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Overexpression of *SlCZFP1*, a Novel TFIIIA-type Zinc Finger Protein from Tomato, Confers Enhanced Cold Tolerance in Transgenic Arabidopsis and Rice

Xin Zhang · Xiuping Guo · Cailin Lei · Zhijun Cheng · Qibin Lin · Jiulin Wang · Fuqing Wu · Jie Wang · Jianmin Wan

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Abstract The C2H2-type (also called TFIIIA-type) zinc finger protein family is a major family of eukaryotic transcription factors. C2H2-type zinc finger proteins in plants play important roles in regulating various developmental processes or responses to abiotic stresses. We isolated a complementary deoxyribonucleic acid (cDNA) clone, designated *Solanum lycopersicum* cold zinc finger protein 1 (*SlCZFP1*), from tomato by analyzing the previous microarray data of the low-temperature transcriptome of tomato. *SlCZFP1* encodes a novel TFIIIA-type zinc finger protein, which contains two typical zinc finger motifs, CX_{2-4} $CX_3FX_3QALGGHX_{3-5}H$, and a potential nuclear localization signal (NLS). The SlCZFP1–GFP fusion protein was localized to the nucleus in a transient expression assay. Expression of *SlCZFP1* was strongly induced by cold stress, dehydration, and salt treatment, but not by abscisic acid (ABA). Overexpression of *SlCZFP1* in transgenic Arabidopsis and rice induced constitutive expression of cold-regulated (COR) or cold-responsive genes and conferred enhanced tolerance to freezing or cold treatments for non-acclimate transgenic plants, compared with wild-type plants. However, there was no obvious enhancement observed in drought and salt tolerance. Our data suggest that *SlCZFP1* plays an important role in plant responses to cold stress by regulating cold-responsive gene expression and that *SlCZEP1* might be a useful gene for improving cold tolerance in crop plants.

Keywords Tomato · C2H2-type zinc finger protein · SlCZFP1 · Cold stress

X. Zhang · X. Guo · C. Lei · Z. Cheng · Q. Lin · J. Wang · F. Wu · J. Wang · J. Wan (✉)
National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Science,
Chinese Academy of Agricultural Sciences,
Beijing 100081, People's Republic of China
e-mail: wanjm@caas.net.cn

J. Wan
National Key Laboratory for Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University,
Nanjing 210095, China

Introduction

Cold stress is one of the major environmental factors limiting the agricultural productivity of crop plants. Plants vary greatly in their abilities to survive freezing temperatures (Sakai and Larcher 1987). At one extreme are plants from tropical and subtropical regions including tomato and rice, which suffer injury when exposed to chilling temperature between 0 and 12°C. In sharp contrast, plants from temperate regions are not only chilling-tolerant, but many of them, such as Arabidopsis and wheat, can also survive freezing after exposure to low nonfreezing temperatures, a process known as "cold acclimation" (Guy 1990; Thomashow 1998). Understanding what accounts for the differences in freezing tolerance between those plant species and the molecular basis of cold acclimation is a fundamental biology question and has the potential to provide new approaches to improve the freezing or chilling tolerance in crop plants, an important agronomic trait (Jaglo et al. 2001). At present, one well-characterized pathway with a role in cold acclimation is the CBF cold response pathway (Thomashow 2001). Key components of the pathway include rapid cold-induced expression of three homologous genes encoding transcriptional activators, CBF1, 2 and 3 (also known as DREB1b, c and a, respectively), followed by expression of CBF-targeted genes, the CBF regulon, i.e., those cold-regulated (COR)

genes that are responsive to CBF expression (Seki et al. 2001; Fowler and Thomashow 2002; Maruyama et al. 2004), and increased freezing tolerance. In addition, recent transcriptome profiling and mutagenesis studies have suggested additional cold-responsive pathways (Xin and Browse 1998; Fowler and Thomashow 2002; Vlachonasios et al. 2003). However, signaling intermediates and the underlying molecular mechanisms involved in these pathways remain poorly characterized.

The C2H2-type zinc finger protein family is one of the largest families of eukaryotic transcription factors. Plant proteins of this family have one to four finger motif(s) within each molecule and two-fingered proteins constitute the major class of this family (Takatsuji 1999). They are mostly plant-specific and contain a conserved QALGGH sequence within their zinc finger domain. Recent studies have revealed that C2H2 zinc finger proteins can function as a key transcriptional regulator involved in the defense and acclimation response of plants to different environmental stress conditions (Ciftci-Yilmaza and Mittler 2008). For example, SCOF-1, a soybean C2H2 zinc finger protein, acts to enhance the binding efficiency of SGBF-1, a bZIP-type transcription factor, to abscisic acid-responsive element (ABRE) and mediates cold tolerance in transgenic plants (Kim et al. 2001). The Arabidopsis C2H2 zinc finger protein STZ/ZAT10 functions as a transcription repressor under abiotic stresses; its constitutive expression leads to enhanced tolerance to salinity, heat, and osmotic stress associated with elevated expression of reactive oxygen defense transcripts in transgenic Arabidopsis (Sakamoto et al. 2004; Mittler et al. 2006). Zat12, another C2H2 protein of Arabidopsis, plays a central role in reactive oxygen and cold stress signaling in Arabidopsis (Rizhsky et al. 2004; Davletova et al. 2005; Vogel et al. 2005). Expression of ZPT2–3, a petunia C2H2 zinc finger protein, was induced by cold and drought; transgenic petunia expressing *ZPT2–3* conferred enhanced drought tolerance (Sugano et al. 2003). Mt-ZFP1 in *Medicago truncatula* is implicated in cytokinin, abscisic acid (ABA), and methyl jasmonate mediated stress responses (Xu and Ma 2004). Three C2H2-type zinc finger protein genes, ZFP245 (Huang et al. 2009), ZFP182 (Huang and Zhang 2007), and ZFP252 (Xu et al. 2008), have been isolated from rice, and their expression was induced by various abiotic stresses. Overexpression of those genes in rice and tobacco resulted in enhanced tolerance to cold, drought, and salt stress. So far, most of the identified stress-responsive C2H2-type zinc finger proteins in plants play important roles in drought or salt stresses. However, only few of them confer tolerance to cold stress in transgenic plants, especially in crop plants (Kim et al. 2001; Huang et al. 2009).

Tomato belongs to the Solanaceae family that includes several other economically important crops, such as potato, pepper, and eggplant, and, as such, represents the most valuable plant family in terms of vegetable crops. Little is known about the mechanisms involved in responses to cold stress in tomato. Our previous transcriptome profiling of cold-treated tomato plants, using a complementary deoxyribonucleic acid (cDNA) microarray representing approximately 8,700 tomato genes, identified several cold-responsive transcription factors (Zhang et al. 2004b). In this study, we present isolation and functional characterization of one of the factors, which is a novel C2H2-type zinc finger protein, designated *Solanum lycopersicum* cold zinc finger protein 1 (SlCZFP1). We provide evidence that SlCZFP1 plays important roles in cold stress tolerance in plants.

