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A novel *CaAbsi1* gene induced by early-abiotic stresses in pepper

*Eun Soo Seong & Myeong-Hyeon Wang**
School of Biotechnology, Kangwon National University, Chuncheon, Korea

The full-length cDNA of *CaAbsi1* encodes a presumptive protein of 134 amino acid residues that has homology to a putative zinc finger protein in its C-terminus. The deduced amino acid sequence has 50% homology to *Oryza sativa* NP001049-274, the function of which is unknown. Expression of *CaAbsi1* was reduced in response to inoculation of non-host pathogens. On the other hand it was induced one hour after exposure to high concentrations of NaCl or mannitol, and six hours after transfer to low temperature. Induction also occurred in response to oxidative stress, methyl viologen, hydrogen peroxide and abscisic acid. Our results suggest that *CaAbsi1* plays a role in multiple responses to wounding and abiotic stresses. [BMB reports 2008; 41(1): 86-91]

INTRODUCTION

Plant productivity is severely affected by abiotic stresses such as high salinity, drought, high and low temperature, and heavy metals; physiological and biochemical responses are altered and cellular aqueous and ionic equilibria are disrupted. The expression of hundreds of genes is affected by these stresses (1-3), and understanding the functions of these stress-inducible genes should help to clarify mechanisms of stress tolerance. The application of multi-parallel techniques such as expression profiling by microarrays can provide important clues for characterizing stress responsive genes and stress tolerance mechanisms. Biotic stress studies have demonstrated significant overlaps between plant responses to osmotic stresses (4). Dehydration, salinity, and low as well as high-temperature stresses damage metabolism and lead to the generation of reactive oxygen species (ROS) and inhibition of photosynthesis (5). At the molecular level, abiotic stress tolerance can be achieved via gene transfer by altering the accumulation of osmoprotectants, producing chaperones, increasing superoxide radical scavenging mechanisms, and extruding or sequestering ions by means of transport and symport systems (5-8).

One of the pathways that plays a significant role in plant responses to drought and osmotic stress mediated by abscisic acid (ABA). Sequences encoding MYC and MYB are essential for the ABA- and drought-responsive expression of rd22 (9). In both cases, the ABA-responsive element (ABRE) and MYC/MYB systems seem to function after the accumulation of endogenous ABA (10). Drought tolerance is enhanced in transgenic plants overexpressing AtNCED3, a key enzyme in ABA biosynthesis and three ABA-induced NAC transcription factors (11, 12). Transcription factors containing the AP2 domain increased drought tolerance when overexpressed in *Arabidopsis* (13). Thus, these and many other reports suggest that although stress-responsive genes are under the control of complex regulatory networks, individual genes can have major effects on stress tolerance (14). Molecular characterization of such gene families implicated in stress responses is needed to improve our understanding of how plants cope with adverse environmental conditions.

In this study we describe a novel family of proteins with 50% homology to the family of Cys2-His2 (C_2H_2) putative zinc finger domain proteins in *Oryza sariva*. The homology extends over only of the amino acid sequences. The strong conservation of zinc fingers in several plant species suggests that they share a common or closely related biological function. RT-PCR and Northern blot analyses showed that *CaAbsi1* expression increased 1-3 hours after exposure to high salt, mannitol or cold as well as in response to oxidative stresses and ABA treatment. These observations indicate that this novel gene plays an important role in multiple signaling pathways activated by abiotic stresses.

RESULTS AND DISCUSSION

Isolation of pepper *CaAbsi1*

Sequence analysis of the 354 bp EST clone of *CaAbsi1* revealed that the cDNA was truncated, because the predicted open reading frame did not contain a start codon (NCBI accession No. EF373541). The complete full length sequence of *CaAbsi1* contained an open reading frame encoding a 118 amino acid protein (Fig. 1A).

