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Molecular Characterization of the *Capsicum annuum* RING Zinc Finger Protein 1 (*CaRZFP1*) Gene Induced by Abiotic Stresses

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A cDNA clone, *CaRZFP1*, was isolated from high temperature-stressed hot pepper plants. This gene contains an open reading frame (ORF) encoding a RING zinc finger protein of 219 amino acids. Our C3HC4 type protein has the C-X2-C-X11-C-X1-H-X2-C-X2-C-X13-C-X2-C sequence motif. Phylogenetic analysis based on the deduced amino acid sequence of *CaRZFP1* cDNA revealed significant sequence similarity (87.2%) to the *Capsicum chinense* zinc finger protein. The putative protein, hydrophilic by nature, encoded by *CaRZFP1* also shared 66.9% homology with an *Arabidopsis thaliana* RING zinc finger protein and 61.2% homology with an *Oryza sativa* RING finger protein. Transcripts were preferentially induced in response to high or low temperatures, drought, and high salinity, but were not detectable in response to heavy-metal treatment. When plants were exposed to high temperature, two strong signals appeared, one for *CaRZFP1*, the other apparently from a family gene of *CaRZFP1*. This demonstrates that high temperatures induce a zinc finger type putative transcription factor from red pepper.

Keywords: abiotic stress, *Capsicum annuum*, high temperature-induced, RING zinc finger protein

Abiotic stresses adversely affect the growth and productivity of crops worldwide (Hoerling and Kumar, 2003; Peters et al., 2004; Suzuki et al., 2005; Mittler, 2006)). High and low temperatures, drought, high salinity, and heavy metals commonly disrupt life cycles in higher plants (Cho et al., 2006; Mittler, 2006). Discovering the genes involved in plant responses to these environmental stresses can provide new tools for genetic engineering of more tolerant field crops. However, the acclimation of organisms to such stresses involves a complex network of transcription factors and genes that are components of signal transduction pathways (Fujita et al., 2006).

The RING (Really Interesting New Genes) domain is a protein interaction domain that has been implicated in a range of biological processes, including oncogenesis, development, signal transduction, and apoptosis (Borden and Freemont, 1996; Saurin et al., 1996; Liu et al., 1999; Vij and Tyagi, 2006). Two variants, the C3HC4- and C3H2C3-types, are clearly related despite having different cysteine/histidine patterns. The C3H2C3 type zinc-finger, sometimes called the 'RING-H2 finger'. The C3HC4 type zinc finger (RING finger) is a cysteine-rich domain of 40 to 60 residues that coordinates two zinc ions. It contains the consensus sequence of C-X2-C-X(9-39)-C-X(1-3)-H-X(2-3)-C-X2-C-X(4-48)-C-X2-C, where 'X' is any amino acid (Hatakeyama and Nakayama, 2003; Alexandrov et al., 2006). Metal ligand Pairs 1 and 3 coordinate to bind one zinc ion, while Pairs 2 and 4 bind the second. The 3D structure of the zinc ligation system is unique to the RING domain, and is referred to as the 'cross-brace' motif (Borden and Freemont, 1996). The latter is probably involved in mediating protein-protein interactions (Borden and Freemont, 1996; Saurin et al., 1996). The RING domain, the most conserved region within genes of the same family, is responsible for DNA-binding and oligomerization (Liu et al., 1999).

A number of zinc finger protein genes have been cloned and characterized in relation to different types of stress or developmental stages in various plants. These include hot pepper *CaZFP1*, a C2/H2-type zinc finger transcription factor involved in promoting both resistance to infection by *Pseudomonas syringae* and tolerance to drought (Kim et al., 2004), CaWRKY2, which contains two WRKY domains with zinc finger motifs, is a pathogen-inducible transcription factor that functions in early defense responses to biotic and abiotic stresses (Oh et al., 2006). Overexpression of a zinc finger protein *OSISAP1* from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco (Mukhopadhayay et al., 2004; Vij and Tyagi, 2006). Zinc fingers also have been isolated in response to treatments with salt (Zhu, 2000) and chilling (van der Krol et al., 1999). From these results, we conclude that plant zinc finger proteins are essential to stress responses and developmental processes.