Materials and Methods

Plant Materials and Experimental Treatments

Seeds of tomato (*Solanum lycopersicum* var. D. Huang) were sterilized with 20% bleach solution followed by three rinses in sterile distilled water and were germinated in Magenta boxes containing Murashige and Skoog (MS) medium (Life Technologies, Gaithersburg, MD) supplemented with 3% sucrose and solidified with 0.7% phytagar (Life Technologies). Seedlings were grown for 3 weeks with 16 h light/8 h dark at 26°C/20°C. The chamber was lighted with cool-white fluorescent illumination at 100 μmol m^{-2} s^{-1}. Abiotic stress treatments, including cold, drought, NaCl and ABA, were performed as previously described (Zhang et al. 2004b). Plants were harvested at various time points after stress treatments and immediately frozen in liquid nitrogen. Plant material was stored at -80°C prior to RNA extraction.

Arabidopsis thaliana (L.) Heynh. (ecotype Wassilewskija, Ws-2) and transgenic Arabidopsis plants in the Ws-2 background were grown in pots. The pots were placed in controlled environment chambers at 20°C under continuous, cool-white fluorescent illumination of 100 μmol m^{-2} s^{-1} for 2–3 weeks.

Rice (*Oryza sativa L.* cultivar Kita-ake) seeds and transgenic rice plants were immersed in water for 3 days and then grown in controlled conditions at 28°C/26°C (day/night) with 16 h photoperiod for about 4 weeks. Four-leaf stage seedlings were used in various stress treatments.

Constructs and Plant Transformation

The full-length cDNA of sense-*SlCZFP1* was amplified from a cDNA EST clone (accession no. AW031185) and was inserted into the *Xba*I and *Sac*I sites of PBI121 vector to replace the GUS coding region. The resulting plasmid,

pXIN3, which contains the *SlCZFP1* coding sequence under control of the CaMV 35S promoter, was transformed into the *Agrobacterium tumefaciens* strain EHA105 by electroporation. Arabidopsis plants were transformed with plasmid pXIN3 using the floral dip method (Clough and Bent 1998). Transformed plants were selected on the basis of kanamycin resistance. T1 plants were selfed and homozygous T2 or T3 plants were selected and used for further study.

The plasmid pXIN3 was also introduced into tomato (var. D. Huang) and rice (var. Kita-ake), respectively, by *Agrobacterium*-mediated transformation (McCormick et al. 1986; Hiei et al. 1994). T2 or T3 transgenic plants were used for subsequent experiments.

Subcellular Localization

The *SlCZFP1* cDNA with the termination codon removed was fused in-frame to the GFP reporter gene in the pCAMBIA1302 vector, resulting in pSlCZFP1–GFP. Both pSlCZFP1—GFP and the control plasmid, which contains the GEP reporter only, were introduced into onion (*Allium cepa)* epidermis cells by particle bombardment according to the protocol described (Varagona et al. 1992). The transformed cells were cultured on MS medium at 25°C for 2 days (16 h light and 8 h dark) and examined under a Zeiss LSM510 confocal laser microscope.

DNA and RNA Hybridization

Tomato genomic DNA was isolated from mature leaves using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). Southern blotting was performed using 10 μg of genomic DNA digested with restriction enzymes, which was transferred to nylon membrane. Total RNA was isolated from Arabidopsis (Gilmour et al. 2000), tomato and rice leaves as described (Howe et al. 1996). Northern transfers (10 μg total RNA) were prepared, hybridized, and washed as described (Stockinger et al. 1997). The probe for tomato *SlCZFP1* transcripts was the full-length cDNA insert from EST AW031185. Full-length cDNAs of *Arabidopsis CBF1, 2, 3* and COR genes were labeled with ^{32}P with the random primers DNA labeling system (Life Technologies/Gibco-BRL, Cleveland) following the manufacturer instruction.

Semi-quantitative RT-PCR Analysis

Total RNA was extracted from seedlings of wild-type (WT) and transgenic rice lines as described previously. First strand cDNA was synthesized with 2 μg of purified total RNA using PrimeScritpt™ reverse transcriptase (RT, Takara Bio, Shiga, Japan). Oligo (dT) was used as a primer and the RT reaction was incubated at 42°C for 1 h in a total volume of 25 μl. The reaction included an initial 5 min of denaturation at 94°C, followed by 40 s at 94°C, 40 s at 58°C, and 40 s at 72°C with 30 cycles, and a final 10-min extension at 72°C. The Actin gene was used as an internal control with the same polymerase chain reaction (PCR) conditions, except that the reaction was reduced to 28 cycles. Primer sequences used for the RT-PCR experiments are listed in Table 1. The expression analysis for each gene was repeated three times.

Phylogenetic Analysis

The Clustal W. Pole BioInformatique Lyonnasis (PBIL) program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) was used to perform multiple sequence alignments. A phylogenetic tree was constructed with the aligned plant two-fingered C2H2-type zinc finger proteins using MEGA software version 4.0 via the neighbor-joining method (Tamura et al. 2007).

Analysis of Abiotic Stress Tolerance in Transgenic Rice Plants

The T2 or T3 of transgenic rice lines carrying *SlCZFP1* were used in the stress assays. To evaluate their tolerance to cold stress, transgenic and WT seedlings at the four-leaf stage were incubated at 4°C for 6 days, and then moved to 26°C for recovery for another 6 days. For drought treatment, seedlings were grown in water until the four-leaf stage, followed by a 14-day-long withholding of water. The plants were then rewatered and allowed for a 7-day recovery. For salt treatment, rice plants were grown in ½ MS medium for 4 weeks and then transferred to the solution containing 250 mM NaCl for 4 days. These plants were then transferred to the normal growth condition for 12 days. The survival rate for all the three treatments was calculated as percentage of surviving plants over total plants treated.

Whole-plant Freeze Tests for Arabidopsis Transgenic Plants

Arabidopsis plants were grown on plates as described previously (Gilmour et al. 2000). Non-acclimated plants (13 days) were placed at -2°C in the dark for 3 h after which freezing of the plates was nucleated with ice chips. Then, the plants were incubated at -2°C for 21 h followed by incubation at -6°C for 24 h. The temperature was then changed to 4°C for 24 h. The plants were allowed to recover at 24°C for 48 h in continuous light and then scored for survival. A *t*-test was used in the statistic analysis. Three independent experiments were conducted.

