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Article

Characterization of *CaCOP1* Gene in *Capsicum annuum* Treated with Pathogen Infection and Various Abiotic Stresses

Jia Guo†, Eun Soo Seong† and Myeong-Hyeon Wang*

School of Biotechnology, Kangwon National University, Chuncheon 200-701, Korea

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We characterized a full-length cDNA of *CaCOP1* from pepper. Phylogenetic analysis based on the deduced amino acid sequence of *CaCOP1* cDNA revealed high sequence similarity to the *COP1* gene in *Oryza sativa* (84% identity). *CaCOP1* shares high sequence identity with regulatory protein in *Arabidopsis* (84%), constitutively photomorphogenic 1 protein in *Pisum sativum* (81%) and COP1 homolog in *Lycopersicon esculentum* (79%). *CaCOP1* gene exists single copy in the chili pepper genome. Expression of *CaCOP1* was reduced in response to inoculation of non-host pathogens. The expression of this gene under abiotic and oxidative stresses was investigated, including 200 mM NaCl, 200 mM mannitol, cold (4°C), 100 μM abscisic acid (ABA), and 10 mM hydrogen peroxide (H_2O_2). *CaCOP1* was induced significantly 3 h after low temperature treatment but not by dehydration or high salinity. Moreover, *CaCOP1* was not induced by plant hormone ABA. These observations suggest that *CaCOP1* gene plays a role in abiotic stress and may be belong to ABA-independent regulation system.

Key words: *abiotic stress, CaCOP1, cold stress, pathogen infection, pepper*

Plants have developed a variety of protective mechanisms to overcome diverse abiotic environmental stresses, such as severe temperature changes, drought and salinity [Yi *et al.*, 2004]. Stresses including cold, drought, high salinity, and freezing damage have been induced by similar mechanisms, most notably, dehydration or water stress [Thomashow, 1998]. A number of genes that respond to drought, salt and cold stresses at the transcriptional level have been described [Ingram and Bartels, 1996]. Genes which are induced as the result of these abiotic stresses have not only in the protection of cells, via the generation of important metabolic and cellular protection proteins, but also in the regulation of genes which are involved in the transduction of stress response signals.

Constitutively photomorphogenic1 (COP1) is a negative regulator of photomorphogenesis in *Arabidopsis thaliana* [Oravecz *et al.*, 2006]. COP1 functions as an E3 ubiquitin ligase, targeting select proteins for proteasomal degradation in plants as well as in mammals [Saijo *et al.*, 2003]. One of its substrates is the basic domain/leucine zipper (bZIP) transcription factor ELONGATED HYPOCOTYL5 (HY5). It is one of the key regulators of photomorphogenesis under all light qualities, including UV-B responses required for tolerance to this environmental threat. 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].

COP1 protein plays an important role in light signal transduction pathways of seeding development of higher plants [Zhao *et al.*, 1998]. The *Arabidopsis* protein COP1 is an essential regulatory molecular that plays a role in the repression of photomorphogenic development in darkness and in the ability of light-grown plants to respond to photoperiod, end-of-day far-red treatment, and ratio of red/far-red light [McNellis *et al.*, 1994]. It was reported that COP1 functions as an E3 ubiquitin ligase, and is responsible for targeting a number of photomorphogenesis-promoting factors for proteasomal degradation in plants as well as in mammals, including HY5, LAF1, phyA and HFR1 [Lee and Hwang, 2003; Duek *et al.*, 2004; Yang and Wang, 2006]. In contrast with the situation in visible light, COP1 is a critical positive regulator of responses to low levels of UV-B [Oravecz *et*

†These authors equally contributed to this work.

*Corresponding author
Phone: 82-33-250-6486
Fax: 82-33-241-6480
E-mail: mhwang@kangwon.ac.kr

Abbreviations: ABA, abscisic acid; H_2O_2, hydrogen peroxide; HR, hypersensitivity response; PR, pathogenesis-related; *X. ag* 8ra, *Xanthomonas axonopodis* pv. glycines 8ra.