Hot pepper (*Capsicum annuum*) is an economically important crop that is cultivated widely in East Asia for its hot taste. Here, we isolated a novel high temperature stress-inducible C3HC4-type RING zinc finger protein gene (*CaRZFP1*) from a cDNA library of hot pepper (cv. Bu Gang). We also analyzed mRNA expression patterns in response to various other abiotic stresses.

MATERIALS AND METHODS

Preparation of cDNA Library and Double Negative Screening

We used a previously constructed cDNA library for high temperature-stressed hot pepper (*C. annuum* cv. Bu Gang) (Ashrafuzzaman et al., 2005). Briefly, after the poly (A) RNA

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was purified at 42°C for 30 min, a unidirectional *Eco*RI/*Xho*I cDNA library was constructed in a Uni-λZAP XR vector (Stratagene, USA). The cDNA and vector recombinants were treated with Gigapack III gold packaging extracts (Stratagene). For double negative screening, after amplification and titrating, duplicate plaque lifts were made from 150-mm plates (~5X 10^3 pfu per plate) onto nylon membranes (Hybond-N, Amersham, UK). These lifts were denatured, neutralized, blotted, dried, and exposed to UV for cross-linking. The membranes were then hybridized with two different probes that had been made using poly (A) RNA from either heat-shocked or unstressed (control) hot pepper plants to the ^{32}P-labeled cDNA. The hybridized membranes were washed and exposed to X-ray film overnight (Sambrook et al., 1989). Five hundred plaques showing high-temperature inducibility were randomly selected and subcloned into pBluescript SK(–) via the ExAssist helper phage (M13 stratagene). The cDNAs were amplified in *Escherichia coli* after *in vivo* excision.

Plant Materials and Growing Conditions

Seeds of hot pepper were sown in plastic pots and reared in a growth chamber under controlled conditions of 25°C, 60% relative humidity, and a 16-h photoperiod from white fluorescent lamps (200 μE m^{-2} s^{-1}). Healthy, 4-week-old plants were used for the treatments and nucleic acid extractions.

Application of Abiotic Stresses

Young plants with 4 to 6 leaves were chosen for treatments with high or low temperature, drought, salinity, or heavy metal. For the heat-shock trials, seedlings were placed in an incubator at 42°C for 2 or 4 h. Relative humidity was maintained at 90%, as measured with a hygrometer (UEI, Korea). Control plants were held in a growth room at 25°C, under a 16-h photoperiod. For the drought treatment, 4-week-old seedlings were carefully transferred from their pots onto dry paper in the growth room. To monitor the effect of salt stress, intact plants were removed from the soil before their roots were washed with water and soaked for various periods in a solution containing 250 mM NaCl. As a control, distilled water was substituted for the NaCl. To induce chilling stress, plants were exposed to 4°C for 4, 8, 12, or 24 h. Heavy-metal experiments were performed in the growth room by soaking the roots for various periods in 100 mM solutions containing $CaCl_2$, $MgCl_2$, $MnCl_2$, $LiCl_2$ or $CoCl_2$. The stressed plant tissues from all completed treatments were then frozen in liquid nitrogen and stored at -70°C.

Isolation and Nucleotide Sequencing of *CaRZFP1*

Cloned plasmids were extracted and purified with a DNA-spin plasmid DNA purification kit (iNtRONE, Korea) to yield template plasmid DNA suitable for automatic nucleotide sequencing. Both strands of the *CaRZFP* cDNA inserts in pBluescript SK(–) were sequenced using T3 and T7 primers on an automated DNA sequencer (ABI 3730; Applied Biosystems, USA). Amino acid sequences were deduced from the nucleotide sequences according to the program ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics, which is available at webpage http://au.expasy.org (Gasteiger et al., 2003). These nucleotide and deduced amino acid sequences were searched for homologous genes and proteins in the databases, using the PC/Gene software system and BLAST network services at the National Center for Biotechnology Information (Altschul et al., 1997). Hydropathicity of the deduced amino acid sequences was calculated as described by Kyte and Doolittle (1982). Multiple alignment and construction of a phylogenetic tree were achieved through the Gene Bee-Molecular Biology server, which is managed by the A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Russia.