Fluorescence Measurement

Four-week-old wild-type and transgenic rice plants were incubated at 4°C with 16 h light of 100 μmol m^{-2} s^{-1} for

Table 1 Information about rice stress-responsive genes and respective primers for RT-PCR used in this work

Gene name	Primer sequence	Accession number[a]
OsWCOR413-like	F 5′GAGGTGGAGGAGGCTAG 3′ R 5′GGGGATAATCACATAAGAG3′	AF283006
OsGOLS3	F 5′ACTGGAAGGGCGTCGTG 3′ R 5′TTGGGGATGGGCTTGTAC 3′	AK107065
OsUSP1	F 5′CTCCATCTCGCCACATTCG 3′ R 5′GCCACTACGGTAAGCTCCAA 3′	AK063881
OsCOR410	F 5′AGAAGAAGGGGCTCAAGGAGAAG 3′ R 5′CCATGATCTTGCCCAGTATACCC 3′	NM_001054224
OsDhn1	F 5′AGCTCAAACAAGTCAAGAGC 3′ R 5′AAGCACCAAACTAACACACG 3′	AY786415
Os14-3-3	F 5′CGATGGTATCCTGAAGTTGCTTG 3′ R 5′CTTGGAGGCTTCTTTCACCTCAT 3′	U65957
OsLEA14-2	F 5′ATGGACAAGGCGAAAGGG 3′ R 5′GATCGGAGTTGGTTGATGAGA 3′	AK061818
OsMAT2	F 5′ GGAGTCTGTGAACGAGGGC 3′ R 5′GACACCGATGGCGTATGAT 3′	AK103157
OsLip5	F 5′GGAAGACGAGCACAAGAAGG 3′ R 5′TGCATCACGTATTGCACAGA 3′	AB011368
OsLip9(Dip1)	F 5′ AGAAGGGCTTCCTCGACAAGAT 3′ R 5′TACCCCACACGAAACACAAACTT 3′	AY587109
OsLEA4	F 5′TCGAGCAAAATCCATCAGAGTTC3′ R 5′ATGGCAGAGTCCTTGGTGTACTG 3′	AK119713
OsLit6a	F 5′AGCTGGGAACAAGCAGAAGA 3′ R 5′AAGGTACCCCGGAAGAACAG 3′	AY607689
OsLit6b	F 5′AATACTGCGAGAGAAATTAATCA 3′ R 5′ TAAGAGGGGAGCTTATTCACAC 3′	AY607690
OsRD22	F 5′CTAGGTCTCTCGCTGCTCTCCT 3′ R 5′GCGCAGTAGTGCTTGTGCTTG 3′	NM_001061473
OsP5CS	F 5′TGGATGTCTCGTCATCTCAACT 3′ R 5′AAGCCAAGACAGCAGCCTTCAC 3′	D49714
Actin	F 5′ TGGAACTGGTATGGTCAAGGC 3′ R 5′ AGTCTCATGGATACCCGCAG 3′	D87740

[a] GenBank accession numbers for full-length cDNA sequences of corresponding genes.

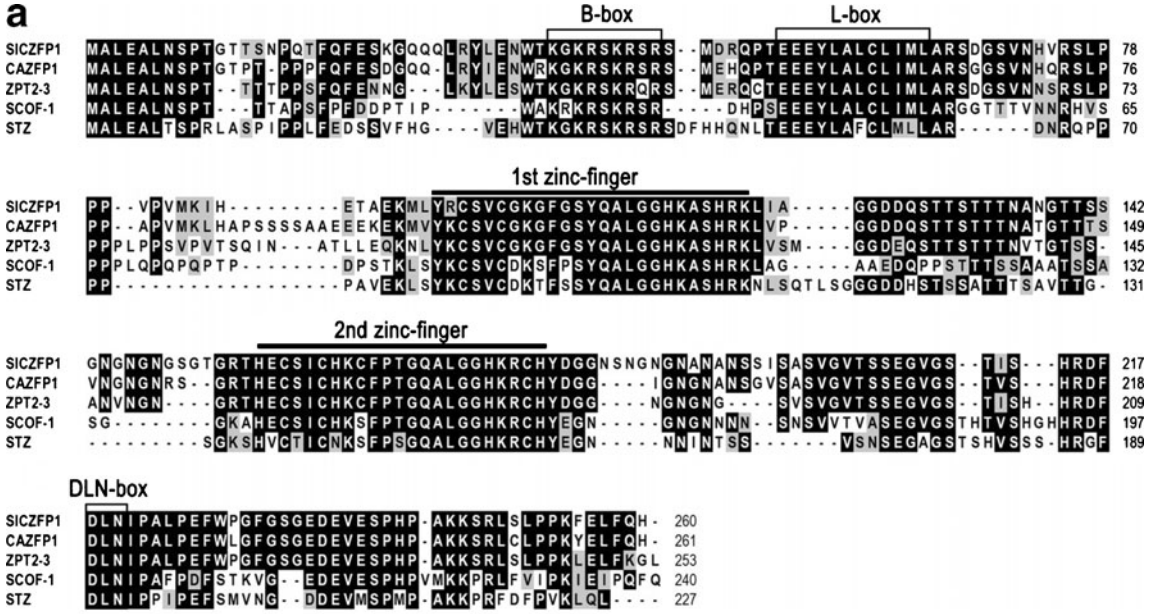
various time intervals. Chlorophyll fluorescence of whole leaves of chilling-treated plants was measured with a fluorescence monitoring system (Hansatech Instruments, Norfolk, UK). The intact leaf was fixed with tape (clip) to light fiber and kept in the dark for at least 15 min before measurements. The fluorescence were calculated as maximum quantum yield of PSII=Fv/Fm, representing the activity of photosystem II (PS II), which was used to assess functional damage to the plants (Genty et al. 1989). Three measurements were conducted on the fourth leaf of a plant and averaged. Five plants were measured for each transgenic event.

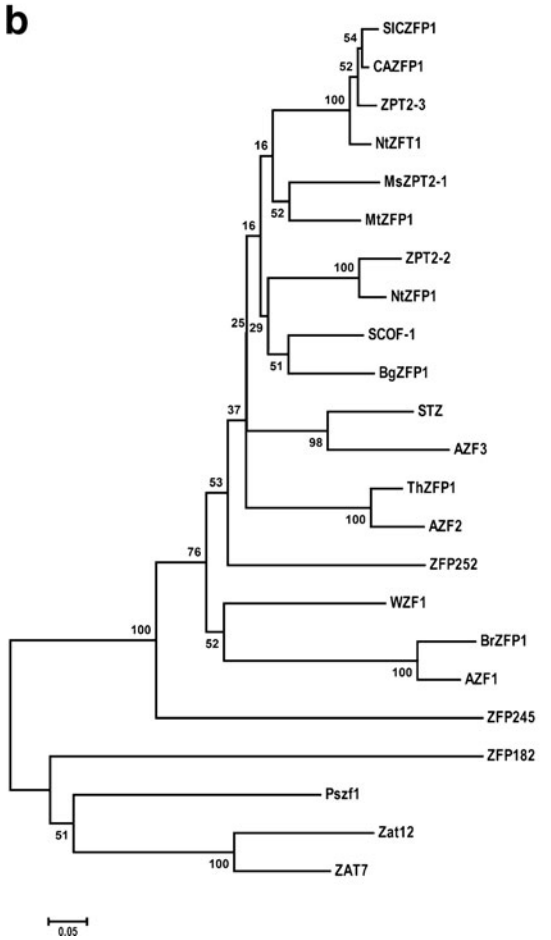
Results

Isolation and Sequence Analysis of SlCZFP1

We previously performed expression profiling using a tomato cDNA microarray chips containing ~8,700 tomato

