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Lycopersicon Eculentum C_2H_2-type Zinc Finger Protein Induced by Oxidative Stress Especially

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ABSTRACT A tomato zinc-finger protein gene, *LeZFP1*, encoding the Cys2/His2-type zinc-finger transcription factor was searched from cDNA microarray analysis of gene expression following induction of the overexpressed tomato transgenic plants showing resistance for pathogen and abiotic stresses. The full-length cDNA of *LeZFP1* encoded a protein of 261 amino acid residues. Analysis of the deduced amino acid sequence of *LeZFP1* revealed that it shares high sequence identity with pepper CAZFP1 (81% identity). We found that single copy of *LeZFP1* gene is present in the tomato genome through southern blot analysis. The *LeZFP1* transcripts were constitutively expressed in the tomato mature and young leaves, but were detectable weakly in the flower, stem and root. The *LeZFP1* transcripts were significantly reduced in treated leaf tissues with NaCl and mannitol. The *LeZFP1* gene was induced by oxidative stress especially. Our results indicated that *LeZFP1* may play a role function involved in oxidative stress signaling pathways.

Introduction

Plant is severely affected by abiotic stress factors which include salinity, drought, high and low temperature, and heavy metals. Recent studies about abiotic stress show considerable overlap of plant responses to osmotic stresses such as drought, and salinity (Chen et al. 2002, Kreps et al. 2002, Buchanan et al. 2005). Dehydration, salinity, low as well as high-temperature stresses lead to metabolic toxicity, membrane disorganization, generation of reactive oxygen species (ROS), inhibition of photosynthesis and altered nutrient acquisition (Hasegawa et al. 2000). At the molecular level, abiotic stress tolerance can be achieved through gene transfer by altering the accumulation of osmoprotectants, production of chaperones, superoxide radical scavenging mechanisms, exclusion or compartmentation of ions by efficient transporter and symporter systems (see reviews by Ingram and Bartels, 1996, Hasegawa et al. 2000, Apse and Blumwald 2002, Zhu 2002, Viswanathan and Zhu 2004, Sangam et al. 2005, Valliyodan and Nguyen 2006).

The expression of CaPIF1, pathogen-responsive transcription factor, increased after pathogen attack or treatment with abiotic stresses (Oh et al. 2005). Homologs to CaPIF1 are found in 14 ZPT2, ZFT1, WZF1, STZ, Pszf1, Mszpt2-1 and SCOF-1 genes (Kubo et al. 1998; Uehara et al. 2005; Sakamoto et al. 1993; Lippuner et al. 1996; Michael et al. 1996; Frugier et al. 2000; Kim et al. 2001). These transcription factors are Cys-2/His-2 zinc finger proteins including a hydrophobic Leu-rich region (L box) that may play a role in protein-protein interactions and a short hydrophobic region in the C terminal part of the protein (DNL motif; Takatsuji 1999). Transgenic plants overexpressing these genes enhance tolerance to various stresses (Oh et al. 2005, Sakamoto et al. 2004, Kim et al. 2001). The gene regulated by the Cys-2/His-2 zinc finger proteins is especially

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important, as the transcription factors regulating stress responses (Sakamoto et al. 2000), flower development (Dinkins et al. 2002) and nitrogen fixation (Frugier et al. 2000). Profiling of changed genes under the overexpression of Cys-2/His-2 zinc finger gene has been identified (Seong et al. 2007).

Here, we report the isolation of a tomato C2H2-type zinc finger gene. Our results suggest that ZFP can play an essential role in oxidative metabolism.

Materials and Methods

Plant Material

'MicroTom' (*Lycopersicum esculentum*) seeds were cultured in MS medium (MS salts including MS vitamins, 3% sucrose, 0.8% agar, pH 5.8). After 2 weeks laters, germinated plants were transferred to pots and kept in a growth chamber at 24℃ for 4 weeks. Several abiotic elicitors were applied to the leaves of the tomato plants. The leaves of tomato plants were detached in 100 μM ABA, 200 mM NaCl, 200 mM Mannitol and 50 μM MV. Tomato leaves were treated with these abiotic elicitors for 0h, 1h, 3h, 6h, 12h and 24h. The treated leaves were frozen in liquid nitrogen and stored at -70℃, until used for RNA extraction.