RNA Isolation and Blot Analysis

Total RNA was isolated from plant tissues that previously had been frozen in liquid nitrogen. These were ground to powder and homogenized in 3 ml of extraction buffer [100 mM LiCl, 100 mM Tris-Cl (pH 8.0), 10 mM EDTA, and 1% SDS (w/v)]. Before extraction, 3 ml of DEPC- treated phenol was added to the extraction buffer and preheated at 80°C for at least 10 min. A mixture of 3 ml of chloroform-isoamyl alcohol (24:1) was added to the homogenate, followed by vortexing and centrifugation at 10000*g* for 25 min at 4°C. Afterward, the supernatant was transferred to a 1.5 ml microcentrifuge tube and the extraction was repeated with a 1.5 ml chloroform-isoamyl alcohol (24:1) mixture. Precipitation was performed in an equal volume of 4 M LiCl at -70°C for 2 h. After centrifugation, the pellets were washed with cold 70% ethanol and dissolved in DEPC-treated distilled water. Equal quantities of total RNA (20 μg) were loaded onto an 1.2% agarose gel with formaldehyde. To check the integrity of the samples, RNAs were visualized by adding ethidium bromide to the sample before loading. After electrophoresis, the RNA was transferred onto nylon membranes (Hybond-N^+; Amersham), (Sambrook and Russel, 2001), followed by cross-linking under irradiation with UV light. To generate *CaRZFP1* gene-specific probes, the coding sequence was PCR-amplified with two primers: 5′-ATATATGCAGAAGTCAACTGCTACG-3′ and 5′-ATATCTAACCAAACAAATATAGGAATAC-3′. PCR was carried out with an initial cycle of 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min; with a final 5 min at 72°C, all performed in a thermal cycler (Perkin-Elmer, USA). The purified PCR products were labeled with α^{32}P-dCTP for hybridization. Both the pre-hybridization for 1 h and hybridization for 16 to 22 h were conducted in solutions containing 5 ml of 1 M dibasic sodium phosphate (pH 7.2), 5 ml of 14% SDS, and 20 μL of 0.5 M EDTA (pH 8.0) at 65°C. After hybridization, the membrane was washed twice with 2X SSPE and 0.1% (w/v) SDS for 15 min, once at room temperature, then at 65°C; and in 1X SSPE and 0.1% SDS at 65°C, before being exposed to X-ray film (Sambrook et al., 1989).

RESULTS

Isolation and Amino Acid Sequence Analysis of *CaRZFP1*

From our expressed sequence tag (EST) analysis of a cDNA library, which was prepared from heat-shocked hot