To determine whether CaAbsi1 was a putative zinc finger

*Corresponding Author. Tel: 82-33-250-6486; Fax: 82-33-241-6480; E-mail: mhwang@kangwon.ac.kr

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protein, the predicted amino acid sequence of CaAbsi1 was compared to those of characterized homologous zinc finger proteins. BLAST analysis showed that CaAbsi1 was more than 41% identical to C_2H_2 zinc finger proteins in several plant species (Fig. 1B), suggesting that this domain might have a significant biological function. The functions of these homologous zinc finger-like families are unknown, as are their responses to various abiotic and biotic stresses. We therefore decided to investigate this novel family of proteins in detail. Several zinc-finger proteins from petunia hybrids, durango root, soybean, alfalfa, *Arabidopsis thaliana*, tobacco and turnip possess different-sized spacers located between zinc-finger motifs, and the variation in the lengths of these spacers may contribute to the selection of their target DNA sequences (15). To examine the genomic organization of *CaAbsi1*, hot pepper genomic DNA was isolated from cv. Bukang and digested with *Xba*I (X) or *Hind*III (H). Southern blot hybridization was carried out using the EST clone of *CaAbsi1* as a full-length probe. Under high-stringency conditions at 65°C, multiple bands were observed in restriction enzyme digests of genomic DNA (Fig. 1C), indicating that there are multiple copies of *CaAbsi1* in the chili pepper genome.

Expression of CaAbsi1 during interaction with a non-host pathogen

We examined the expression of CaAbsi1 during HR. As shown in Fig. 2, the decline of *CaAbsi1* expression monitored using full-length primers started 6 h post-inoculation with *X. ag* 8ra. Rapid high level accumulation of CaAbsi1 mRNA was detected in control plants that were inoculated with 10 mM $MgCl_2$. As a positive control for inoculation of bacterial pathogens we monitored the expression of CaPR1 (16). Its expression was induced 6 h post-inoculation with *X. ag* 8ra. Expression monitored using 3'-UTR primers were similarly reduced.

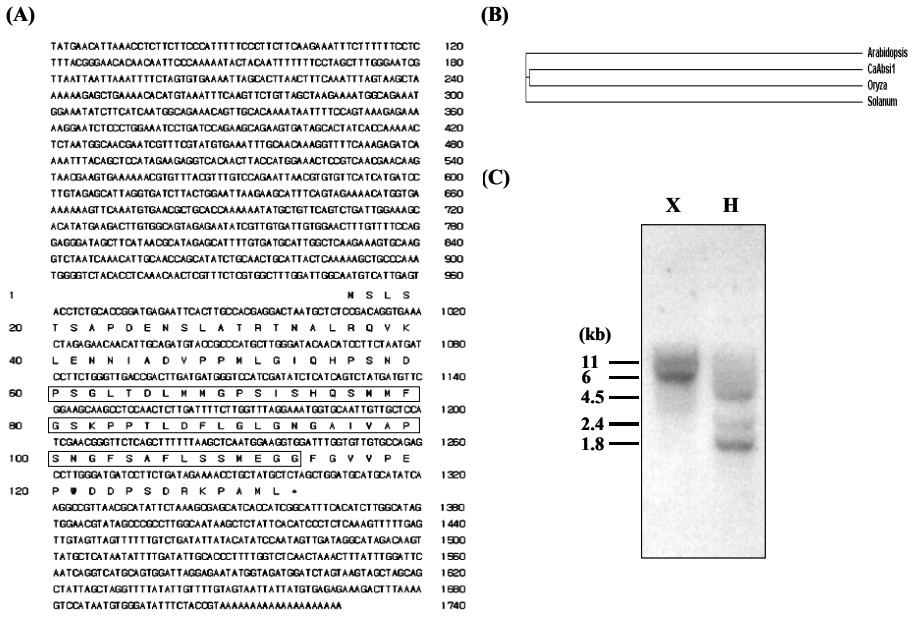

Fig. 1. Isolation and characterization of CaAbsi1. (A) Nucleotide and deduced amino acid sequence of CaAbsi1 (GenBank Accession No. EF373541). Predicted amino acids are represented by one letter codes. The stop codon is marked by an asterisk. Box shows zinc finger domain. (B) Phylogenic tree of CaAbsi1 (EF373541) homologues in *Solanum lycopersicum* (AAG01127), *Arabidopsis thaliana* (NP190639), and *Oryza sativa* (NP001049274). (C) Genomic organization of *CaAbsi1*. Genomic DNA was digested with *Hind*III and *Xba*I, separated on a 0.7% agarose gel, and transferred to a nylon membrane. A ^{32}P-labeled full-length cDNA of *CaAbsi1* was used as probe.