al., 2006]. COP1 may involve a specific control of its nuclear activity in hypocotyls and cotyledons, but not in roots, of developing seedlings [Matsui *et al.*, 1995]. However, COP1 protein is localized primarily in the nucleus while it is depleted from the nucleus and assembles in the cytosol on the set of light. COP1 gene homolog has also been identified from *P. sativum* [Zhao *et al.*, 1998] and COP1 protein from pea is constitutively expressed under light and darkness, which was consistent to the study on *A. thaliana* [Deng *et al.*, 1992]. COP1 homolog isolated from rice is conserved to COP1 proteins from dicots [Raghuvanshi *et al.*, 2001]. Thus OsCOP1 probably acts to constitutively promote coleoptile and leaf elongation, and to inhibit leaf expansion and plastid development during early seeding development in rice [Zhang *et al.*, 2006]. COP1 homologues have been identified in animals, suggesting that mammalian COP1, like its plant counterpart, is involved in ubiquitination and is itself a substrate [Yi *et al.*, 2002]. In mammalian cells, COP1 is significantly overexpressed in breast and ovarian adenocarcinoma since COP1 contributes to the accelerated degradation of p53 protein in cancers and attenuates the tumor suppressor function of p53 [Dornan *et al.*, 2004]. Furthermore, COP1 was found to interact with MVP, also known as lung resistance protein (LRP) under unstressed condition to suppress c-Jun-mediated AP-1 transcription, thereby preventing cells from undergoing the stress response [Yi *et al.*, 2005].

In this study, we examined the interaction with a non-host pathogen and chili pepper plants (*Capsicum annuum* cv. Bukang) to determine the mechanisms regulating defense responses. Moreover, the expressions of *CaCOP1* under abiotic and oxidative stresses were investigated, including H_2O, 200 mM NaCl, 200 mM mannitol, cold (4°C), 100 μM abscisic acid (ABA), and 10 mM H_2O_2.

Materials and Methods

Plant material and treatment. Chili pepper (*Capsicum annuum*) 'Bukang' seeds were cultured in MS (Murashige and Skoog) medium (MS salts including MS vitamins, 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 4 weeks. The bacterial pathogen used for inoculation was *X. ag* 8ra, a soy bean pustule pathogen [Oh *et al.*, 2005]. Bacterial infiltration was accomplished by syringe infiltration of bacterial suspensions (approximately 4×10^8 cfu/mL). The leaves were placed in distilled water and kept in a 4°C cold chamber under dim light for 24 h, and they were incubated in 200 mM NaCl, 200 mM mannitol and 10 mM for various durations. The ABA stock solution was prepared by dissolving ABA in small aliquots of 1 N NaOH. The stock was diluted to 10^{-3} M with distilled water and adjusted to pH 6.0 with 0.1 N HCl. ABA solutions 10^{-4} and 10^{-5} M were concocted by further dilution. The ABA solutions were applied to detached leaves through their petiole.

Multiple amino acid sequence alignment. CaCOP1 cDNA has been isolated from chili pepper [Lee *et al.*, 2004]. Multiple alignments of CaCOP1 homologs were generated using http://us.expasy.org/tools. The accession numbers are: AAA32772 (*Arabidopsis thaliana*); AAK49415 (*O. sativa*); AAK81856 (*Rosa* Hybrid cultivar) and CBA89693 (*P. sativum*).

RNA isolation and RT-PCR analysis. To examine whether the expression of these genes is induced by abiotic stresses, total RNA was isolated from stress-treated and control chili pepper plants using TRI-reagent® according to the manufacturer's instructions (MRC, USA). Total RNA was treated with 1 U DNase for 10 min at 37°C and subjected to a second round of TRI-reagent purification. From the DNase-treated total RNA (1 μg), first-strand cDNA was synthesized using the AccuPower® PCR PreMix containing oligo(dT) primers, and Moloney murine leukemia virus reverse transcriptase (M-MLV RTase, Invitrogen, USA). The primers used for reverse transcriptase-PCR were listed in Table 1.

The PCR reaction was carried out as follows: initial 5 min denaturation at 94°C; followed by 30 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min; and a final 7 min at 72°C. Twelve microliter of the reaction products were separated on 1% agarose gels and visualized after staining with ethidium bromide. All experiments were preformed in triplicate.

DNA isolation and genomic DNA gel blot analysis. Genomic DNA was isolated from mature leaves of pepper cv Bukang. Genomic DNA samples (20 μg) were digested to completion with *Eco*RI, *Hind*III, and *Xba*I.