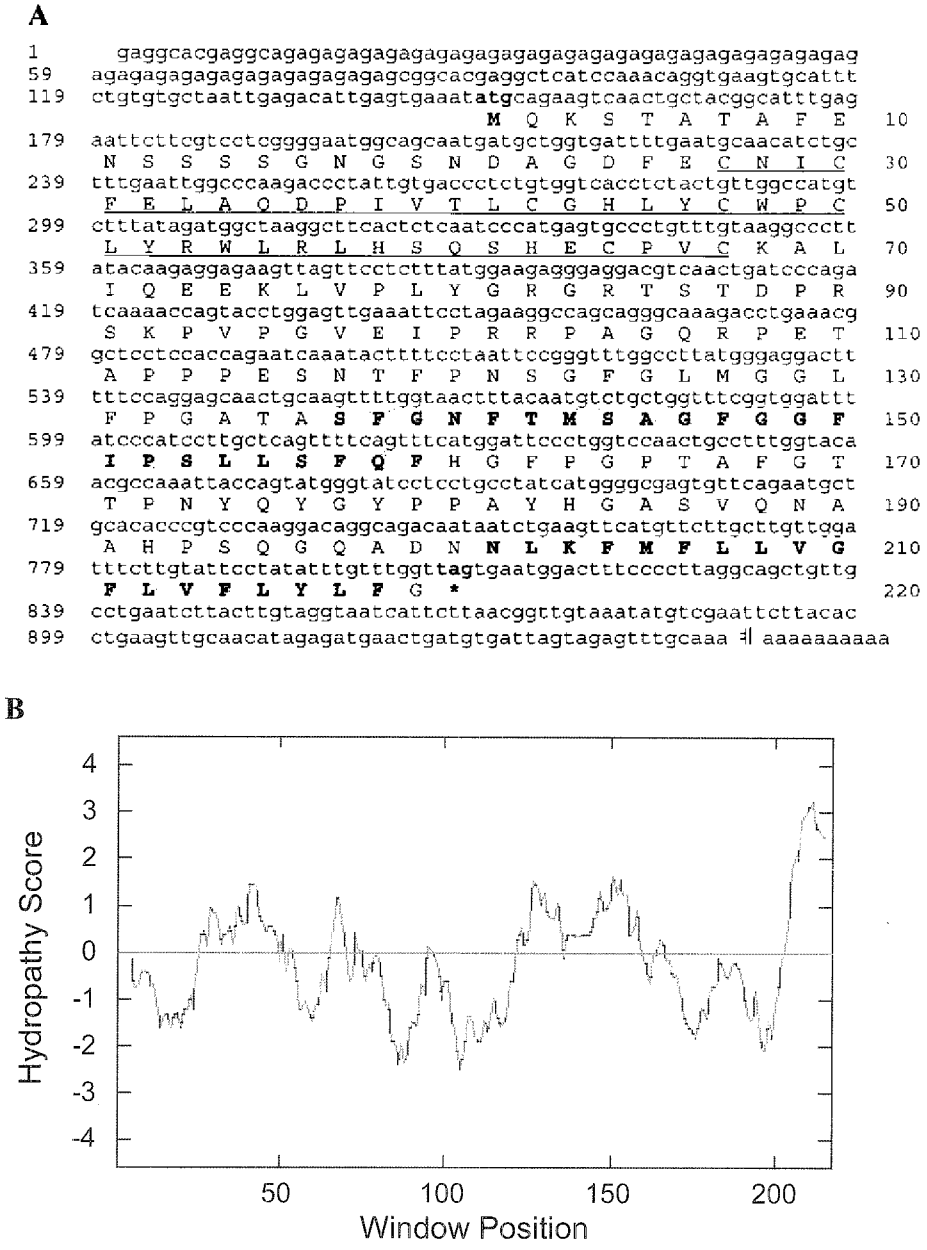

Figure 1. Sequence analysis of *CaRZFP1*. (**A**) Nucleotide sequence and deduced amino acid sequence of *CaRZFP1* cDNA. Residues corresponding to highly conserved RZFP motifs are underlined. Sequence motifs that match consensus sequence for transmembrane domain are represented by shaded boxes. Transcriptional start site is shown in bold face and termination codon is marked by asterisk (*). (**B**) Hydropathic index analysis of *CaRZFP1* deduced amino acid sequence. Hydrophobic domains are indicated by positive numbers; hydrophilic domains are above line and hydrophilic domains are below.

pepper, we identified a cDNA clone, HTI 80 (High Temperature Induced 80) encoding a RING finger type Zinc binding motif. We named this gene *CaRZFP1* (*C. annuum* Ring zinc finger protein 1); its nucleotide sequence is presented in Figure 1. The 958-bp *CaRZFP1* cDNA contains a 148-b 5′ untranslated sequence and a 150-b 3′ untranslated region flanking the putative open reading frame. This ORF encodes a Ring zinc finger protein of 219 amino acids, beginning at the initiation codon ATG (Position 149) and ending at the stop codon TAG (Position 805) of the cDNA. *CaRZFP1* shows one C3HC4-type RING zinc finger domain, between amino acid residues 27 and 67. This C3HC4 type RING zinc finger domain has the C-X2-C-X11-C-X1-H-X2-C-X2-C-X13-C-X2-C sequence motif. *CaRZFP1* also shows two transmembrane domains, which are located from 137-159 and 201-218 amino acid residues. By calculating the hydropathy value by the method of Kyte and Doolittle (1982) over a window of nine amino acid positions, we found that CaRZFP1 is very hydrophilic (Fig. 1B).

