

Silencing of dehydrin *CaDHN1* diminishes tolerance to multiple abiotic stresses in *Capsicum annuum* L.

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Abstract

Key message We cloned a dehydrins gene *CaDHN1* from pepper and the expression of *CaDHN1* was markedly upregulated by cold, salt, osmotic stresses and salicylic acid (SA) treatment.

Abstract Dehydrins (DHNs) are a subfamily of group 2 late embryogenesis-abundant (LEA) proteins that are thought to play an important role in enhancing abiotic stress tolerance in plants. In this study, a DHN EST (Expressed Sequence Tag) was obtained from 6 to 8 true leaves seedlings of pepper cv P70 (*Capsicum annuum* L.) by our laboratory. However, the DHN gene in pepper was not well characterized. According to this EST sequence, we isolated a DHN gene, designated as *CaDHN1*, and investigated the response and expression of this gene under various stresses. Our results indicated that *CaDHN1* has the DHN-specific and conserved K- and S- domain and encodes 219 amino acids. Phylogenetic analysis showed that *CaDHN1* belonged to the SKn subgroup. Tissue

expression profile analysis revealed that *CaDHN1* was expressed predominantly in fruits and flowers. The expression of *CaDHN1* was markedly upregulated in response to cold, salt, osmotic stresses and salicylic acid (SA) treatment, but no significant change by abscisic acid (ABA) and heavy metals treatment. Loss of function of *CaDHN1* using the virus-induced gene silencing (VIGS) technique led to decreased tolerance to cold-, salt- and osmotic-induced stresses. Overall, these results suggest that *CaDHN1* plays an important role in regulating the abiotic stress resistance in pepper plants.

Keywords *Capsicum annuum* L · *CaDHN1* · Abiotic stress response · Expression analysis · Gene silencing

Introduction

Plants are sessile organisms that are unable to escape from unfavorable environmental conditions, such as cold, heat, drought and high salinity stresses. These environmental abiotic stresses can detrimentally affect plant growth and development and ultimately decrease crop productivity. Plants protect themselves from the detrimental effects of abiotic stresses by increasing the expression of a large number of stress-responsive genes. According to their functions during stress, the products of these genes can be classified into two groups (Hirayama and Shinozaki 2010). One group contains proteins involved in stress-inducible gene expression and further regulation of signal transduction, such as protein kinases and transcription factors. The other group includes proteins functioning in direct abiotic stress tolerance, such as osmotin, chaperones, late embryogenesis-abundant (LEA) proteins and detoxification enzymes.

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LEA proteins are highly hydrophilic proteins that play a key role in the response to abiotic stresses in plants (Amara et al. 2012). Plant dehydrins (DHNs) are the group II (D-11 family) of the LEA proteins that are accumulated in the later stages of embryogenesis when the water content in seeds declines (Brini et al. 2007). The DHNs genes are expressed at high levels in response to low temperature, salinity or drought stresses (Szabala et al. 2014). They are believed to stabilize metabolism during cellular dehydration (Kosova et al. 2007), probably via stabilization of membranes by chaperone activity (Kovacs et al. 2008), hydrophobic interactions (Campbell and Close 1997; Koag et al. 2003) or antioxidant activity preventing excessive reactive oxygen species (ROS) formation (Hara et al. 2005; Sun and Lin 2010).

DHNs are classified according to their highly conserved lysine-rich domain (EKKGIMDKIKEKLP), also known as the K-segment, which are present at between 1 and 11 copies near the C-terminus of all DHNs characterized to date (Close 1997; Svenson et al. 2002). The K-segment takes on an amphipathic α -helical structure and is believed to play a role in protein-lipid interactions (Close 1997). Many DHNs include a tract of phosphorylatable serine residues named the S-segment, and the consensus sequence (V/T) DEYGNP, known as the Y-segment, is sometimes present near the N-terminus. Besides these three conserved segments, a second less conserved DHN domain (the Φ -segment) that is rich in polar amino acids may be present. Based on the number and order of these highly conserved segments, DHNs are categorized into five subgroups: Y_nSK_n , SK_n , K_n , Y_nK_n and K_nS (Allagulova et al. 2003; Rorat 2006).

In recent years, several studies have reported a positive correlation between the accumulation of DHN transcripts and/or proteins and tolerance to low temperature, drought and salinity stresses. Borovskii et al. (2002) reported that cold, freezing, drought or exogenous ABA treatment resulted in accumulation of thermostable DHNs in plant mitochondria. Moreover, cryotolerant species such as wheat and rye accumulate more DHNs than cryosensitive species such as maize. The acidic SK3 dehydrin DHN24 from *Solanum sogarandinum* is constitutively expressed, and its expression is associated with cold acclimation (Rorat et al. 2006). In addition, the peach Y2K9-type DHN gene *ppdhn1* is induced by cold, drought or ABA, and the gene product exhibits cryoprotective and antifreeze activities (Artlip et al. 1997; Wisniewski et al. 2006). In blueberries (*Vaccinium* spp.), three DHN cold-responsive genes have been identified, and accumulation of their transcripts was positively correlated with cold hardiness in several different genotypes (Dhanaraj et al. 2005). Hara et al. (2003) isolated WCor410 that encodes a wheat dehydrin and introduced it into strawberry, which resulted in transgenic plants with enhanced tolerance against freezing and chilling stress.

Despite numerous studies on DHNs in various plants, pepper proteins have not been investigated extensively. In our previously studies, we characterized an EST (GenBank no: JZ198814) homologous to DHNs by differential screening of a previously reported cold-associated pepper seedling cDNA library (Guo et al. 2013). So in this study, based on the sequence of this EST sequence, the full-length ORF was obtained using the rapid amplification of cDNA ends (RACE) method, and the designated gene *CaDHN1* showed tissue-specific expression in different organs in pepper plants. Moreover, the possible role of *CaDHN1* in the response against cold, salt, osmotic and heavy metal stresses and plant hormone (abscisic acid and salicylic acid) signaling was investigated using RT-qPCR. Furthermore, *CaDHN1* loss-of-function plants were engineered using a virus-induced gene silencing (VIGS) system (Liu et al. 2002; Chung et al. 2004; Wang et al. 2013). Together, the results suggested that *CaDHN1* is a potentially important player in the regulation of plant defense responses.