Table 1. Nucleotide sequences of the primers used for RT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
CaCOP1	ATGGGATCATTTTTCATCAG	TCAAGTAGCAAGAACGAGTG
CaActin	TTGGATTCTGGTGATGGTGTG	AACATGGTTGAGCCACCACTG
CaPR1	ACTTGCAATTATGATCCACC	ACTCCAGTTACTGCACCATT
Cadhn	ATGGCTGATCAGTATGAACAC	TTAGTGAGATGCTGCTTCTTT

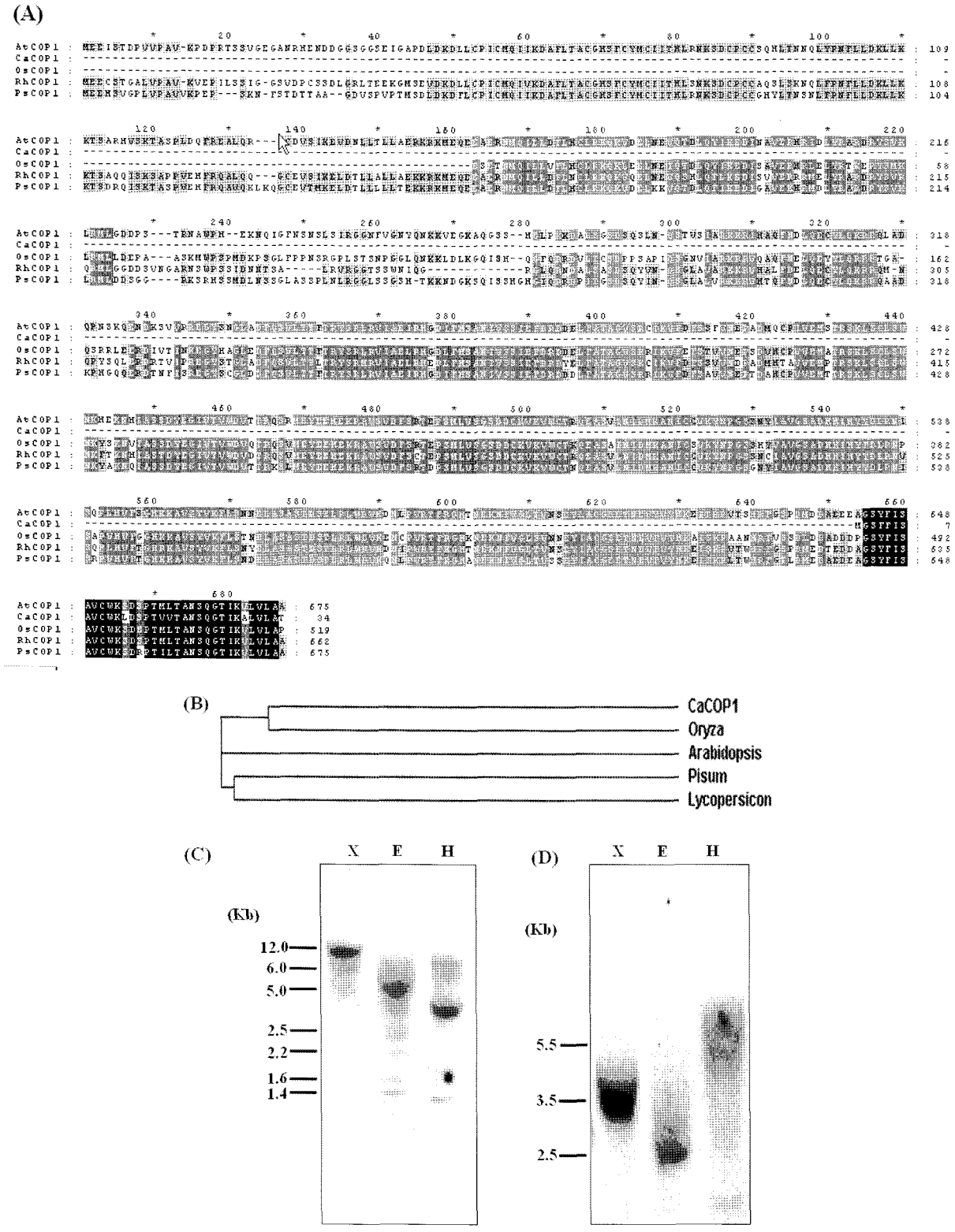

Fig. 1. Characterization of the *CaCOP1* gene. (A) Comparison of derived amino acid sequences of chili pepper *CaCOP1* (EST ID KS01047F10) with COP1 in *Arabidopsis*, *O. sativa*, Rosa hybrid cultivar, and *P. sativum*. Residues shaded in black are identical between the two proteins. The NCBI accession numbers of nucleotide sequences are the following: AAA32772, AAK49415, AAK81856 and CAB89693. (B) Phylogenic comparison of the five COP1-like family protein sequences. Alignments were made in ClustalW using the default parameters. Accession numbers for the four COP1 proteins used are as follows: BAD16847, AAA32772, CAB89693 and AAC98912. (C-D) Genomic DNA gel blots of *CaCOP1* was digested with *Xba*rI (X), *Eco*RI (E), and *Hind*III (H), loaded on agarose gel and hybridized with the ^{32}P-labeled probe corresponding to the full-length (C) and to the 3'UTR region (D) of *CaCOP1* cDNA.

Digested genomic DNA was separated by electrophoresis on a 1% agarose gel, denatured, and blotted onto a nylon membrane (Amersham Pharmacia, Uppsala). DNA gel blotting was conducted and membranes were hybridized with the *CaCOP1* cDNA probe (full length and 3'UTR specific probes) labeled with [α-^{32}P] dCTP.

Results and Discussion

Sequence and genomic DNA gel blot analysis of *CaCOP1*. cDNA of *CaCOP1* was probed with RNA extracted from chili pepper leaves infected by *X. ag* 8ra to isolate pepper genes induced during the non-host bacterial pathogen HR. *X. ag* 8ra is not a pathogen of pepper, but induce the expression of a number of PR genes, as well as does occur an HR in pepper leaves [Lee *et al.