DNA Sequencing

We searched a tomato cDNA clone (SGN-U144010), ZFP1 gene, from (http://www.sgn.cornell.edu/). And full-length primers designed to amplify ZFP1 gene of tomato. Detected band by PCR amplification cloned into pGEM T easy vector (Promega). We confirmed that sequences result match with one at Cornell University's homepage.

Southern Blot Analysis

Genomic DNA was prepared according to Dellaporta et al. Twenty micrograms of total DNA was digested with *Xba* I, *Eco*R 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 LeZFP1 cDNA in a buffer consisting of 1% BSA /1 mM EDTA/0.5 M $NaHPO_4$, pH 7.2/7% SDS at 65℃ 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 NaHPO4, pH 7.2/5% SDS) at 65℃, and the dried blots were placed on X-ray film at −80℃ for a week and developed.

Northern Blot Analysis

Total RNA was isolated from the various organs of tomato (Yi et al. 2004). Plant materials (1−1.5 g) that had been frozen in liquid nitrogen and ground to the powder were homogenized in 10 ml extraction buffer [4 M guanidine isothiocyanate, 25 mM sodium citrate at pH 7.0, 0.55% (w/v) N-laurylsarcosine and 0.1 M 2-mercaptoethanol]. A mixture of 2 M sodium acetate (pH 4.0), water saturated phenol and chloroform−isoamylalcohol (24:1) was added to the homogenate. Precipitation was performed with isopropanol at -20℃ for 2 h. After centrifugation, the pellet was suspended in 2 M LiCl solution and incubated at 4℃ for 18 h. The total RNA concentration and purity were determined by spectrophotometer and staining of the ribosomal RNA with ethium bromide, respectively. Equal quantities of total RNA (10 μg) were loaded into 1% agarose gel, containing 7.4% formaldehyde. After electrophoresis and visualization under UV light, the RNA was transferred onto nylon membranes (Hybond N^+, Amersham), followed by cross-linkage under irradiation with UV light. To generate *LeZFP1* gene-specific probe, each coding sequence was PCR-amplified with two primers: (5'-atggctcttgaagctttgaattct-3' and 5'-aattacaa ccttattaagtttaaa-3') for *LeZFP1*. The purified PCR products were ^{32}P-labeled. Hybridization was performed overnight at 65℃ 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 X SSC and 0.1% SDS for 10 min each at room temperature, and twice with 0.1 X SSC and 0.1% SDS for 5 min each at 65℃.

RT-PCR

First-strand cDNA was synthesized from 1 μg samples of

the DNase-treated total RNA (M-MLV RT, Invitrogen, USA). The primers used for reverse transcriptase-PCR were as follows: (5'-atggctcttgaagctttgaattct-3' and 5'-aattacaaccttattaagtttaaa-3') for *LeZFP1* or (5'-gcagtgtttcccagtattgt-3' and 5'-accgctagcata cagagaaa-3') for *LeActin*. PCR conditions were as follows: initial denaturation for 5 min at 94℃; followed by 25 cycles of 94℃, 1min; 55℃, 1min; and 72℃, 1min; and a final 7 min at 72℃. Twelve 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.