Results

Cloning and sequence analysis of *CaDHN1*

A pepper cDNA clone (GenBank accession no. JZ198814) was selected from the differential screening of the previously characterized cold-associated pepper seedling cDNA library (Guo et al. 2013). Based on the sequence of the fragment, 3'- and 5'-RACE primers were designed and RACE was performed, which generated two fragments of 862 bp and 318 bp, respectively. Following alignment and assembly of these three sequences, the full-length cDNA was deduced, amplified by PCR, and confirmed by sequencing. The 1104 bp full-length cDNA contains a 660 bp ORF and encodes a 219 amino acid polypeptide (Fig. 1) with a calculated isoelectric point of 5.41 and a theoretical molecular mass of 24.7 kDa. This gene was designated as *CaDHN1* and submitted to GenBank with accession No. JX402924.

An NCBI blast search indicated that the deduced *CaDHN1* amino acid sequence showed moderate homology with other dehydrin proteins from *Solanum tuberosum* (C17, cold stress dehydrin inducible protein, GenBank accession no. AAB53203.1, 84.9 % identity), *Solanum sogarandinum* (25 kDa dehydrin protein, GenBank accession No. AAp44575.1, 83.1 % identity), *Solanum peruvianum* (dehydrin protein, GenBank accession no. ADQ73992.1, 75.8 % identity), *Nicotiana tabacum* (dehydrin protein, GenBank accession no. BAD13499.1, 78.7 % identity), and *Solanum commersonii* (dehydrin 2 protein, GenBank accession no. AAK66763.1, 65.3 % identity).

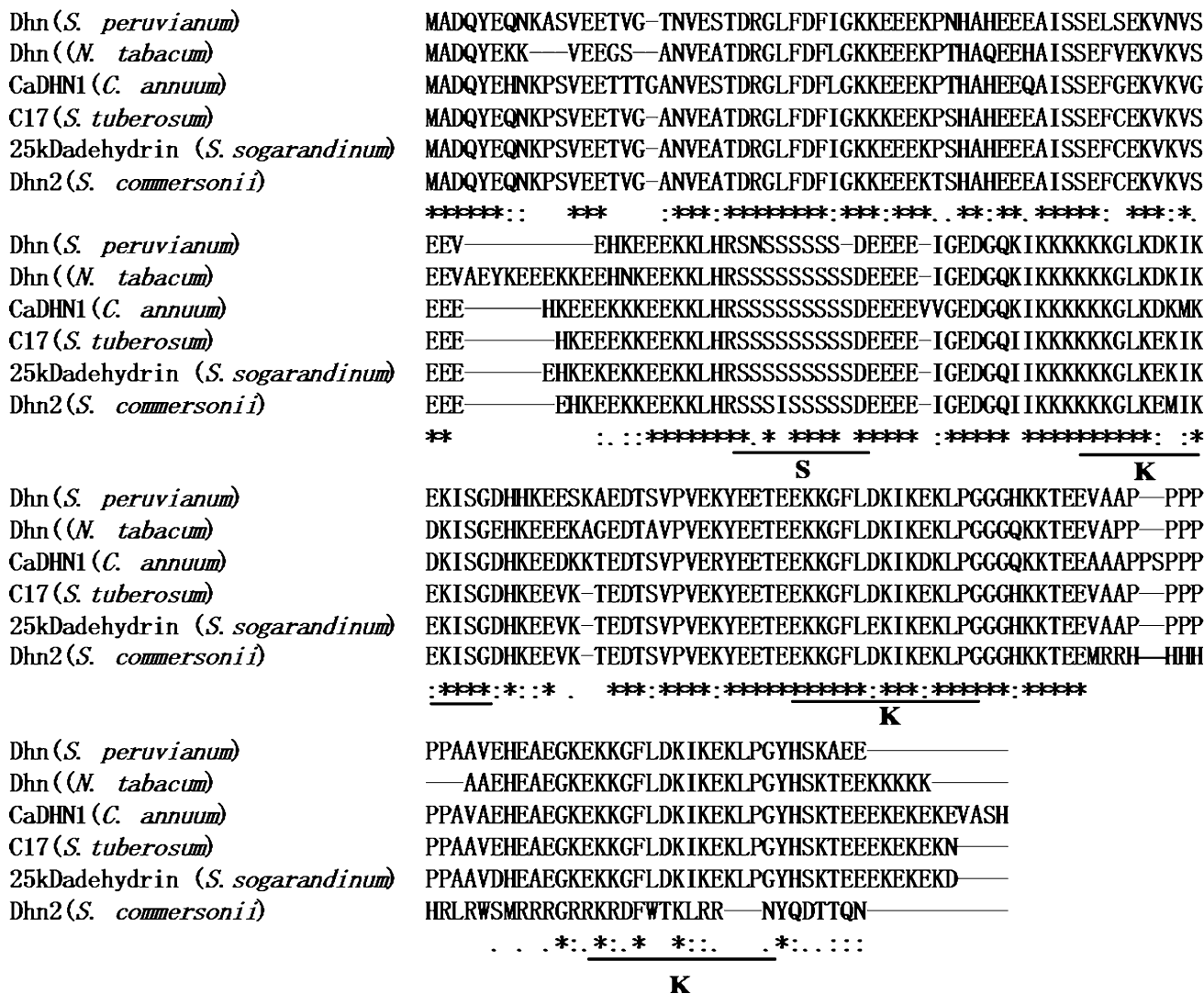


Fig. 1 Multiple sequence alignment of CaDHN1 with the amino acid sequences of *Solanum tuberosum* C17 cold stress dehydrin inducible protein, (GenBank accession no. AAB53203.1), *Solanum sogarandinum* 25 kDa dehydrin protein (GenBank accession no. AAP44575.1),

Solanum peruvianum dehydrin protein (GenBank accession no. ADQ73992.1), *Nicotiana tabacum* dehydrin protein (GenBank accession no. BAD13499.1), *Solanum commersonii* dehydrin 2 protein (GenBank accession no. AAK66763.1). S and K motifs are underlined

Following comparison CaDHN1 with other members of the DHN family, CaDHN1 was shown to contain characteristic DHN motifs. There is one S-segment near the N-terminus and three K-segments near the C-terminus (Fig. 1). CaDHN1 belongs to the SKn-type dehydrin subfamily, and the K-segment closely matches that of the reported consensus sequence (EKKGIMDKIKEKLPG) with only a few conservative substitutions (Campbell and Close 1997).