*, 2004], suggesting that one of the down-regulated genes showed *CaCOP1*, exhibited sequence similarity with rice constitutive photomorphogenesis 1. To determine the structure of *CaCOP1* cDNA, we sequenced the 886 bp insert of *CaCOP1*. This clone contains a single open reading frame of 105 nucleotides and the deduced polypeptide was 34 amino acids in length (Fig. 1A). The overall amino acid sequence identity between CaCOP1 and rice constitutive photomorphogenesis 1 is 80% at the amino acid level. However, the deduced amino acid of open reading frame of CaCOP1 (2-33) and C-terminal domain of AtCOP1 (residues 643-674), OsCOP1 (489-518), RhCOP1 (630-661) and PsCOP1 (643–674) display high sequence identity (Fig. 1A), which reveals that the C-terminal domains of COP1 in different species are highly conserved. The evolutionary relationships among CaCOP1 and OsCOP1, *Arabidopsis*, *P. sativum* and *L. esculentum* COP1 were analyzed (Fig. 1B). CaCOP1 and OsCOP1 were most similar (84%). CaCOP1 shares high sequence identity with regulatory protein in *Arabidopsis* (84%), constitutively photomorphogenic 1 protein in *P. sativum* (81%) and COP1 homolog in *L. esculentum* (79%).

To assess the copy number of the *CaCOP1* gene in chili pepper, DNA gel blot analysis was performed on pepper genomic DNA digested by *Xba*rI, *Eco*RI, and *Hind*III using ^{32}P-labeled *CaCOP1* full-length cDNA or 3' UTR as probes. Hybridization of the genomic DNA blot with a probe encompassing the full-length cDNA of *CaCOP1* resulted in multiple bands (Fig. 1C). In contrast, single hybridizing bands were detected from hybridization with a 3' UTR gene-specific probe (Fig. 1D). Some bands are not from the presence of the endogenous restriction-enzyme sites, such as *Xba*I or *Hind*III, since the 3' UTR region does not contain any of these. This finding indicates the presence of other *CaCOP1*-related genes. However, *CaCOP1* gene exists single copy in the chili pepper genome.