Fig. 1 Amino acid sequence comparison and phylogenetic analysis of SlCZFP1 with those of the related plant C2H2-type zinc finger proteins. **a** Alignment of the amino acid sequence of tomato (*Solanum lycopersicum*) SlCZFP1 with zinc finger proteins from petunia ZPT2-3 (D26086), soybean SCOF-1 (*Glycine max*, U68763), pepper CAZFP1 (*Capsicum annuum* L., AAP41717), *Arabidopsis thaliana* STZ/ZAT10 (X95573). The two zinc finger motifs, the putative NLS, L-Box and DLN-Box are boxed-in. The gaps introduced to maximize alignment are indicated by dashes. Identical and conserved amino acids are displayed in black and gray backgrounds, respectively. **b** Phylogenic relationship between SlCZFP1 and its related zinc finger proteins. In addition to the members cited in Fig. 1**a**, the following protein sequences were incorporated into the analysis: petunia ZPT2-2 (D26084); *Arabidopsis thaliana* AZF1 (AB030731), AZF2 (AB030730), AZF3 (AB030732), ZAT7 (X98676), ZAT12 (X98674); salt cress ThZFP1 *(Thellungiella halophila*, ABI74621); alfalfa Mszpt2-1 (*Medicago sativa*, Y18788); MtZFP1(*Medicago truncatula*, AAP81810); tobacco NtZFT1 (*Nicotiana tabacum*, ABI86899), tobacco NtZFP1 (T01985); turnip BrZFP1 (*Brassica rapa*, T14409); durango root DgZFP1 (*Datisca glomeratarice*, AF119050); rice ZFP182 (*Oryza sativa*, AAP42461), rice ZFP245 (AAQ95583), rice ZFP252 (AAO46041); wheat WZF1 (*Triticum aestivum*, D16416); pea Pszf1 (*Pisum sativum*, CAA60828)


a
B-box
L-box
1st zinc-finger
2nd zinc-finger
DLN-box
SlCZFP1
CAZFP1
ZPT2-3
SCOF-1
STZ


b
SlCZFP1
CAZFP1
ZPT2-3
NtZFT1
MsZPT2-1
MtZFP1
ZPT2-2
NtZFP1
SCOF-1
BgZFP1
STZ
AZF3
ThZFP1
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Zat12
ZAT7
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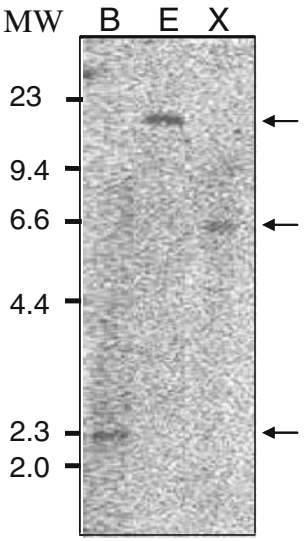


Fig. 2 Southern blot analysis of *SlCZFP1*. The genomic DNA was digested with *Bam*HI (*B*), *Eco*RI (*E*), or *Xba*I (*X*) and hybridized using *SlCZFP1* as a probe. Lambda phage DNA was digested with *Hin*dIII and served as the molecular marker. *Arrow* indicates the hybridized band

genes (Tom1 cDNA array http://bti.cornell.edu/CGEP/CGEP.html) with low-temperature-treated tomato seedlings. We identified several transcription factors which showed 2.5-fold or more induction upon cold treatment (unpublished data). In this study, we chose the one with strong induction for further functional analysis. The corresponding expressed sequence tag (EST; accession no. AW031185) was obtained and the complete DNA sequence of the insert was determined (accession no. HM061128). The deduced amino acid sequence has sequence similarity with the C2H2-type zinc finger proteins. The protein is 260 amino acids long and contains two C2H2-type zinc finger motifs, CX_{2-4} $CX_3FX_3QALGGHX_{3-5}H$, separated by 40 amino acids (Fig. 1a). We named this gene as *SlCZFP1* for *Solanum lycopersicum* cold zinc finger protein 1. SlCZFP1 has a putative nuclear localization signal (NLS), with a consensus sequence, KXKRSKRXR, and a Leu-rich region (L-box) (Fig. 1a). Comparisons of the amino acid sequences between SlCZFP1 and some previously reported two zinc finger proteins of other plants are presented in Fig. 1. SlCZFP1 shares a high degree of similarity to pepper CAZFP1 (87.7%) and petunia ZPT2-3 (84.7%). To investigate the evolutionary relationship among plant C2H2 zinc finger proteins involved in stress responses, a phylogenetic tree was constructed with the full-length amino acid sequences (Fig. 1b). The result revealed that SlCZFP1 is more similar to tobacco NtZFT1, pepper CAZFP1, petunia ZPT2-3 than to other plant C2H2 zinc finger proteins.

To determine whether *SlCZFP1* represents a single locus in the *Solanum lycopersicum* genome or whether it is a member of a multigene family, a Southern blot analysis was performed using the *SlCZFP1* cDNA as a probe. The results consistently showed presence of one major band, regardless of three restriction enzymes used (Fig. 2), indicating that *SlCZFP1* is a single copy gene in the genome of tomato.

The Cellular Localization of SlCZFP1

The deduced SlCZFP1 protein contains a stretch of basic residues KGKRSKR, which may function as a potential NLS. To examine if SlCZFP1 is indeed targeted to the nucleus, we performed an in vivo targeting experiment using green fluorescent protein (GFP) as a marker. We generated the SlCZFP1–GFP fusion using the pCAMBIA1302 vector. Both the SlCZFP1–GFP fusion vector and the control vector containing the GFP reporter alone were introduced into onion epidermis cells by particle bombardment. As shown in Fig. 3, the fusion protein SlCZFP1::GFP was localized to the nucleus of onion epidermal cells, whereas GFP itself was targeted to both nucleus and cytoplasm, confirming that SlCZFP1 is a nucleus-localized transcription factor.