In order to evaluate the molecular evolutionary relationships between CaDHN1 and other DHN proteins, a phylogenetic tree was generated using Mega5.1 (Fig. 2). Based on our phylogenetic results, the DHNs could be divided into five groups, corresponding to SKn-, YK-, YnSKn-, Kn-, and KS-type proteins. CaDHN1 falls into the SKn-type DHN protein family.

Expression of *CaDHN1* in response to abiotic stress and plant hormones

In order to investigate the expression pattern of *CaDHN1* in different tissues of pepper cv P70 plants, RT-qPCR was performed on RNA from pepper roots, stems, leaves, flowers, immature fruits and seeds (Fig. 3). Clear differences were apparent, and expression of *CaDHN1* was highest in fruits, followed by flowers and stems. However, *CaDHN1* was expressed at very low levels in roots.

The transcription expression of *CaDHN1* was further investigated by abiotic (cold, salt, osmotic, and heavy metal) stresses and abscisic acid (ABA), salicylic acid (SA) treatments in seedlings. When seedlings of pepper exposed to cold stress, the expression of *CaDHN1* was not

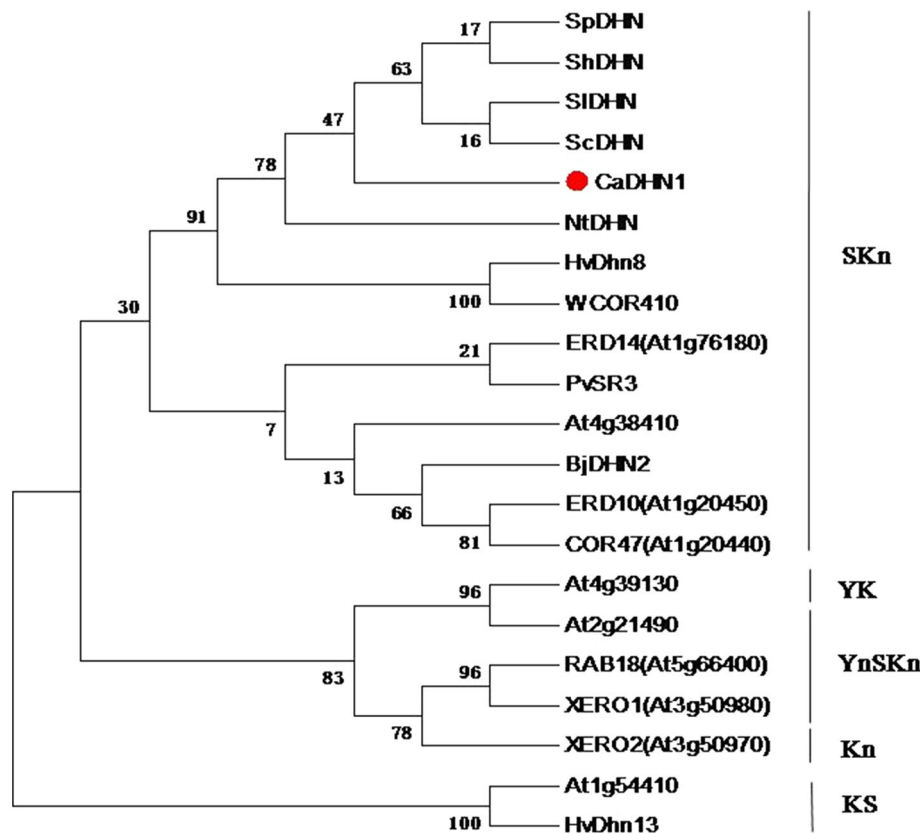


Fig. 2 Phylogenetic tree of *CaDHN1* and homologous DHN proteins from other species. The tree was constructed with MEGA 5.1 program using neighbor-joining method. Genbank accession numbers are as follows: *CaDHN1* [JX402924], *COR47* (At1g20440) [AY114699], *ERD10* (At1g20450) [AF360351], *At1g54410* [NM_104319], *ERD14*(At1g76180) [AF339722], *At2g21490* [BT000900], *XERO2*

(At3g50970) [NM_114957], *XERO1*(At3g50980) [NM_114958], *At4g38410* [NM_120003], *At4g39130* [NM_120073], *RAB18* (At5g66400) [AY093779], *HvDhn8* [AF181458], *HvDhn13* [AY681974], *ShDHN* [AHB20199.1], *ScDHN* [AK224734], *SIDHN* [M97211], *SpDHN* [ADQ73992], *NtDHN* [BAD13499], *WCOR410* [XM_006647600], *PvSR3* [U54703], *BjDHN2* [DQ441470]