Expression of *CaCOP1* mRNA in response to bacterial pathogen. The transcription level of *CaCOP1* gene was analyzed in the pepper leaves inoculated with *X. ag* 8ra (Fig. 2). However, *X. ag* is not a pathogen of chili pepper but a casual agent of pustule disease on bean [Oh *et al.*, 2005]. It elicits a hypersensitive response (HR) in pepper leaves as well as inducing expression of a number of pathogenesis-related (PR) genes [Kim *et al.*, 2002; Lee *et al.*, 2004]. As shown in Fig. 2, control plants were infiltrated with 10 mM $MgCl_2$ buffer only. *CaCOP1* gene transcript reduced after pathogen inoculation, suggesting it was not induced by non-host pathogen infection. Under same conditions, *CaPR1* mRNA level were investigated as a positive marker of pathogen inoculation. The transcript of *CaPR1* was detected 6 h after pathogen inoculation, and increased continuously over the time-course of the study. Our result showed that *CaCOP1* gene transcript reduced after non-host pathogen inoculation, however whether it is involved in host resistance response would be further analyzed.

Expression of *CaCOP1* mRNA in response to various abiotic and oxidative stresses. To determine whether *CaCOP1* gene in chili pepper affects the

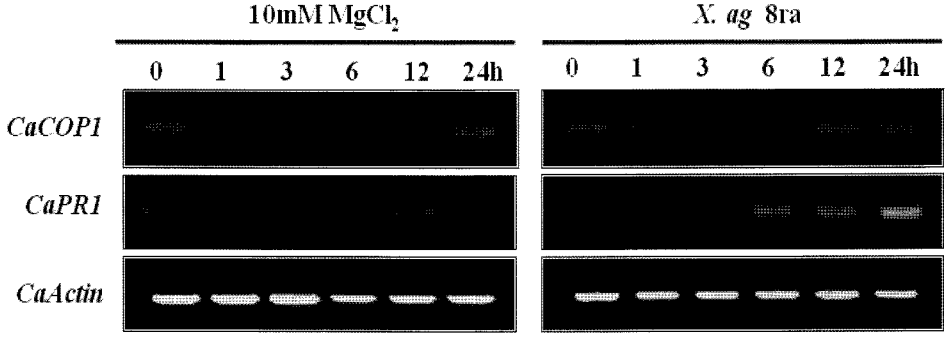

Fig. 2. Expression of *CaCOP1* mRNA in response to bacterial pathogen. (A) Chili pepper plant leaves (cv. Bukang) were infiltrated with bacterial suspensions (1×10^8 cfu/mL) of the bean pustule pathogen, *X. ag* 8ra, or 10 mM $MgCl_2$ as a buffer control.