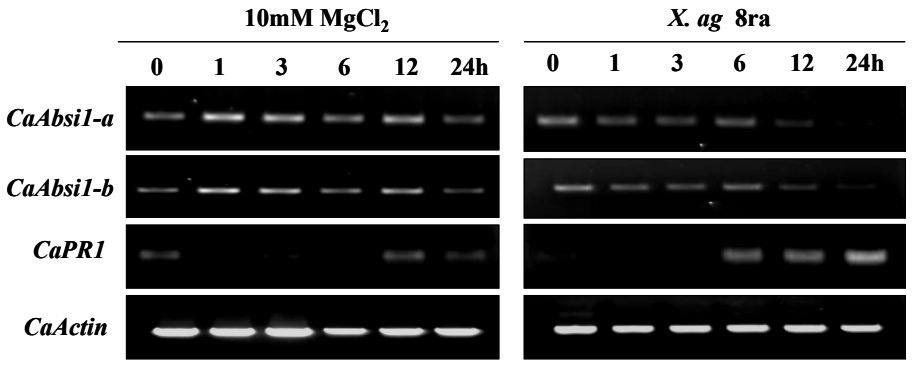

Fig. 2. Expression of *CaAbsi1* for 10 mM $MgCl_2$ and a bacterial pathogen. Chili pepper plants (cv. Bukang) were inoculated with the non-host bacterial pathogen, *X. ag* 8ra. Total RNA was isolated from leaves harvested at the indicated times after bacterial challenge and performed RT-PCR analysis. CaAbsi1-a was used primers containing of full sequence and CaAbsi1-b was used 3'-UTR primers.

Responses to mechanical wounding in plants involve the induction of numerous defense-related genes (17). In tomato plants, signal pathway genes are expressed within 0.5 h of wounding, whereas defense genes are expressed within 4 h (18). In our study, *CaAbsi1* transcription was strongly induced within 1 h by $MgCl_2$ infiltration wounding and continued to increase up to 12 h. Evidently *CaAbsi1* is implicated in a signal pathway triggered by wounding. Wounding also induces ethylene biosynthesis and ethylene acts in conjunction with JA to regulate PIN expression (19). In addition to these signals, the WR is known to involve ROS (20).

The CAZFP1 gene containing two Cys2/His2-type zinc-finger motifs is induced to a low level by 18 h after inoculation in compatible interactions in pepper. Transcript levels of *CAZFP1* are higher in incompatible interactions than in compatible ones (21). and transcripts of CaWRKY2 with two putative zinc finger motifs (C-X4-C-X22-23-HX1-H) are preferentially induced during incompatible interactions in plants exposed to PMMoV, *Pseudomonas syringae* pv. syringae 61, and *Xanthomonas axonopodis* pv. vesicatoria race 3 (22).

Expression of CaAbsi1 in response to abiotic stresses

We examined the expression of CaAbsi1 in response to treatment with elicitors related to abiotic stresses. CaAbsi1 expression was induced within an hour of exposure to NaCl and mannitol (Fig. 3C, D), and in response to cold CaAbsi1 transcription began after 3 h and reached a maximum by 6 h (Fig. 3B). Increase of biomass productivity under water deficit conditions in transgenic lines overexpressing the barley HVA1 gene, and transgenic *Arabidopsis* plants overexpressing *CAZFP1* had enhanced resistance to bacterial infection and drought. CAZFP1 is an early-defense gene enhancing disease resistance and drought tolerance (21). Transgenic plants overexpressing the stress-induced NAC1 transcription factor (SNAC1) had enhanced drought tolerance under field conditions (23).

ABA is an important plant hormone involved in the adaptive responses of plants to various environmental conditions (24). We found that CaAbsi1 mRNA accumulated within 3 h of ABA treatment (Fig. 3E) but transcript levels were lower than in plants subjected to high salt or water-deficit stress (Fig. 3C, 3D). Exposure of plants to osmotic stress results in elevated ABA biosynthesis, and the increased ABA induces a number of genes (25, 26). These findings suggest that *CaAbsi1* is responsive to both osmotic stress and ABA.

CaAbsi1 is induced by oxidative stresses

Reactive oxygen species (ROS) act as signal molecules in the defense responses of plants (27). To examine the influence of ROS on *CaAbsi1* expression, we treated plants with 50 μM methyl viologen (MV). *CaAbsi1* transcripts were induced within an hour and declined soon after (Fig. 4A). *CaAbsi1* expression was also induced within three hours by exposure to H_2O_2 (Fig. 4B). The expression level of the zinc finger protein