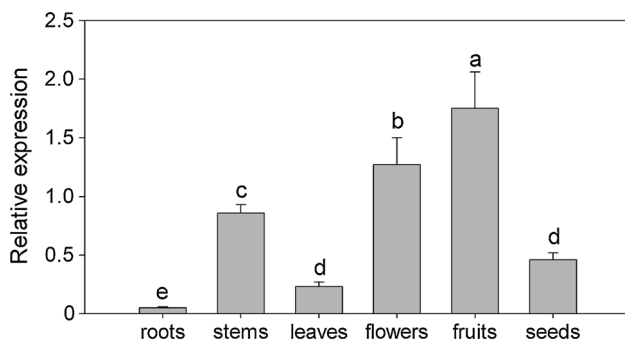


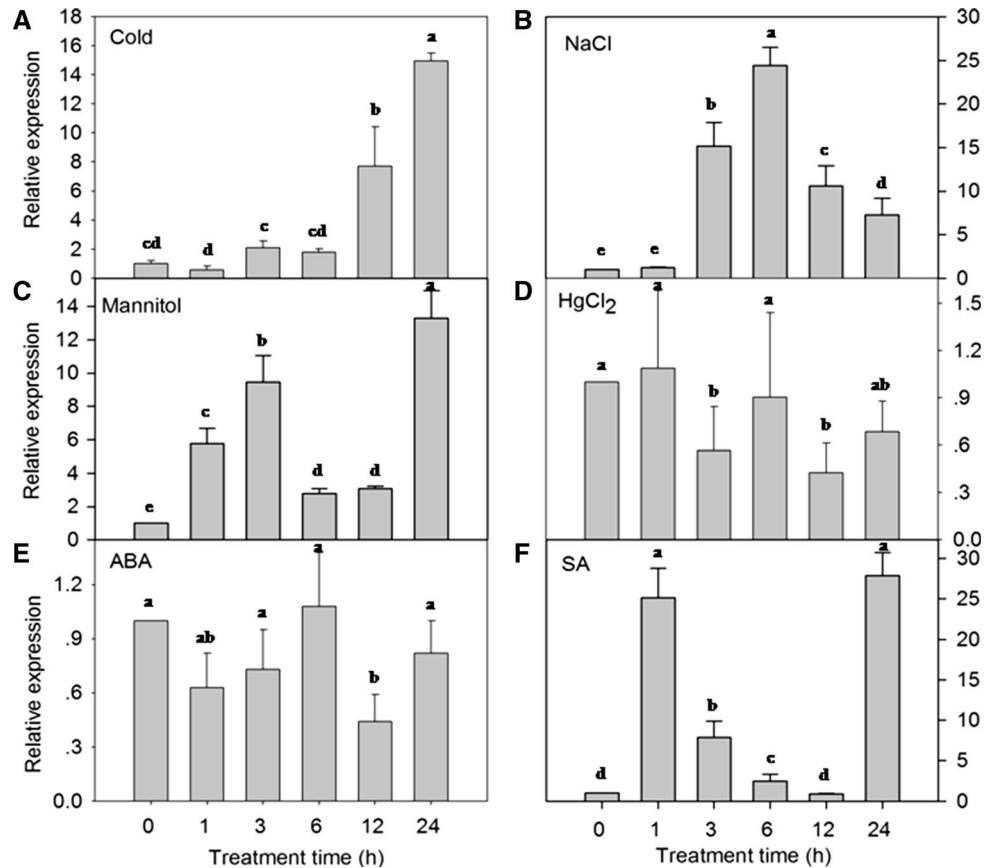
Fig. 3 Tissue-specific expression of *CaDHN1* in different tissues of pepper plants. Bars with different lower case letters in each group were significantly different, as determined using Duncan's multiple range tests ($p < 0.05$)

significantly affected in the initial stages (0–6 h) 6 °C treatments, but was upregulated threefold 12 h after treatment (Fig. 4a). The strongest response to cold treatment was highest at 24 h, at which point expression levels were elevated 15-fold. Similar to the results for cold stress,

CaDHN1 expression was also significantly enhanced in pepper leaves following salt and osmotic stress (Fig. 4b, c). By salt stress, the transcript of *CaDHN1* was dramatically increased with a 15-fold increase at 3 h and reached a peak of 25-fold at 6 h, then subsequently decreased to a 7-fold elevation by 24 h. By osmotic stress, expression of *CaDHN1* was strongly and rapidly upregulated with a sixfold elevation at 1 h after a 0.3 M mannitol treatment. Upregulation continued and reached tenfold by 3 h, before diminishing slightly. By 24 h, expression of *CaDHN1* was again increased by 13-fold. However, in this study, expression of *CaDHN1* was not significantly affected by heavy metal stress (Fig. 4d).

To investigate the possible involvement of *CaDHN1* in defense-associated signaling, expression was analyzed in pepper leaves treated with 0.57 mM ABA or 5 mM SA using RT-qPCR. *CaDHN1* expression was not significantly changed between control plants and following exogenous ABA treatment plants (Fig. 4e). Following treatment with 5 mM SA, however, the transcription levels of *CaDHN1*

Fig. 4 RT-qPCR analysis of *CaDHN1* expression in the leaves of pepper plants following abiotic stresses and plant hormone treatments. The results are the mean \pm standard error (SE), replicated thrice. Different lower case letters indicates significant difference when compared with the control at a p value <0.05



were strongly increased by 25-fold at 1 h, before expression levels fell sharply and were maintained at low levels between 3 and 12 h (Fig. 4f). By 24 h, expression increased again and peaked at 28-fold.

Suppression of *CaDHN1* diminishes tolerance to abiotic stresses

To examine further the role of *CaDHN1* in the response to cold, salt and osmotic stress, virus-induced gene silencing (VIGS) was performed on pepper cv P70 using the tobacco rattle virus (TRV)-induced gene silencing technique (Wang et al. 2013; Chen et al. 2014). A fragment from the 3' end of the *CaDHN1* ORF was cloned into the pTRV2 vector to generate the pTRV2:*CaDHN1* construct (empty pTRV2:00 was used as a negative control). The pTRV2:*CaPDS* construct silences the phytoene desaturase (*PDS*) gene and induces a bleaching phenotype (about 70 percent plants have these phenotype), and was used as a positive control to determine the success of gene silencing (Fig. 5a).