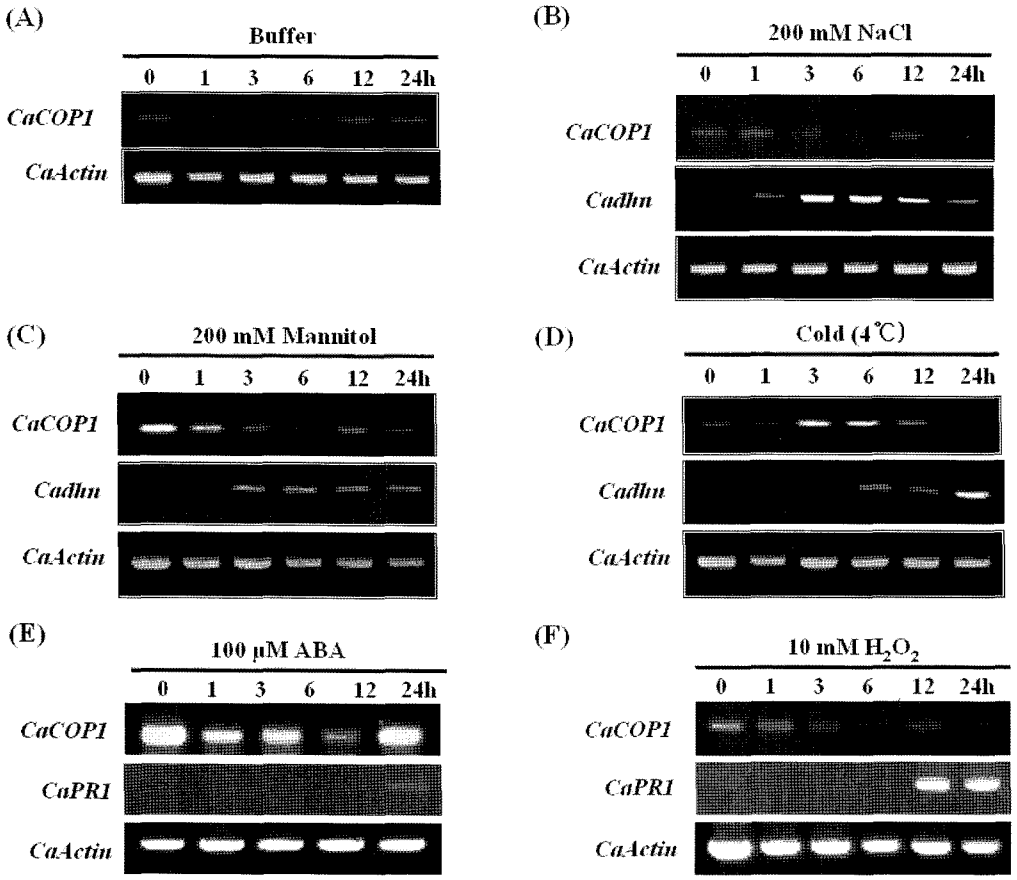

Fig. 3. The expression of the *CaCOP1* gene in the pepper leaf tissues, exposed to abiotic and oxidative stresses. (A) Buffer treatment was used as control, (B) NaCl (200 mM), (C) mannitol (200 mM), (D) cold treatment at 4°C, (E) ABA (100 μM), (F) H_2O_2 (10 mM). Total RNA (20 μg) from leaf samples at various time points after treatment was loaded into each lane. *CaCOP1* cDNA inserts in pBluscript SK was used as probes. To ensure equal loading of RNA, a duplicate gel was stained with ethidium bromide as an RNA loading control. The *Cadhn* gene was used as a positive control.

responses of plants to abiotic stresses, the expression of this gene under abiotic stresses was investigated by RT-PCR, including 200 mM NaCl, 200 mM mannitol, cold (4°C), 10 mM H_2O_2, and following 100 μM ABA treatment (Fig. 3). As a response to NaCl treatment, *CaCOP1* transcript increased slightly at 1 h after treatment (Fig. 3B). In the mannitol-treated pepper leaves, the *CaCOP1* transcripts were not induced but decreased after treatment (Fig. 3C). By contrast, *CaCOP1* gene transcripts were induced 3 h after cold treatment, and then began to decline gradually 12-24 h after treatment (Fig. 3D). However, *CaCOP1* transcript level is not induced by ABA (Fig. 3E) and decreased after H_2O_2 treatment (Fig. 3F). Buffer treatment was used as control condition of all abiotic stresses. *Cadhn* transcripts were strongly induced in all abiotic stresses. These results suggest that *CaCOP1* gene is not involved in mannitol, ABA and H_2O_2 stresses signal pathway but is related to cold mediated signal transduction at the transcriptional level. *Cadhn* used as marker gene was osmotic stress regulated *dehydrin* gene of chili pepper [Chung *et al.*, 2003].

Studies of abiotic stress signal transduction have identified a pathway which leads to a response to both cold and drought stresses, and appears to function via members of the ethylene responsive element binding factor (ERF, known as also EREBP) transcription factor family [Hwang *et al.*, 2005]. It was also demonstrated that transgenic tobacco and *Arabidopsis* plants which overexpressed SCOF-1 also exhibited enhanced cold tolerance properties, which were attributed to the increased expression of cold-regulated genes [Kim *et al.*, 2001; Huang *et al.*, 2005]. A total of 317 cold inducible genes were isolated in the chili pepper (*Capsium annuum*) using cDNA microarray analysis and Northern blot analysis [Hwang *et al.*, 2005].

Most of the genes that respond to drought, salt, and cold stress are also induced by exogenous application of ABA [Shinozaki and Yamaguchi-Shinozaki, 1996]. However, several genes that are induced by water stress are not responsive to exogenous ABA treatment. These findings suggest 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]. In our study, *CaCOP1* was specifically induced 3 h after low temperature treatment after but not by ABA, which confirmed this result that at least two separate regulatory systems function in gene expression during drought and cold stress.

In the present study, we investigated the expression of *CaCOP1* gene under abiotic stresses by RT-PCR. *CaCOP1* was induced significantly in cold stress but not by dehydration or high salinity. Furthermore, *CaCOP1* was not induced by plant hormone ABA. These observations collectively provide initial evidence that *CaCOP1* gene was related to cold stress and maybe belong to ABA-independent regulation system. The mechanisms underlying the activation of cold response by *CaCOP1* remain to be elucidated in detail.

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