Expression of the SlCZFP1 under Various Stress Conditions or Hormone Treatments

SlCZFP1 was highly induced by cold treatment in our microarray experiments. We further performed Northern

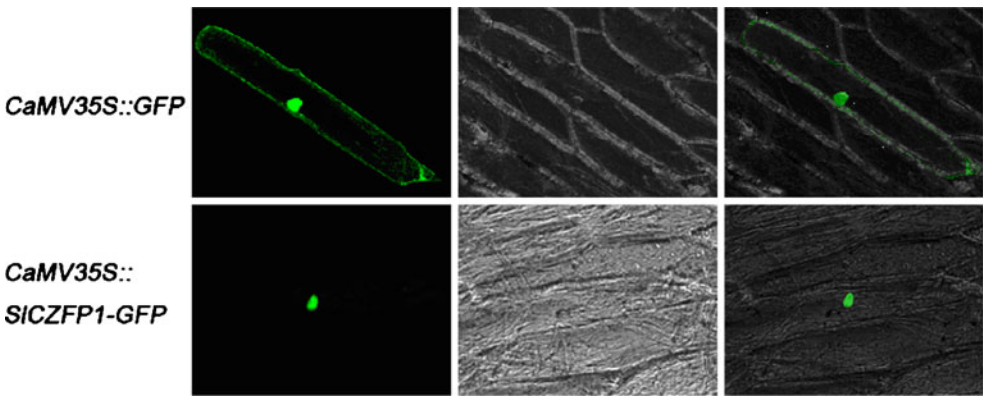


Fig. 3 Nuclear localization of the SlCZFP1 protein. Constructs carrying 35S–GFP (*upper panel*) or 35S–SlCZFP1–GFP (*lower panel*) was introduced into onion epidermal cells. Transformed cells were observed by fluorescence microscopy (*left*), optical (*middle*), and merged (*right*)

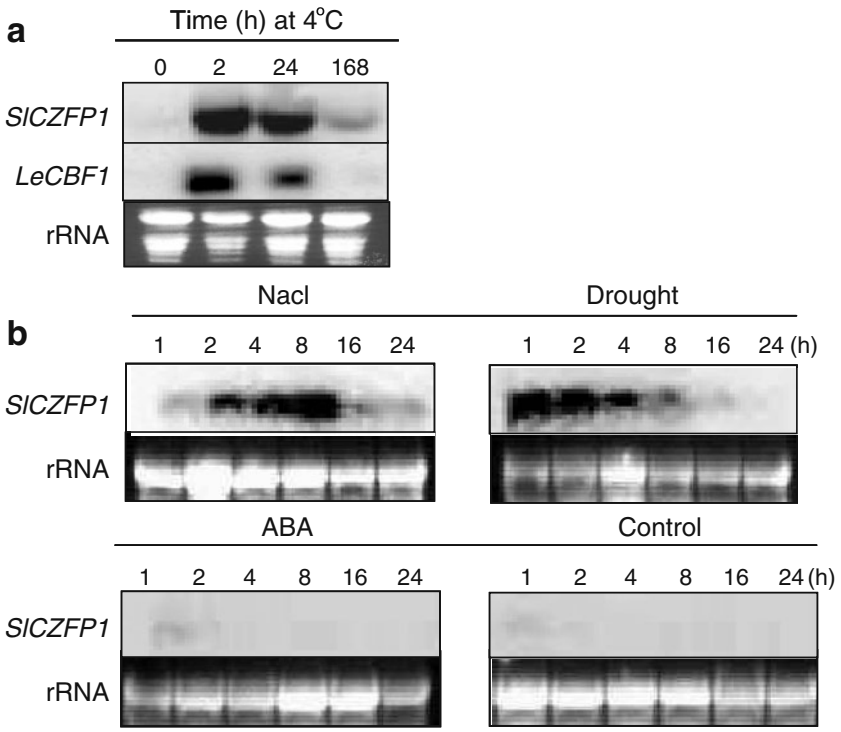


Fig. 4 Accumulation of *SlCZFP1* transcripts in response to abiotic stresses. Tomato seedlings were grown at 26°C and subjected to a variety of abiotic stresses. RNA gel blots were prepared from total RNA and hybridized with gene probe for *SlCZFP1*. rRNA served as a control for RNA loading. **a** RNA was isolated from plants grown in a 16 h photoperiod (16:8) then treated at 4°C for the time points indicated. The *LeCBF1* gene served as a positive control for cold treatments. **b** Plants were grown in a 16 h photoperiod. For drought, NaCl and ABA treatments, the plants were placed on dry filter paper or transferred to filter paper saturated with MS solution supplemented with 250 mM NaCl or 100 μM ABA, respectively, for various durations as indicated. For the control samples, the plants were transferred to filter paper saturated with MS solution and incubated in parallel with the experimental samples

blot analysis to determine the time course of its induction. As shown in Fig. 4a, transcription of *SlCZFP1* was induced by a 4°C treatment and peaked at 2 h. The induction remained high till 24 h and dropped to almost the background level at 168 h. In contrast, expression of the *LeCBF1* gene (Zhang et al. 2004b) peaked at 2 h and then decreased dramatically at 24 h after cold treatment (Fig. 4a). It is interesting to notice that induction of *SlCZFP1* transcription by cold was higher and stayed longer than that of *LeCBF1*.

Next, we investigated if *SlCZFP1* also responds to other stresses, such as salt, drought, and ABA. As shown in Fig. 4b, under drought treatment, the expression level peaked at 1 h and dramatically decreased at 4 h after drought treatment. The induction dropped to almost the background level at 8 h. When treated with 250 mM NaCl, *SlCZFP1* transcripts were detected at 2 h and reached a peak at 8 h, but the induction disappeared at 16 h. In contrast, there was no induction of *SlCZFP1* transcription detected in seedlings treated with ABA, as seen in the control (Fig. 4b). These results suggested that expression of *SlCZFP1* in response to cold, drought, and salt may be mainly induced via an ABA-independent pathway.

Enhanced Cold Tolerance in Transgenic SlCZFP1 Plants

To understand function of *SlCZFP1* in plants under stress conditions, we generated transgenic plants in tomato, Arabidopsis and rice overexpressing *SlCZFP1* under control of the CaMV 35S promoter. We had low transformation efficiency in tomato and finally were able to produce only six PCR-positive events. However, we were not able to detect significant expression of *SlCZFP1* in any of the six transgenic plants by RNA gel-blot analysis (data not shown). Therefore, we performed functional analysis only in Arabidopsis and rice.