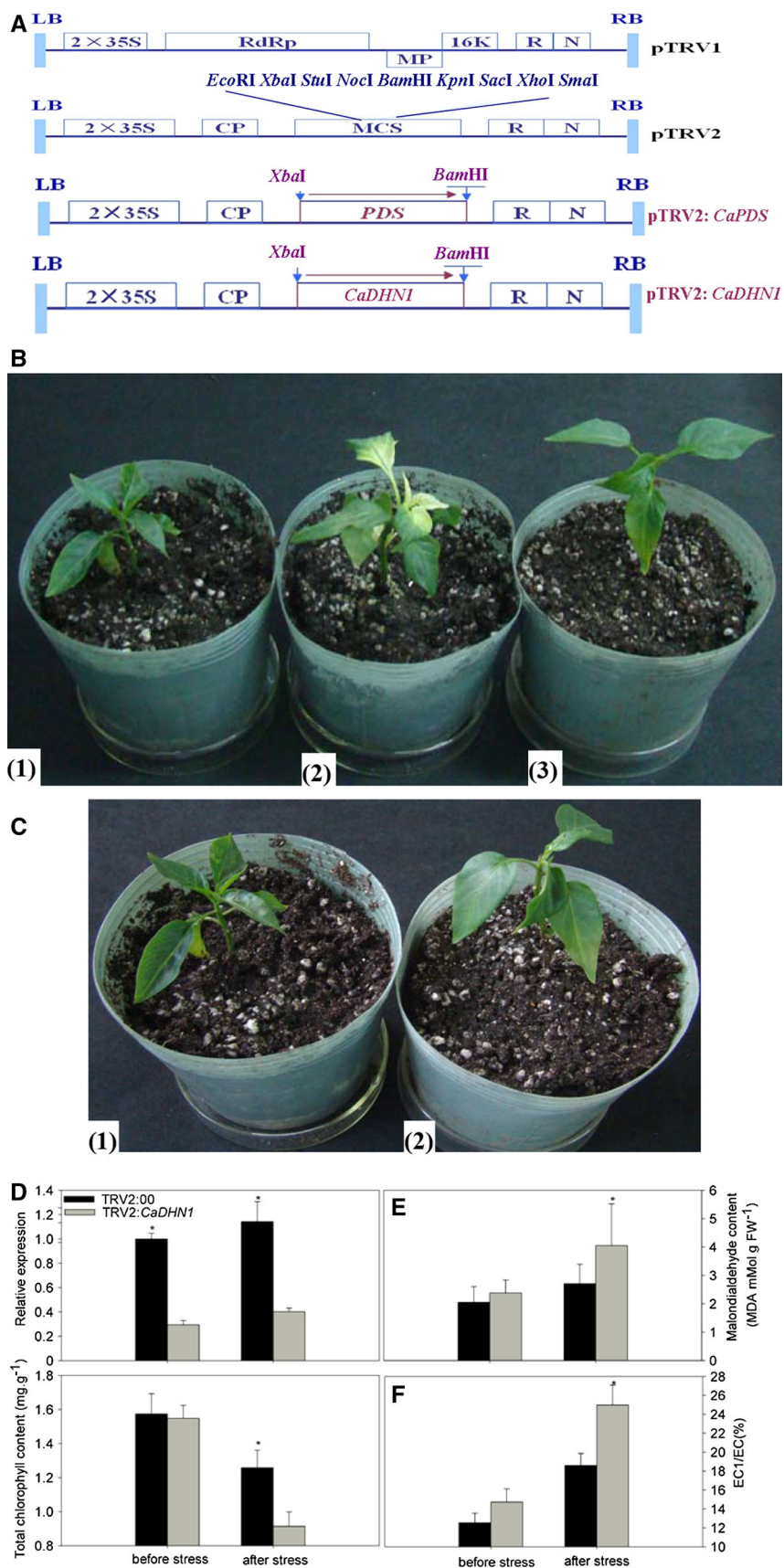
At 35 days after the induction of TRV-mediated gene silencing, pepper plants infiltrated with pTRV2:*CaDHN1* showed no visible phenotypic differences from negative

control plants (Fig. 5b), but bleaching was evident on the leaves of *CaPDS*-silenced plants. To verify that the *CaDHN1* transcript was effectively downregulated by VIGS, RT-qPCR was carried out (Fig. 5c). *CaDHN1* expression was dramatically reduced by 70 % in the new leaves of silenced pepper plants grown at 22 °C under non-stress conditions, suggesting that VIGS was successful and effective for *CaDHN1* gene silencing.

Under 24 h 6 °C cold stress, the pTRV2:*CaDHN1* plants aggravated the visible symptoms of leaf damage in seedlings. There was more seriously wilting appeared in pepper seedlings in pTRV2:*CaDHN1* plants than that of pTRV2:00 plants (Fig. 5c). In order to confirm the influence of silencing of *CaDHN1* in the cold stress defense response, malondialdehyde (MDA), chlorophyll and electrical conductivity were measured in control and silenced pepper plants. After 24 h of 6 °C cold treatments, the MDA and electrical conductivity measurements were both significantly increased, while chlorophyll levels were markedly decreased in *CaDHN1*-silenced plants (Fig. 5e–g).

To determine whether the silencing of *CaDHN1* led to reduced tolerance to salt and osmotic stresses, leaf discs

Fig. 5 VIGS of *CaDHNI* in pepper. **a** Schematic representation of the pTRV1, pTRV2, pTRV2:*CaPDS* and pTRV2:*CaDHNI* constructs. **b** Phenotypes of gene-silenced pepper plants. (1), control (pTRV2:00); (2), *CaPDS*-silenced (pTRV2:*CaPDS*); (3), *CaDHNI*-silenced (pTRV2:*CaDHNI*). **c** Phenotypes of gene-silenced pepper plants after 24 h of 6 °C cold treatments. (1), control (pTRV2:00); (2), *CaDHNI*-silenced (pTRV2:*CaDHNI*). **d** RT-qPCR analysis of *CaDHNI* expression in gene-silenced (pTRV2:*CaDHNI*) and control (pTRV2:00) plants 35 days after inoculation. **e** Effects of low temperature stress on MDA, chlorophyll (f) and EC1/EC2 (g) in *CaDHNI*-silenced pepper seedlings. Asterisk indicates significant differences compared with the control at a *p* value <0.05



from empty vector control (pTRV2:00) and *CaDHN1*-silenced (pTRV2:*CaDHN1*) plants were exposed to 300 mM NaCl and 300 mM mannitol solution, respectively, with continuous lighting for 3 days (Fig. 6). *CaDHN1*-silenced leaf discs were more yellow than those of control plants, and some were blackened after 3 days of salt treatment, and the same was true of mannitol-treated plants. Chlorophyll levels were also significantly reduced in *CaDHN1*-silenced plants compared with controls.

To elucidate the mechanism of the reduced tolerance to cold stress in *CaDHN1*-silenced plants, the expression patterns of cold stress- and antioxidant system-related genes (*KIN*, a cold-induced gene, *Mn-SOD*, *POD*) were monitored in control (pTRV2:00) and *CaDHN1*-silenced (pTRV2:*CaDHN1*) plants by RT-qPCR (Fig. 7). The result indicated that cold stress induced *Mn-SOD*, *POD* and *KIN1* expression in both control and *CaDHN1*-silenced plants (Fig. 7). However, after cold treatment, expression of these genes in *CaDHN1*-silenced plants was noticeably lower than in control plants.