Results and Ddiscussion

Isolation of Tomato *LeZFP1* Gene

To study one of tomato clones up-regulated by *CaPIF1*-overexpressed transgenic tomato showing resistance against with pathogens and abiotic stress, the tomato EST cDNA database was searched to identify annotated *LeZFP1* cDNA clone (http://www.sgn.cor nell. edu/) (Seong et al. 2007). Sequence had similarity with the Cys2/His2-type zinc-finger proteins, was designated *LeZFP1* (*Lycopersicon esculentum* zinc-finger protein 1). The *LeZFP1* cDNA contains 783-bp in length. The predicted open reading frame (ORF) encodes a zinc-finger protein of 261 amino acids (Figure 1). The LeZFP1 gene contains two Cys2/His2-type zinc-finger motifs, separated by 38 amino acids (Fig. 1). In most of the Cys2/His2-type zinc-finger proteins, consists of putative transcription factors that contain, within the same region, two separate zinc-finger motifs bearing QALGGH motifs. These factors also contain putative NLS sequences near their N-termini (Pavletich and Pabo 1991, Takatsuji et al. 1992). The *LeZFP1* gene has a potential nuclear localization signal (NLS) located in the N-terminal region. There are also a hydrophobic leucine-rich region with a core sequence EXEXXAXCLXXL (L-Box), and a DLN-Box in the C-terminal region (Figure 1). The predicted amino acid sequence of the LeZFP1 protein has high similarity to other HMGB proteins from *Nicotiana benthamiana* (82% identity), *Capsicum annuum*

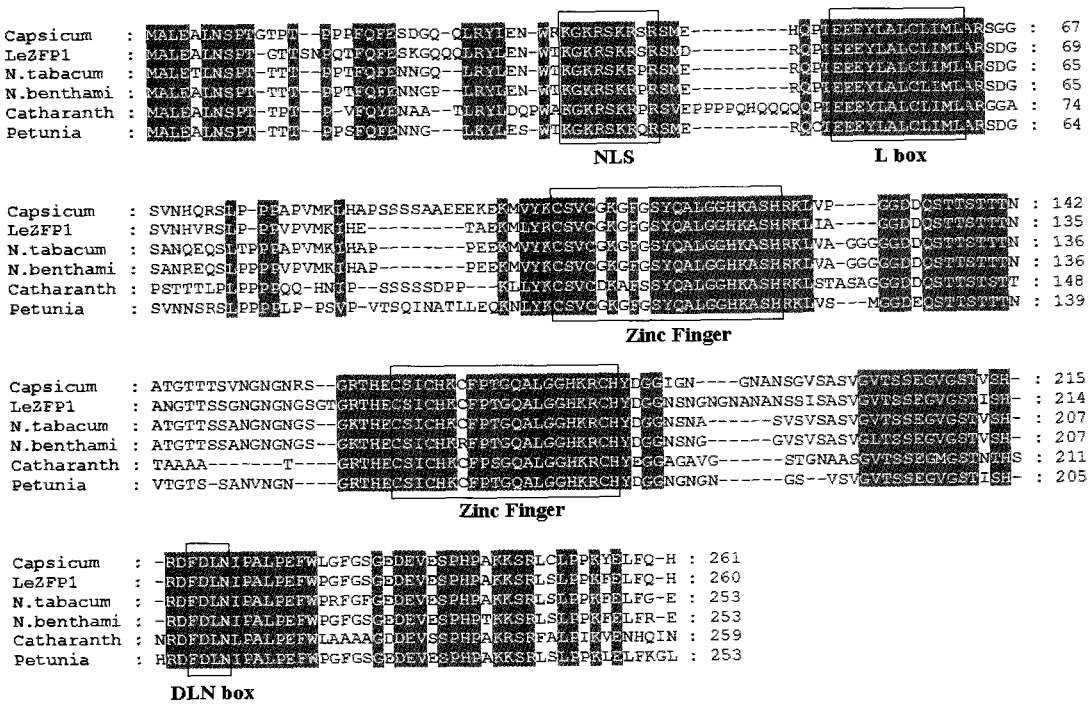

Figure 1. Comparison of the deduced amino acid sequence of the tomato (Lycopersicon esculentum) zinc-finger protein *LeZFP1* with zinc finger proteins from pepper (Accession no. AAQ10954), tobacco (Accession no. AAQ54303, BAE17114), petunia hybrida (Accession no. BAA05079), and Catharanthus roseus (Accession no. CAF74935). The two zinc finger motifs, the putative nuclear localization signal (NLS), L-Box and DLN-Box are boxed-in. The gaps introduced to maximize alignment are indicated by dashes.. Shade shows the same amino acid residues in six zinc finger proteins.

(81% identity), *Nicotiana tabacum* (79% identity) and *Petunia* x *hybrida* (72% identity).