A total of ten independent transgenic Arabidopsis plants were obtained. An RNA gel-blot analysis showed that *SlCZFP1* was over-expressed in five of them (Fig. 5a). We selfed the three plants (nos. 2, 4 and 10 in Fig. 5a), which had the highest level of expression, to generate T2 homozygous plants. Apparently, no difference was seen in overall growth and morphology between transgenic and wild-type plants (Fig. 5b). Those three lines were tested for their capacity to tolerate a freezing temperature. We performed a Petri dish whole plant freeze assay with T3 seedlings. In this assay, non-acclimated wild-type and

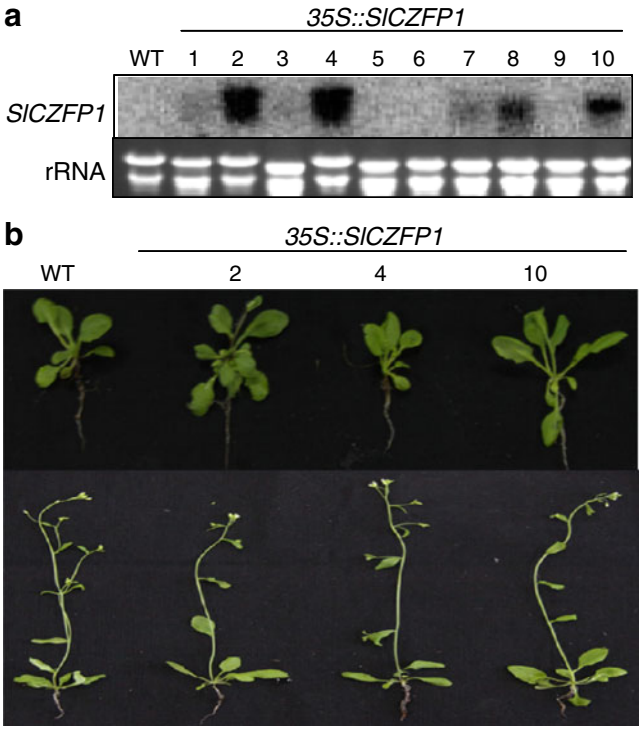
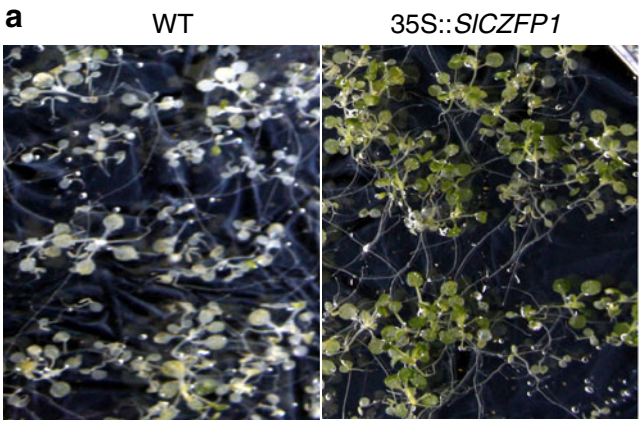


Fig. 5 Molecular characterization and phenotypes of *SlCZFP1* transgenic Arabidopsis plants. **a** Expression of independent transgenic plant lines of Arabidopsis by RNA gel-blot analysis. Each lane was loaded with 10 μg total RNA isolated from 3-week-old seedlings of transgenic Arabidopsis. The RNA blot was hybridized with a ^{32}P-labeled *SlCZFP1* cDNA probe. Ethidium bromide-stained rRNA was used as a RNA-loading control. **b** The phenotypes of the T2 generation of independent lines of overexpressed *SlCZFP1* transgenic Arabidopsis. Wild-type and *SlCZFP1* overexpressed lines after 3 weeks growth at 22°C

SlCZFP1 transgenic plants were frozen at -6°C in dark for 24 h, followed by 2 days recovery at 22°C. As shown in Fig. 6a, most of the *SlCZFP1* transgenic plants survived the shock, whereas nearly all the wild-type plants were killed or severely damaged. The survival rate in transgenic plants was 70.0%, which is significantly higher than the rate of 21.1% in wild-type (*t*-test, $P<0.01$, see Fig. 6b). Thus, overexpression of *SlCZFP1* can enhance the tolerance of transgenic plants to freezing stress.

We performed RNA gel-blot analysis with 18 transgenic rice plants selected based on PCR genotyping. Four of them (nos. 1, 8, 14, and 16) had the highest *SlCZFP1* expression (Fig. 7a). The event 16 failed to produce seeds. We were able to get enough seeds for the other three events. Those plants looked morphologically similar to wild-type plants (Fig. 7b). To investigate the effect of *SlCZFP1* overexpression on cold tolerance, T2 transgenic and wild-type seedlings at four-leaf stage were moved to a growth chamber set at 4°C with illumination of 16 h light/8 h dark for 6 days. During the cold treatment, there was no obvious phenotypic difference between transgenic plants and the wild type. When the cold-stressed plants were transferred back to normal growth conditions, however, the transgenic plants showed much less damage than WT (Fig. 7b, c). After 6 days of recovery, survival rate was 24.2% for the WT and 64.2–83.2% for transgenic lines (Fig. 7d). Similar result (improved tolerance to low-temperature stress) was obtained by using homozygous T3 transgenic plants (data not shown). These results suggested that overexpression of *SlCZFP1* could enhance tolerance of transgenic plants to cold stress. We also tested tolerance of *SlCZFP1* transgenic plants to drought and salt but did not see any enhancement by the *SlCZFP1* expression (data not shown).

The increased cold tolerance of the 35S::*SlCZFP1* plants was further confirmed by measuring changes in chlorophyll fluorescence. The ratio of Fv to Fm was used to estimate the quantum yield of PSII (Genty et al. 1989). The PS II stability reflects the level of cellular damage after chilling treatments. As shown in Fig. 7e, at warm condition (26°C), the *SlCZFP1*-overexpressing plants exhibited values of Fv/Fm comparable to that of the WT, indicating that the *SlCZFP1*expression did not affect the PS II efficiency. During chilling stress (4°C), both transgenic and WT plants had a slight decrease Fv/Fm at 2 days. At the end of 6-day chilling stress, the Fv/Fm in wild-type plants reduced to 0.522±0.049, whereas the Fv/Fm values for the over-



b

	r / n	% survival	*P*-value
wild type	3/672	21.1%± 2.91%	
35S::SlCZFP1	3/770	70.0%± 3.04%	3.58E-05

Fig. 6 Freezing tolerance of *SlCZFP1* transgenic Arabidopsis plants. **a** Non-acclimated *SlCZFP1* transgenic plants (*35S::SlCZFP1*, right) and wild-type (WT, left) plants were frozen at -6°C using the protocol described under experimental procedures. The photograph was taken 2 days after the return to original growth conditions. **b** Table of results of multiple freeze tests including the percentage of plants scored as surviving (% survival), *r*/*n*=number of replicates of freeze test (*r*)/ number of plants examined (*n*), and the *P*-value from a *t*-test