Homology of Proteins Coded by *CaRZFP1*

The amino acid sequence for CaRZFP1 was compared with those of previously reported zinc finger proteins (Fig. 2A). The putative protein encoded by *CaRZFP1* shares 87.2% homology with a *Capsicum chinense* zinc finger protein (Accession no. CAJ13712), 66.9% homology with an

A

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                    *.+....+..+..++++.+.++.*+******+**********************+**+.*.
CaRZFP1      (      1) MQKSTATAFENSSSSGN-GSNDAGDFECNICFELAQDPIVTLCGHLYCWPCLYRWLRLHS
C.chinense   (      1) MQDSTTRAFASSSSSSGDGNNDAGDFECNICFELAQDPIVTLCGHLYCWPCLYRWLRLHP
A.thaliana   (      1) MVNGESSTSTSYSDSNNDTNDQGGDFECNICFELAQDPIVTLCGHLFCWPCLYRWLHHHS
O.sativa     (      1) MENRVGESSATAVDGGG-GAKDSGSFECNICLELAQDPVVTLCGHLFCWPCLYEWLHVHA

                    +++*******.+************+..******.+++*++**.**+*****+* *+++*+..
CaRZFP1      (     60) QSHECPVCKALIQEEKLVPLYGRGRTSTDPRSKPVPGVEIPRRPAGQRPET-APPPES--
C.chinense   (     61) QCHECPVCKALIQEEKLVPLYGRGRTFTDPRSKPIPGLEIPSRPAGQRPET-APQPEP--
A.thaliana   (     61) HSQECPVCKAVVQDDKLVPLYGRGKNQTDPRSKRYPGLRIPNRPTGQRPETaAPPPQPEa
O.sativa     (     60) HSRECPVCKAGLEEEKLVPLYGRGKASTDPRSRSVAGVQIPSRPAGQRPAT-ASQPDHH-

                     +.++* +.+.***. .*  *+.+****++.+++**++*+*++**+***+.+++*+.+.+
CaRZFP1      (    117) -NTFPNSGFGLMGGL-FP-GATASFGNFTMSAGFGGFIPSLLSFQFHGFPGPTAFGTTPN
C.chinense   (    118) -NNFPNLGFGHMGGF-FP-TATARFGNFSMFAGFGGLLPSLLSFQFHGFPGPTAYPTTSN
A.thaliana   (    121) ASNFFNYGIGLMGGI-MPmMATTRFGNFSM--GFGGLLPSLFNFQFHGFHDATLYGSTPG
O.sativa     (    118) HDHLPHHDPWFMGGAgAP-VAGGRWGNYTFSAAIGGLFP-LLSFQVHGFPQAAAYGPAAG

                    ++* ** ..***.. ++ .*...+ . +.*..**.++.+**++*.  ++++.
CaRZFP1      (    174) YQY-GYPPAYHGASVQNAAHP-SQ--GQADNNLKFMFLLVGFLVF--LYLFG
C.chinense   (    175) HPF-GYTPAYHGAHVRN-AHDTAQ--VQADSNLKFMFLLVGFLVL--IYLLG
A.thaliana   (    178) YPYgGYHNGFRGVPPRGQERPMARgGNQSDAFLKNILFFVGICVV--IFLIW
O.sativa     (    176) FPY-GYGHSFHGWHGHGFPHQAPQ-GQHVDVFLKVLLVLVGVLVIasLIVF-
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B