Discussion

In this study, we isolated a dehydrin gene from *Capsicum annuum* leaves, designated *CaDHN1*. Amino acid alignment of this gene has the DHN-specific and conserved K- and S-domain and belongs to the SKn-type dehydrin subfamily. Tissue distribution expression indicated that *CaDHN1* was expressed in all tested tissues and high level in fruits (Fig. 3). The relatively high expression in fruits may be due to the fruits have the developing seeds which contain high levels of LEA family transcripts and proteins, and DHNs belong to the LEA group II gene family (Chung et al. 2003).

Temperature stress is one of the most important abiotic factors limiting the growth, development and geographical distribution of plants (Nguyen et al. 2009), and the expression of many DHNs is altered in response to low or high temperature stress. In this study, expression of *CaDHN1* was upregulated under cold stress (6 °C treatments) (Fig. 4a) and reached a peak of 5-fold at 48 h. This expression pattern was reminiscent of the findings of Qiu

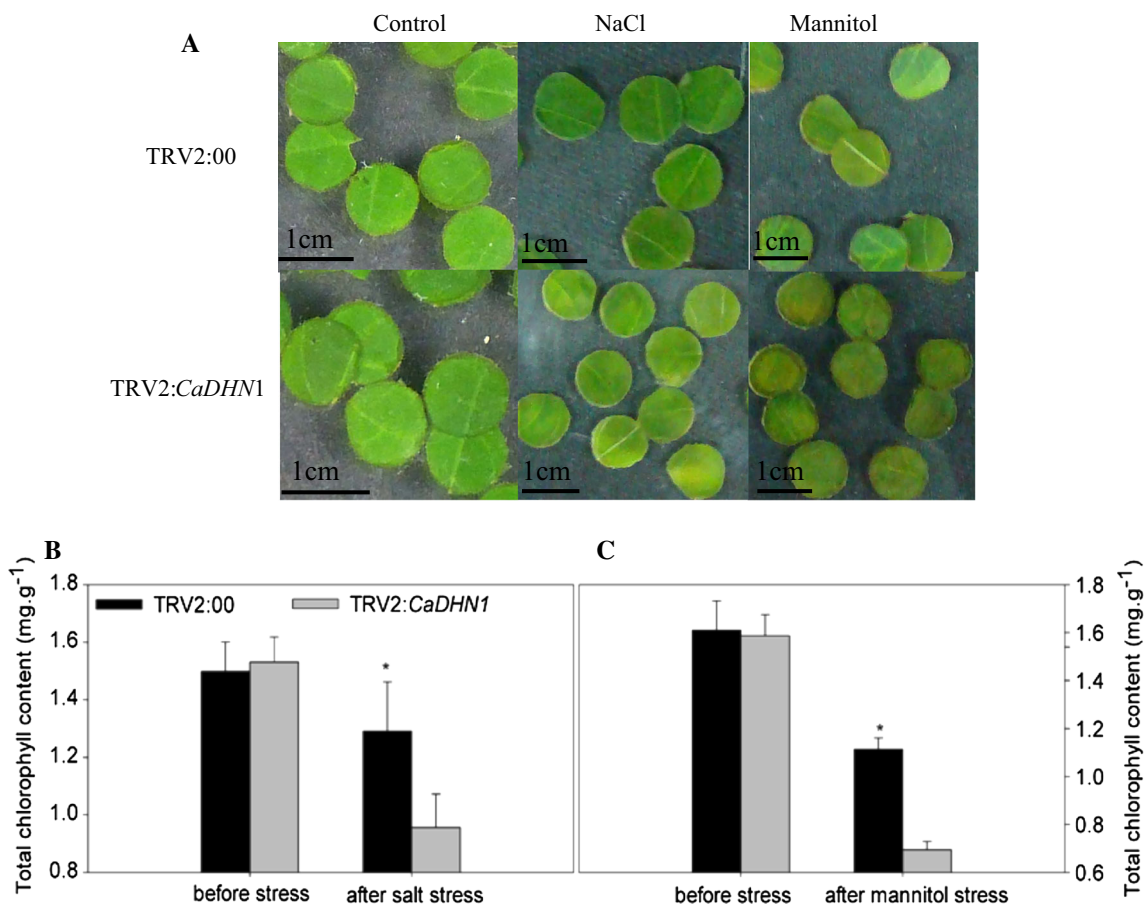


Fig. 6 Reduced tolerance of *CaDHN1*-silenced pepper plants to salt and mannitol stress. **a** Phenotypes; **b** chlorophyll content in leaf discs of 3 days after NaCl and **c** mannitol treatments in gene-silenced and

control plants. The results are the mean \pm standard error (SE), replicated thrice. Asterisk indicates significant differences compared with the control at a p value < 0.05