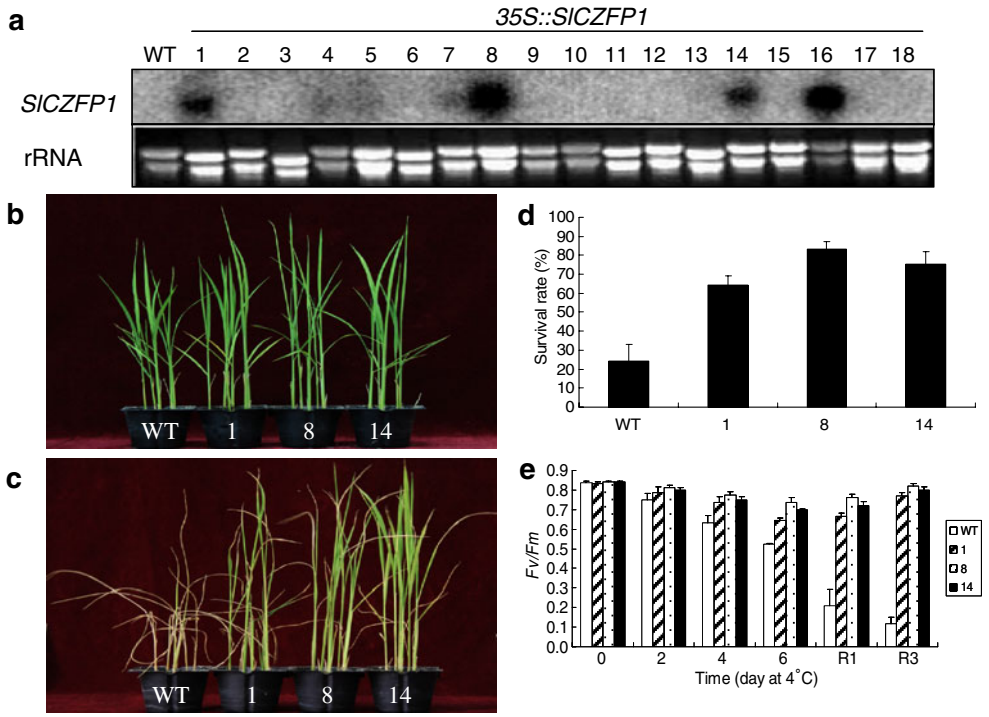


Fig. 7 Identification and cold tolerance testing of *SlCZFP1*-overexpressing rice. **a** RNA gel-blot analysis of the transgenic plants and the wild type. **b** *SlCZFP1*-expressing lines (1, 8, and 14) and wild-type (WT, *left*) plants before cold treatment. **c** Performance of cold tolerance of three transgenic lines and wild-type plants after cold treatment (4°C for 6 days) and recovery (26°C for 6 days). **d** Survival rate of three transgenic lines and the wild type after cold treatment. *Error bar* represents the standard deviation among three replicates. **e** Chlorophyll fluorescence measurement and chilling tolerance of *SlCZFP1*-overexpressing plants. Both wild-type and *SlCZFP1*-expressing lines (1, 8, and 14) were incubated at 4°C for 6 days and recovery at 26°C. Fv/Fm values were measured at various time points of days (0, 2, 4, 6) during cold treatment and 1 and 3 days of recovery (R1, R3). Results were shown as average of five measurements with less than 5% SD

expressors 1, 8, and 14 were between 0.643±0.013 and 0.736±0.025. In addition, it was found that the recovery of Fv/Fm in transgenic plants was also quick. In contrast, there was no recovery in WT (Fig. 7e). This suggested that photoinhibition of PS II during chilling stress was alleviated in transgenic plants and the PS II reaction center was less damaged compared with similarly treated WT plants.

Overexpression of *SlCZFP1* Induced Expression of COR or Stress-responsive Genes

In response to dehydration stress, such as cold and drought, a set of COR genes were induced in wild-type Arabidopsis plants (Seki et al. 2001). It has been shown previously that overexpression of CBF1/DREB1b (Jaglo-Ottosen et al. 1998) or CBF3/DREB1a (Kasuga et al. 1999; Gilmour et al. 2000; Seki et al. 2001) is sufficient to induce expression of the downstream CRT/DRE element-containing COR target genes. To investigate whether the *SlCZFP1* transcription factor could also activate the COR genes, expression levels for *COR6.6, COR15a,* and *COR47* known as downstream target genes of CBF/DREB1 proteins were analyzed and shown in Fig. 8a. Constitutive expression of the *SlCZFP1* gene resulted in activation of *COR6.6*, *COR15a*, and *COR47*, in absence of a low-temperature stimulus. Moreover, the expression level of the *SlCZFP1* in individual transgenic Arabidopsis lines correlated well with the concomitant trans-activation of COR genes (Fig. 8a). However, the transcription levels of Arabidopsis *CBF1, 2, 3* and *COR78*, did not show any changes in *SlCZFP1*-overexpressing transgenic lines under normal conditions, as compared with the WT (Fig. 8a), indicating that *CBFs* and *COR78* are not the downstream genes of *SlCZFP1*.

To further understand the mechanisms of chilling tolerance conferred by overexpressing *SlCZFP1*, we analyzed expression of 15 known stress-responsive or rice homologs of CBF/DREB target genes (Dubouzet et al. 2003) (Table 1) under normal conditions in *SlCZFP1*-overexpressing lines by RT-PCR analysis. As shown in Fig. 8b, the expression levels of *OsUSP1* (AK063881), *OsWCOR413*-like (AF283006), and *OsGOLS3* (AK107065) were increased in *SlCZFP1* transgenic rice plants compared with the levels in wild-type rice. There was no significant difference in the expression levels of

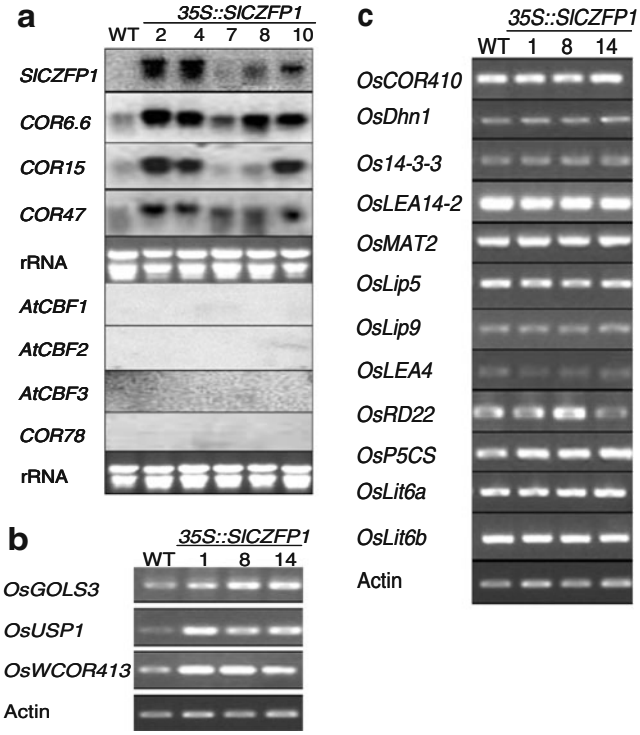


Fig. 8 Overexpression of *SlCZFP1* in Arabidopsis and rice modulates the expression of COR or stress-responsive genes. **a** Northern analysis of Arabidopsis COR genes and *CBF1, 2, 3* in *SlCZFP1* transgenic and wild-type Arabidopsis plants. Leaves from non-acclimated wild-type plant and the T2 progenies of five individual lines of transgenic plants were harvested. The total RNA (10 μg) was analyzed for *SlCZFP1*, *COR* and *AtCBFs* gene transcription by RNA blots analysis with ^{32}P-labeled cDNA probes, respectively. **b** and **c** RT-PCR analysis of 15 stress-responsive genes in *SlCZFP1* transgenic rice plants. Total RNAs from 2-week-old seedlings of both wild-type and *SlCZFP1* transgenic rice were isolated and reverse-transcribed. Transcript levels of 15 stress-responsive genes were determined by RT-PCR using gene primer pairs of the corresponding genes (Table 1). Actin was used as an internal control. WT: wild type; 1, 8, and 14: *SlCZFP1*-overexpressing lines. The experiments were repeated three times. The results shown were representative of the three replicates. **b** Expression of *OsWCOR413*, *OsGOLS3,* and *OsUSP1* in WT and *SlCZFP1* transgenic rice plants under normal condition. **c** Transcript levels of other tested stress-responsive genes in WT and *SlCZFP1* transgenic rice plants