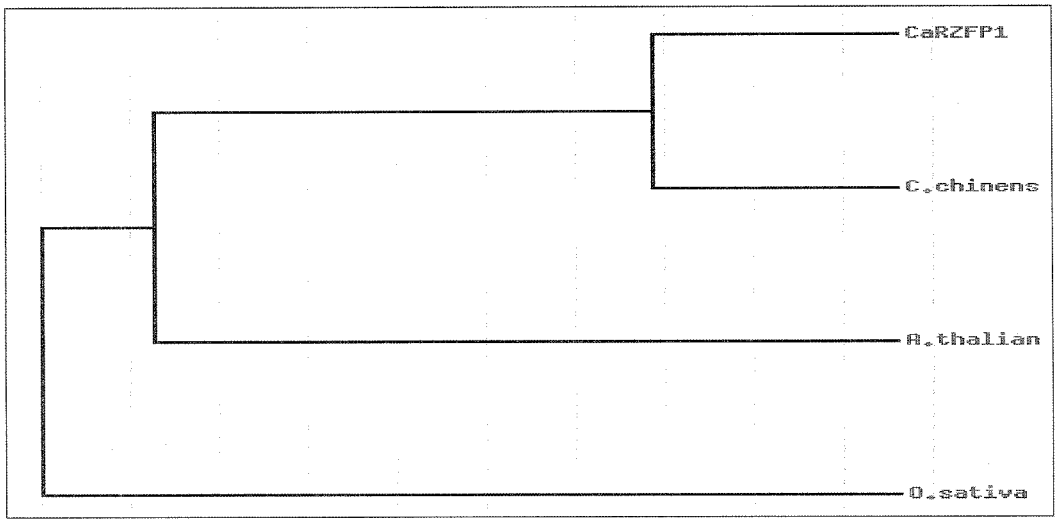

Figure 2. Sequence homology and phylogenetic analysis of hot pepper *CaRZFP1* with other zinc finger proteins. (**A**) Comparisons of deduced amino acid sequence of *CaRZFP1* with putative zinc finger protein from *C. chinense* (Accession no. CAJ13712), putative RING Zinc finger protein from *A. thaliana* (Accession no. AAM62942; Haas et al., 2002; Alexandrov et al., 2006), and putative RING finger protein from *O. sativa* (Accession no. BAD53343; Sasaki et al., 2002). *Represents conserved amino acid. Hyphen was inserted within amino acid sequence to denote gap. (**B**) Phylogenetic tree of *CaRZFP1* and zinc finger proteins from other species: *C. chinense* zinc finger protein (220 amino acids), *A. thaliana* RING zinc finger protein (227 amino acids), *O. sativa* RING finger protein (224 amino acids), and *CaRZFP1* (219 amino acids). Tree was obtained from amino acid sequences.

Arabidopsis thaliana RING zinc finger protein (Accession no. AAM62942; Haas et al., 2002; Alexandrov et al., 2006), and 61.2% homology with an *Oryza sativa* RING finger protein (Accession no. BAD53343; Sasaki et al., 2002). Phylogenetic analyses showed that *CaRZFP1* and the zinc finger protein from *C. chinense* are most similar to each other, and are not allied with the *Arabidopsis* RING zinc finger protein and rice RING finger protein (Fig. 2B).

Temporal Expression of *CaRZFP1* Gene in Response to Abiotic Stresses

To analyze the induction kinetics of *CaRZFP1* transcripts, 4-week-old hot pepper plants were treated with several abiotic stresses. After 2 h of heat-shock at 42°C, two bands appeared, with transcript levels of both being maintained at a steady state until Hour 4; no expression was induced in the control plant. The northern band for the smaller transcript was approximately 1 kb, which corresponded to the expected transcript size for *CaRZFP1*. The second northern band, appearing at about 3.8 kb, indicated that hot pepper has a RING zinc finger protein gene highly homologous to the high temperature-expressed *CaRZFP1* (Fig. 3A). We next examined the expression profile of *CaRZFP1* under drought conditions. Transcript was first detected, and at its peak level, after 30 min of dehydration stress. Expression continued to be strong until Hour 2, after which it declined until Hour 12 (Fig. 3B). At a low temperature (4°C), induction was first detected in plants treated for 4 h, with the expression level remaining

