activation of cold response by *LeCOP1* remain to be elucidated in detail.

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