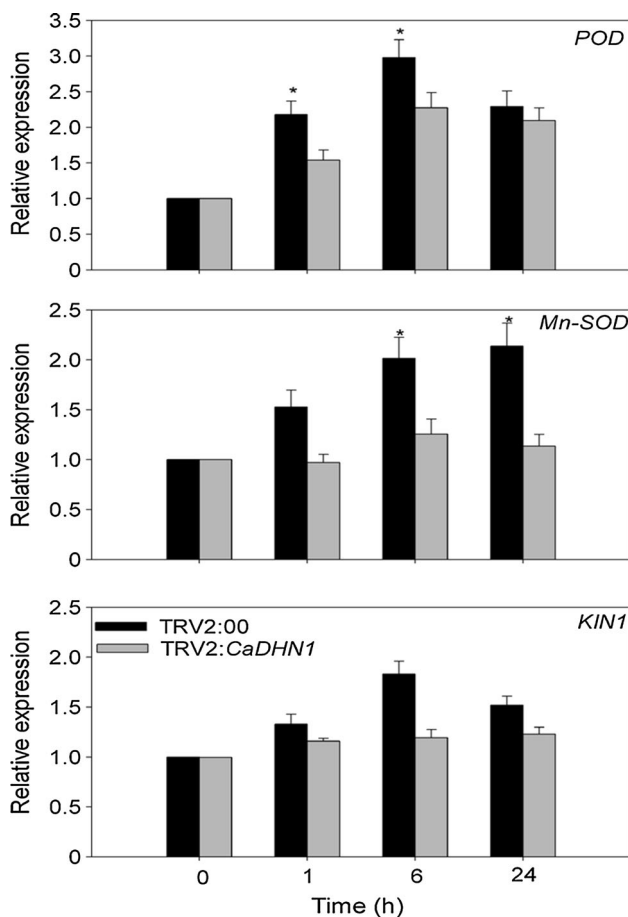


Fig. 7 RT-qPCR was used to assess the transcript levels of cold stress- and antioxidant system-relative genes (*KIN1*, *POD* and *Mn-SOD*) in control and *CaDHN1*-silenced plants subjected to cold stress. Results are the mean \pm standard error (SE), replicated thrice. Asterisk indicates significant differences when compared with the TRV2:00 leaves at a p value <0.05

et al. (2014) who found that the expression of *SiDhn2*, a DHN protein-encoding gene from *Saussurea involucreata*, was increased under low temperature stress conditions. After low temperature treatment for 6 h, expression of *SiDhn2* was enhanced 3-fold compared to controls. In another study, five *DHN/LEA/RAB* (dehydrin, late embryogenesis-abundant, responsive to ABA)-related genes (*COR47*, *DHNX*, *LTI30*, *LTI45* and *RAB18*) from *Arabidopsis thaliana* exhibited different expression levels in response to chilling stress, and *LTI30*, *LTI45* and *COR47* primarily responded to low temperature stress (Welin et al. 1994). However, expression of *RAB18* was strongly induced both in water-stressed and ABA-treated plants, but was only slightly responsive to cold stress. Together, these results suggest that different DHN genes exhibit different expression patterns under cold stress conditions.

Many DHNs are upregulated in response to salt and osmotic stress, and transgenic plants overexpressing exogenous DHNs are highly tolerant to this abiotic stress

(Brini et al. 2007; Santos and Mazzafera, 2012). In this study, expression of *CaDHN1* was strongly and rapidly upregulated by salt and osmotic stresses (Fig. 4b, c). The result is in accordance with previous studies on the DHNs *PpDHNA* and *PpDHNB* from *Physcomitrella patens*, which were strongly upregulated by NaCl and mannitol treatment (Ruibal et al. 2012). Choi and Close (2000) found that the barley dehydrin *DHN3* was induced by drought, ABA and salt treatment, whereas expression of *DHN12* was embryo-specific and not induced by dehydration, cold or salt stress, or ABA treatment. These results suggested a possible role for *CaDHN1* in response to salt and osmotic stress in pepper.

Some Dehydrin gene transcript could be induced by heavy metals. For example, the *Brassica juncea* dehydrin genes *BjDHN2* and *BjDHN3*, which were both upregulated by heavy metals, and transgenic tobacco plants overexpressing *BjDHN2/BjDHN3* exhibited enhanced tolerance of heavy metal stress (Xu et al. 2008). Similarly, Zhang et al. (2006) isolated the heavy metal-inducible SK2-type dehydrin gene *PvSR3*, and found that *PvSR3* was strongly induced by heavy metals. However, in this study, expression of *CaDHN1* was not significantly affected by heavy metal stress (Fig. 4d). These results suggest that different DHNs may have different functions, and pepper *CaDHN1* may not be involved in response to heavy metal stress.

The plant hormone ABA plays a key role in a broad array of developmental processes and adaptive stress responses to environmental stimuli (Cutler et al. 2010; Fujita et al. 2011). SA acts as an important endogenous signaling molecule, and is involved in establishing the local and systemic disease response to pathogen attack, as well as in the induction of defense-related genes in response to abiotic stress (Chini et al. 2004; Li et al. 2004). Numerous dehydration-responsive genes including DHNs have been identified, and many are induced by exogenous ABA and SA application (Yamaguchi-Shinozaki and Shinozaki 2006; Ochoa-Alfaro et al. 2012). However, in this study, *CaDHN1* expression was slightly lower than that of control plants following exogenous ABA treatment, but rapidly and strongly increased by SA treatment (Fig. 4e, f). This was consistent with a previous study in which *PvSR3*, encoding an acidic DHN from bean (*Phaseolus vulgaris* L), was not responsive to ABA, but strongly induced by SA (Zhang et al. 2006). These results indicated that *CaDHN1* may operate through ABA-independent but SA-dependent signaling pathways.

It is well documented that the primary site of stress injury in plants is the cell membrane, and electrolyte leakage is a typical symptom of stress-associated injury following membrane damage causing expansive lysis or phase transitions (Thomashow 1999; Xu et al. 2008). Additionally, dehydration has been suggested to cause decreased membrane fluidity and lipid peroxidation, which can be estimated from the MDA content. MDA and

electrical conductivity measurements are established indicators of the extent of cell membrane injury and electrolyte leakage, which are known to correlate well with the severity of the visual damage index (Li et al. 2011). Similarly, chlorophyll levels can be used to estimate the degree of leaf senescence. In this study, cold stress significantly increased MDA levels and electrical conductivity, and reduced the chlorophyll levels in *CaDHNI*-silenced plants. Addition, leaf discs were more yellow and chlorophyll levels were also significantly reduced in *CaDHNI*-silenced plants than those of control after 3 days of salt and osmotic treatment. This suggests loss of *CaDHNI* reduced the tolerance of pepper seedlings to cold, salt and osmotic treatment and accelerated leaf senescence, indicating a potential role for *CaDHNI* in resistance to these abiotic stresses.

Moreover, in plant cells, cold stresses induce the production of the reactive oxygen species (ROS). Cell membrane lipid peroxidation is mainly induced by ROS and/or free radicals. To protect cell membrane during oxidative stresses, plants possess very efficient enzymatic antioxidant defense systems, such as SOD, POD (Choudhury et al. 2013; Liu et al. 2015). Meanwhile, earlier studies found *KIN1*, a cold-induced gene, has similar functions to fish antifreeze proteins (AFPs) genes (Kurkela and Franck 1990). In this study, we found that knock-down of *CaDHNI* suppressed the expression of *Mn-SOD*, *POD* and *KIN1* genes transcripts (Fig. 7). These changes maybe lead to more ROS accumulation in the gene knock-down lines than the wild-type under stress conditions and this result indicated that *CaDHNI* may act as a positive regulator of cold stress-responsive gene expression, consistent with the results of the leaf chilling assays described above.