Gel Blot Analysis of Genomic DNA and Tissue-Specific Expression of *LeZFP1*

DNA gel blot analysis revealed that one band was detected in the DNA digested with *Xba*I or *Eco*RI that could not cut *LeZFP1*, and two bands were observed in the DNA subjected to double digestion with *Hind*III (Figure 2a), suggesting a singlecopy gene for *LeZFP1*. RNA gel blot analysis showed that the *LeZFP1* was more abundantly expressed in the young or mature leaves than in stems, flowers and roots (Figure 2b). Expression of EPF-type gene is involved in flower-specific and flower development (Takatsuji et al . 1994; Takatsuji 1998). The genes with C_2H_2-type putative zinc finger domain were expressed in all organs investigated (Rodriguez-Milla et al. 2006). The CaPIF1 gene is more strongly expressed in pepper roots and fruits than in flowers, and is undetectable in stem and leaf tissues (Oh et al. 2005). In constant, *LeZFP1* mRNA from tomato was detected in all examined organs. Further characterization of *LeZFP1* will be required to determine its protein localization in each tissue.

LeZFP1 Gene Expression Responds to Various Environmental Stresses in Tomato

Fig. 3 shows the pattern of *LeZFP1* mRNA accumulation over time in the tomato plants exposed to different abiotic stresses including methyl viologen, drought, ABA and high salinity. The transcript levels of *LeZFP1* decreased markedly in tomato by drought (mannitol) or high salinity stress. The decrease in *LeZFP1* expression in tomato was rapid in that its mRNA level at 3 hours after salt or drought treatment. After osmotic stress (ABA) treatment, the transcript level of *LeZFP1* decreased and then increased at 6 hours. *LeZFP1* was reduced after 12 hours ABA treatment. Whereas the transcript level of *LeZFP1* induced after oxidative stress (methyl viologen) treatment.

Function of this gene family confers enhanced tolerance to low temperature (Kim et al. 2001), dehydration (Sugano et al. 2003), high salt and drought (Lippuner et al. 1996, Sakamoto et al. 2004) to plants or yeast cells, confirming their involvement in abiotic stress responses. The hot pepper CAZFP1 gene, belonging to this gene family was known to a pathogen- induced early defense gene (Kim et al. 2004). A few plant Cys2/His2-type zinc-finger factors are known to be involved in the regulation of floral development, seed or pollen development (Dinkins et al. 2002; Kapoor et al. 2002; Takatsuji et al. 1994; Takatsuji 1998) and tolerance to abiotic stresses (Frugier et al. 2000, Kim et al. 2001, Lippuner et al. 1996, Sakamoto et al. 2004). A massive change of CaPIF1 gene on the microarray through overexpressed tomato is reported that it turned on genes involved in metabolic pathways, and stress and defense responses (Seong et al. 2007). Our results showed that transcript level for *LeZFP1* gene is specifically upregulated by oxidative

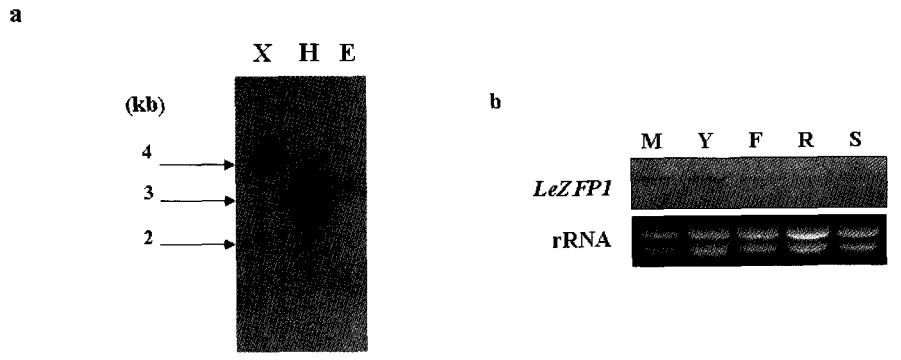

Figure 2. Genomic DNA gel blot analysis of *LeZFP1* and RNA expression of *LeZFP1* in the different tissues. **a** Genomic DNA blot anfalysis of tomato *LeZFP1*. Each lane was loaded with 20 μg of tomato genomic DNA digested with *Xba* I (X) *Hind* III (H),or *Eco* RI (E). The membranes were hybridized with 32[P]-dCTP-labelled full-length *LeZFP1* cDNA. The sizes of the molecular weight markers are indicated in kilobases to the left. **b** Tissue-specific expression of the *LeZFP1* gene in tomato plants. Total RNA was isolated from various tissues and the RNA gel blot was hybridized with full-length *LeZFP1* cDNA. M: mature leaf, Y: young leaf, F: flowers, R: roots, S: stems.