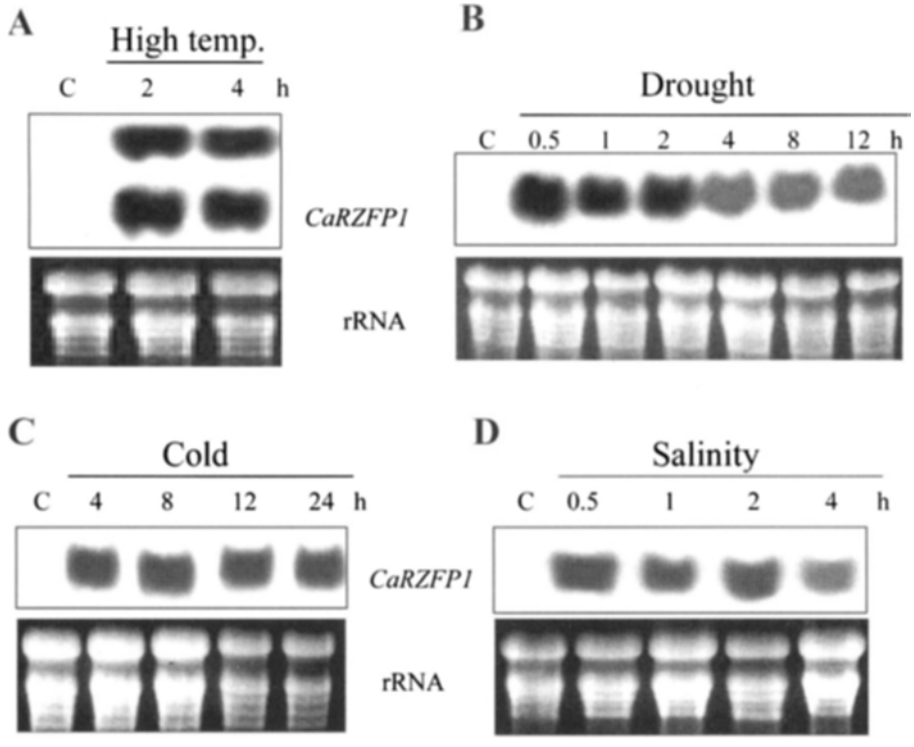

Figure 3. Induction kinetics of *CaRZFP1* in response to adverse environmental conditions in hot pepper. Four-week-old plants were subjected to (**A**) high temperature (42°C), (**B**) drought, (**C**) low temperature (4°C), or 250 mM NaCl. Treated plants were harvested at indicated time periods and total RNAs were isolated. Total RNAs (20 μg) were separated by electrophoresis on 1.2% formaldehyde–agarose gel and blotted to Hybond-N^+ nylon membrane. To ensure equal loading of RNA, gel was stained with ethidium bromide after electrophoresis. To confirm transfer of RNA to membrane filter, both gel and membrane were viewed under UV light at end of transfer. Filter was hybridized to ^{32}P-labeled PCR amplified coding sequence of *CaRZFP1* probe, washed, and visualized by autoradiography.

steady until Hour 24 (Fig. 3C). In the case of salinity stress, *CaRZFP1* mRNA accumulated at 0.5 h and remained steady until 2 h before declining. Except for those plants exposed to heat shock, no upper signal appeared from any other stressed tissues. We also examined *CaRZFP1* expression following treatment with various heavy metals, but found no accumulation in response to any of those compounds (data not shown).