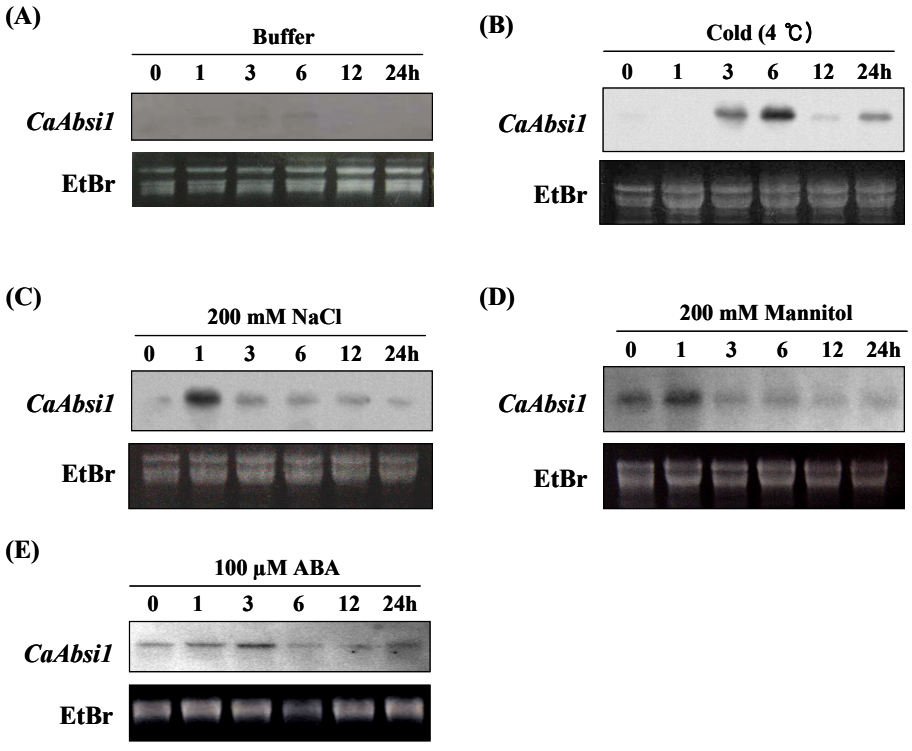

Fig. 3. Expression of *CaAbsi1* mRNA in response to abiotic stresses. (A) Expression of *CaAbsi1* mRNA following treatment with buffer, (B) low temperature, (C) high salt, (D) mannitol (drought mimic) or 100 μM ABA (E). Total RNA was prepared from 2-month-old pepper plants transferred to 4°C, to a 200 mM mannitol solution, or to a 200 mM NaCl solution.

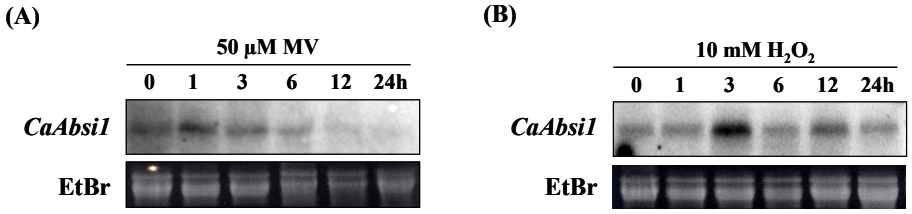

Fig. 4. Expression of *CaAbsi1* in pepper plants treated with chemicals related to oxidative and osmotic stresses. Total RNA was extracted from leaves exposed to 50 μM MV (A) or 10 mM H_2O_2 (B) at the indicated times after treatment.

increased after pathogen attack or treatment with ethephon, salicylic acid, methyl jasmonic acid and cold (28). Homologs to CaPIF1 were found in many plant species, e.g. 14 ZPT2 genes in petunia (29), ZFT1 in tobacco (30), WZF1 in wheat (31), STZ in *Arabidopsis* (32), Pszf1 in pea (33), Mszpt2-1 in alfalfa (34) and SCOF-1 in soybean (35). These Cys2/His2 zinc finger proteins (also called TFIIIA-type, or classical zinc finger) were located in the nucleus and could bind to DNA. The Cys2/His2 zinc finger protein was known to work as repressors, regulating alkaloid biosynthesis transcripts in *Catharanthus roseus* (36). The spermine-mediated signal transduction pathway in *Nicotinana* was indicated by drought, cold and high-salinity stress conditions *in Arabidopsis* (37). Our observations show that *CaAbsi1*, a homologue of a Cys2/His2-type zinc-finger transcription factor gene of *Arabidopsis* (NP190639), is a novel abiotic stress gene induced at early times in pepper. *CaAbsi1* transcripts were rapidly induced by abiotic stresses and fell soon after. *CaAbsi1* expression also responded to wounding. In further work we intend to test whether overexpression of *CaAbsi1* in transgenic tobacco plants enhances their resistance to abiotic stresses. Further *in vivo* analyses, such as examination of gene silencing phenotypes, should help to clarify the role of *CaAbsi1* in responses to abiotic stresses.