other tested stress-responsive genes between *SlCZFP1* transgenic lines and WT plants (Fig. 8c). These results suggest that *SlCZFP1* may be one of upstream regulators of these genes and act as a master switch in cold stress tolerance and was involved in the complicated network controlling expression of cold-responsive genes.

Discussion

Low temperature is a major abiotic factor that limits crop productivity in many areas around the world. A number of studies have demonstrated that overexpression of cold-induced genes can confer cold tolerance to transgenic plants (Puhakainen et al. 2004; Zhang et al. 2004a). In this report, we have cloned and characterized *SlCZFP1*, a novel cold-inducible gene in tomato. *SlCZFP1* encodes a C2H2-type zinc finger protein, which is localized in the nucleus (Fig. 3). In addition, we showed that the expression of *SlCZFP1* could be induced by cold, salt, and drought, but not by exogenously applied ABA (Fig. 4).

Notably, expression-pattern analysis shows that the activation pattern of *SlCZFP1* under cold stress differs from that of *LeCBF1* (Zhang et al. 2004b). *SlCZFP1* transcript accumulated rapidly upon exposure to low temperature reaching a peak level at about 2 h of cold treatment, remained elevated at a constant level for 24 h, and then decreased at 7 days, but remained elevated over the level found in the warm grown plants. In contrast, the transcript level of *LeCBF1* increased rapidly to a high level (not as high as that of *SlCZFP1*) at 2 h, then declined, and returned to levels found in warm grown plants after about 7 days of cold treatment (Fig. 4a). Compared with the case of *LeCBF1*, the induction of *SlCZFP1* occurred more strongly and was more prolonged. In addition, *SlCZFP1* is induced by drought and salt stress (Fig. 4b), which is dissimilar to *LeCBF1* that was induced only by cold stress (Zhang et al. 2004b). The expression pattern of *SlCZFP1* is of prime interest because it suggests that *SlCZFP1* may represent a novel cold-regulated transcription factor from tomato which may control the expression of a distinct set of downstream genes other than CBF-regulated regulons.

To understand the function of *SlCZFP1* under abiotic stress conditions, we generated several lines of transgenic tomato plants overexpressing *SlCZFP1* under the control of the constitutive CaMV 35S promoter. Unfortunately, for unknown reasons, the expression of transgenes was not elevated in all transgenic tomato plants (data not shown). We showed that constitutive expression of *SlCZFP1* gene in Arabidopsis and rice plants conferred enhanced freezing or chilling tolerance compared with wild-type plants (Figs. 6 and 7), but not salt and drought tolerance (data not shown). There were no significant changes in morphology between *SlCZFP1*-ox plants and WT plants (Figs. 5b and 7b). Further determination of photosynthetic efficiency revealed that the Fv/Fm ratio was less affected by low-temperature treatment in transgenic rice plants (Fig. 7e). These results confirmed that *SlCZFP1* plays a role in low-temperature stress response.

Our molecular analysis revealed that the expression of some COR genes, such as *COR6.6*, *COR15a* and *COR47*, was affected by altered expression of *SlCZFP1* in transgenic Arabidopsis plants (Fig. 8a). It is interesting to note that soybean SCOF-1 protein is one of the homologues of SlCZFP1 and its overexpression in Arabidopsis also

induced the expression of some COR genes, resulting in enhanced cold tolerance (Kim et al. 2001). This suggested that *SCOF-1*, *SlCZFP1*, and some COR genes may function in a common signaling pathway.

Previously, we found that *SlCZFP1* was not responsive to overexpression of *LeCBF1* in tomato plants, suggesting that *SlCZFP1* is not a target gene of *LeCBF1* (Zhang 2004b). In this study, we found that *AtCBF1, 2*, *3* were not response to *SlCZFP1* (Fig. 8a), suggesting that CBFs are not the downstream genes of *SlCZFP1* either. These results suggest that *SlCZFP1* and *LeCBF1* may act in separate parallel pathways to regulate cold acclimation (Fowler and Thomashow 2002; Zhu et al. 2004). Notably, we observed that overexpression of *SlCZFP1* also resulted in enhanced expression of some stress-responsive genes including *OsGOLS3*, *OsUSP1*, and *OsWCOR413* like in *SlCZFP1*-ox transgenic rice plants (Fig. 8b). This suggested that these three stress-inducible genes may function in *SlCZFP1*-mediated cold signaling pathway. The expression of these genes is induced by external stimuli and plays important roles in plant response to abiotic stresses (Sauter et al. 2002; Taji et al. 2002; Breton et al. 2003; Reyes et al. 2003). For example, *Cor413* gene encode transmembrane protein and act to stabilize plasma membrane as well as perceive external signals (Breton et al. 2003; Reyes et al. 2003;). Galactinol synthase (*GolS*) is a key enzyme in the synthesis of raffinose family oligosaccharides that function as osmoprotectants in plant cells (Taji et al. 2002) and *AtGolS3* is a DREB target gene that is induced only by cold stress (Gilmour et al. 2000; Taji et al. 2002). *OsUSP1*, a USP family protein, has been identified to be regulated by ethylene and may play a role in the adaptation of rice to submergence stress (Sauter et al. 2002). These data suggested that the induction of *OsGOLS3*, *OsWCOR413*, and *OsUSP1* in the transgenic rice by *SlCZFP1* may contribute to enhanced tolerance to cold stress. However, no significant changes were observed for the expression of some other cold-responsive genes (Fig. 8c) such as *OsLti6b* (Kim et al., 2007), 14-3-3-like protein (Jarillo et al. 1994), *lip5 and lip9* (Aguan et al. 1991), *OsDhn1* (Close 1997), and *OsP5CS* (Igarashi et al. 1997), suggesting that these genes might function in a different cold signaling pathway.

In conclusion, this study has identified a novel TFIIIA transcription factor gene *SlCZFP1* that can be induced by various abiotic stresses. The enhanced freezing or chilling stress tolerance of the *SlCZFP1*-overexpressing Arabidopsis and rice suggest a promising utility of this gene in genetic improvement of cold tolerance in economically important crops.

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