DISCUSSION

Here, we report the isolation of a novel C3HC4-type RING zinc finger protein gene (*CaRZFP1*) from a cDNA library for hot pepper plants treated at 42°C. This C3HC4-type zinc finger domain contains a sequence of C-X2-C-X11-C-X1-H-X2-C-X2-C-X13-C-X2-C, where a Zn-finger of 41 amino acid residues binds two atoms of zinc (Haas et al., 2002; Sasaki et al., 2002; Hatakeyama and Nakayama, 2003; Alexandrov et al., 2006). Alignment of the zinc finger domain sequences (Fig. 2A) indicated that the motif of the CaRZFP1 contains a sequence highly conserved with other putative RING zinc finger proteins. The conserved ring motifs are located at the N-terminus (Fig, 2A), as is standard for most of these types of proteins. Because proteins carrying such a motif have been proven to function as transcription factors, it would be reasonable to assume that CaRZFP1 also is a transcription factor. Although CaRZFP1 lacks the typical NLS, this is not uncommon with other plant transcription factors as well, so that an NLS is thought to be imported into the nucleus by dimerizing with proteins that possess those signals (Liu et al., 1999).

Although high temperature is a major abiotic stress that severely damages crop productivity, most previous research on plant responses at the molecular level has centered on heat shock proteins (HSPs) (Cho and Hong, 2004; Yoon et al., 2005) and heat shock transcription factors (HSFs). HSFs are constitutively present and, under non-stressed conditions, they exist as monomer forms that usually bind to heat shock protein 70 (HSP70). HSFs can be activated by heat shock, a process that usually accompanies the dissociation of HSP70 and homotrimerization of HSFs (Ahn et al., 2001). The structure of CaRZFP1 differs significantly from that of the HSFs. The latter are ubiquitous in eukaryotes, carrying several defined domains in common, i.e., an amino-terminal DNA-binding domain, an adjacent coiled-coil trimerization domain, a central regulatory domain, a second coiled-coil domain, and a transcriptional activation domain (Morano and Thiele, 1999). The amino terminal DNA-binding domain is highly conserved among the HSFs, and the helix-turn-helix motif with loop domain define the site for binding to a heat shock element (HSE), as well as the specificity of binding between the HSF and HSE (Ahn et al., 2001). CaRZFP1, as a zinc finger protein, shows a zinc finger domain for binding to DNA (Fig. 1A, 2A). Although it is not surprising to see a zinc finger protein related to gene expression control and to spec-

ulate on the possibility that CaRZFP1 is under the control of an HSF, we are the first to describe a putative transcription factor with a zinc finger domain that probably functions under high-temperature stress in plants.

The molecular and cellular processes underlying the acclimation of hot pepper to abiotic stresses have attracted much interest, because the response of this economically important crop to adverse environmental factors is not as well understood as in other crop plants. Here, several stress treatments caused early, strong induction of *CaRZFP1* mRNA. Thus, it is reasonable to suggest that, in hot pepper, CaRZFP1 is functional in those initial stages of the tolerance response. Zinc finger transcription factors have been analyzed as they relate to tissue development in plants, and stress-related zinc fingers also have been isolated in response to treatments with salt (Zhu, 2000) and chilling (van der Krol et al., 1999). Moreover, the genome-wide analysis of the SAP (Stress Associated Protein) family, which encodes A20/AN1 zinc-finger proteins, has been completed in rice and *Arabidopsis*. All members present in the rice genome show inducibility under stress from cold, salt, or dehydration (Vij and Tyagi, 2006). Furthermore, our transcript-level analysis demonstrated that CaRZFP1 is inducible under most of the abiotic stresses tested here. *CaRZFP1* typically is not transcribed under non-stressed conditions, but was quickly and strongly induced when plants were variously challenged (Fig. 3).

Interestingly, two bands of transcripts were highly induced by our heating treatment. The lower band, at about 1.0 kb, also appeared in response to other abiotic stresses. Based on its nucleotide sequence, we determined that the transcript was for *CaRZFP1*. In contrast, the second, upper band appeared at about 3.8 kb only after the high-temperature exposure (Fig. 3). This suggests that a family gene member of *CaRZFP1* in hot pepper is uniquely induced only under that type of abiotic stress.

Although we can conclude that *CaRZFP1* probably codes a zinc finger type putative transcription factor, functional analysis is still necessary. We are also attempting to access the family member of CaRZFP1 that has been shown here to be expressed specifically under high-temperature stress, and which has a transcript size of about 3.8 kb.

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