MATERIALS AND METHODS

Treatment with pathogens, abiotic elicitors and environmental stresses

Chili pepper (*Capsicum annuum*) 'Bukang' seeds were cultured in MS medium (MS salts with MS vitamins, 3% sucrose, 0.8% agar, pH 5.8). 2 weeks 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. axonopodis* pv. glycines 8ra (*X. ag* 8ra), a soy bean pustule pathogen (38). Bacterial infiltration was accomplished by syringe infiltration of bacterial suspensions (approximately 4×10^8 cfu/ml). Inoculated leaves were collected at various times and used as source of RNA for further analyses. The abiotic elicitors were applied to the leaves of the pepper plants at the 6-leaf stage. For the abscisic acid (ABA) and NaCl treatments the pepper plants were removed from the soil. Their roots were carefully washed and then soaked in 100 μM ABA, 200 mM NaCl, or 200 mM mannitol solutions. The low temperature treatment was performed at 4°C. Hydrogen peroxide (H_2O_2, 10 mM) and methl viologen (MV, 50 μM) were sprayed onto the pepper leaves. The plants were exposed to the elicitors and stress treatments for various times and the treated leaves were cut from the plants, frozen in liquid nitrogen and stored at −70°C for later extraction of RNA.

Southern hybridization

Genomic DNA was prepared as described by Dellaporta *et al* (39). Twenty micrograms of total DNA was digested with *Xba*I or *Hind*III and the digested DNA was fractionated by size on 0.8% (w/v) agarose gels. Membranes were hybridized overnight with a ^{32}P-labeled fragment of the PCR product of the 3'UTR of the *CaMYB* cDNA in a buffer consisting of 1% BSA /1 mM EDTA/0.5 M $NaHPO_4$, pH 7.2/7% SDS at 65°C and washed in 0.5% BSA/1 mM EDTA/40 mM $NaHPO_4$, pH 7.2/5% SDS at room temperature for 5 min. The blot was then washed three times with high stringency wash buffer (1 mM EDTA/40 mM $NaHPO_4$, pH 7.2/5% SDS) at 65°C, and the dried blots were placed on X-ray film at −80°C for a week and developed.

RNA extraction and RT-PCR analysis

Total RNA was isolated from the leaves of treated sample with TRI-reagent® (MRC, USA). It was treated with 1 U DNase for 10 min at 37°C and subjected to a second round of TRI-reagent purification. First-strand cDNA was synthesized from 1 μg samples of the DNase-treated total RNA using AccuPower® PCR PreMix (Bioneer, Korea) containing oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen, USA). The primers used for reverse transcriptase-PCR were as follows:

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
CaAbsi1-a	ATGGATCGGGTTAAAGGTCCA	CTAATCAAATTTGCTTATTCC
CaAbsi1-b	CTGGATGCATGCATATCAAGG	TTACGGTAGAAATATCCCACA
CaActin	TTGGATTCTGGTGATGGTGTG	AACATGGTTGAGCCACCACTG
CaPR1	ACTTGCAATTATGATCCACC	ACTCCAGTTACTGCACCATT

PCR conditions were as follows: initial denaturation for 5 min at 94°C; followed by 25 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min; and a final 7 min at 72°C. Twenty μl samples of the reaction products were separated on 1% agarose gels and visualized by staining with ethidium bromide. All experiments were preformed in triplicate.

Northern blot analysis

Total RNA concentrations and purity were determined by spectrophotometer and staining of ribosomal RNA with ethidium bromide, respectively. Equal quantities of total RNA were loaded on 1% agarose gels containing 7.4% formaldehyde. After electrophoresis and visualization under UV light, the RNA was transferred to nylon membranes (Hybond N^+, Amersham), and baked for 2 h at 80°C. To generate a *CaAbsi1* gene-specific probe, each coding sequence (ORF) was PCR-amplified with T_3 and T_7 primers for CaAbsi1 cloned into pBluscript. The purified PCR products were ^{32}P-labeled, using a random primer kit (Boehringer Mannheim, Mannheim, Germany). 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 filter 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.

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