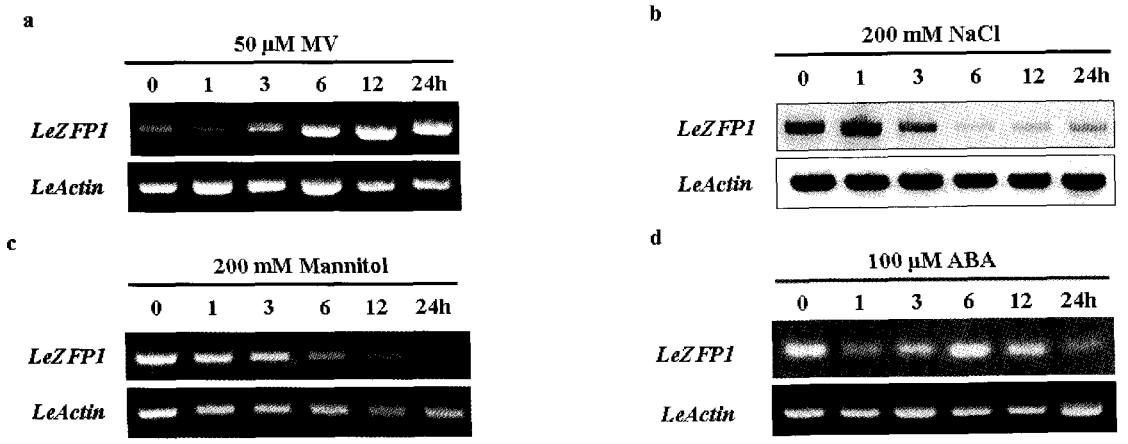

Figure 3. Expression of *LeZFP1* mRNA in response to treatment with abiotic stresses. Detached chili pepper leaves submerged in MV, NaCl, Mannitol or ABA solutions for various time points. Treated leaves were harvested at the indicated times, and total RNAs were isolated. To carry out RT-PCR, cDNA was synthesized from total RNAs. RT-PCR analysis was performed as described in Materials and methods.

stress, whereas it reduced salt and drought stress. Therefore, we also cannot rule out the possibility that *LeZFP1* maybe a negative regulator in signaling pathways related to salt and mannitol stress.

It is evident from other studies that C_2H_2-type zinc finger protein functions to integrate plant responses to abiotic stresses and plant-pathogen interactions (Seong et al. 2007). A key player in both these stresses is ROS, such as superoxide radical and H_2O_2. In fact, many of the genes induced in response to constitutive C_2H_2-type zinc finger levels in defense mechanism. However, the functional mechanism at a molecular level of C_2H_2-type zinc finger is unknown involved in oxidative metabolism. Oxidative stress or plant-pathogen interactions could activate the protein, either transcriptionally or through covalent modification, leading to the upregulation of transcripts encoding pathogenesis-related proteins and other proteins involved in antioxidant metabolism.

In summary, the cloning and stress-related expression of a *LeZFP1* gene is presented here for the first time from tomato. A few is known about the regulation of C_2H_2-type zinc finger itself by environmental factors. The present report not only adds to the database a cDNA encoding C_2H_2-type zinc finger protein in tomato but also provides information about its regulation by various abiotic stresses including ABA, drought, MV and high salt, which will be useful to further study its function in stress adaptation process in tomato. These findings suggest that *LeZFP1* plays an important role in the oxidative metabolism involved in response to environmental stresses in plants. Clearly, understanding the role of *LeZFP1* can be applied to develop oxidative stress-resistant plants.

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