Experimental section

Plant materials and growth conditions

Pepper cv P70 was provided by the pepper breeding group in Northwest A&F University, China. Seeds were treated with warm water (55 °C) for 20 min to promote germination, then placed on moist gauze in an incubator at 28 °C, 60 % relative humidity in darkness and rinsed twice per day. When seeds were at least 80 % germinated, they were sown in pots containing compost. Pepper seedlings were grown in a growth chamber with temperature conditions at a 25/20 °C day/night temperature cycle, 75 % relative humidity, and a 16 h light and 8 h dark photoperiod cycle.

Bacteria Strains, Vectors and Restriction Enzymes

pTRV2 (Liu et al. 2002; Chung et al. 2004) and Vector pMD19-T (TaKaR, Dalian, China) were used to engineer

constructs, and *Agrobacterium tumefaciens* strain GV3101 was used for subsequent plant transformation. *Pfu* DNA polymerase (Promega Corporation, Madison, WI, USA) was used for PCR, and products were subsequently sequenced. *Xba* I and *Bam*H I restriction enzymes and T4 DNA ligase (TaKaR, Dalian, China) were used for vector construction.

Cloning of *CaDHNI* Gene and Sequence Analysis

The *DHN*-homologous EST R031 (GenBank accession no.: JZ198814) characterized from the differential screening of a cold-related pepper seedling cDNA library was previously reported (Guo et al. 2013). The full-length *CaDHNI* ORF was obtained using the rapid amplification of cDNA ends (RACE) method. First-strand cDNA synthesis was performed using the Smart RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). Gene-specific primer GSP1 (5'-ACTTCCTACAATATTCACGAC-3') was used for 3'-RACE and GSP2 (5'-GGAATGAATGTTTATTTGTTG-3') was used for 5'-RACE. Universal primers for 5' and 3' RACE were provided in the kit. The full-length cDNA sequence of *CaDHNI* was obtained by PCR amplification using forward (5'-GTCAAACTTTTAC TTTAGTGATCAT-3') and reverse (5'-GAAATCAAAA TCACTGCATTTTAC-3') primers. PCR products were cloned into the pMD19-T vector (TaKaR, Dalian, China) and sequenced (Shanghai GeneCore Biotechnologies Co. China). The sequence analysis was performed as described by Chen et al. (2014).

CaDHNI Gene Expression Patterns Analysis

Tissue-specific Expression of CaDHNI Gene

To evaluate the expression levels of *CaDHNI* in different tissues under normal conditions, roots, stems, leaves, flowers, fruits and seeds were collected from pepper cv P70 plants, frozen in liquid nitrogen and stored at –80 °C until needed for gene expression analysis.

Stress treatments

Pepper plants at the sixth leaf expansion stage were treated with abiotic stress and plant hormone. The abiotic stress (cold, salt, osmotic and heavy metal) and plant hormone (ABA and SA) treatments were performed as described previously (Chen et al. 2014). After 0, 1, 3, 6, 12 and 24 h treatment, pepper leaves were harvested for examination of *CaDHNI* expression pattern under various stress conditions. At each time point, three or four upper young leaves from four separate seedlings were collected to form one sample, wrapped with aluminum foil, immediately frozen in liquid nitrogen and stored at –80 °C.

Isolation of RNA and RT-qPCR analysis

RNA isolation, cDNA preparation and RT-qPCR were performed as described by Chen et al. (2014). The relative fold difference in mRNA levels was determined using the $2^{-\Delta\Delta CT}$ method. The *CaUbi3* gene (GenBank accession no. AY486137.1) encoding the ubiquitin-conjugating protein was amplified from pepper plants as a reference gene for normalization of *CaDHN1* cDNA samples (Wan et al. 2011). The cold stress- and antioxidant system-related genes-specific gene primers (*Mn-SOD*, *POD*, and *KINI*) are presented in Table 1.

Virus-induced gene silencing (VIGS) assay of *CaDHN1* in pepper

The pTRV2: *CaDHN1* construct was engineered to include a 338 bp fragment of *CaDHN1* cloned from a pepper cDNA template using gene-specific forward (5'-GGAA TGAATGTTTATTTGTTG-3') and reverse (5'-CGC GGATCCGAAATCAAATCACTGCATT-3') primers containing a *BamH* I restriction site (underlined). The resulting PCR product was cloned into vector pMD19T (Takara, Dalian, China), the resultant construct was digested with *Xba*I and *BamH* I, and the *CaDHN1* fragment was inserted into the *Xba*I–*BamH* I site of pTRV2 to form pTRV2:*CaDHN1*. *Agrobacterium tumefaciens* GV3101 containing either pTRV1 or pTRV2:*CaDHN1* were injected into pepper and plants were grown as described by Wang et al. (2013). 50 plants were used for the silencing assay.

MDA, electrolyte leakage and chlorophyll level assay

The amount of lipid peroxidation in the chloroplast membranes was estimated by measuring MDA produced by the

thiobarbituric acid reaction as described previously by Dhindsa et al. (1981). To assess membrane permeability, electrolyte leakage was measured according to the method described by Dionisio-Sese and Tobita (1998). Total chlorophyll was extracted from the treated samples and calculated by a spectrophotometric method as described by Arkus et al. (2005).

Statistical Analysis

RT-qPCR data analysis was carried out using Sigma Plot 10. The relative expression levels of DHN under various abiotic stress and hormonal treatments are mean \pm SE of three biological replicate samples. Each replicate sample was a composite of leaves from three individual seedlings. For each treatment, the expression level at time point 0 was defined as 1.0. Statistical analysis were performed using the Statistical Analysis System software (SAS 8.2, North Carolina State University, Cary, NC, USA), and the means were compared using Duncan's multiple range test, taking $p < 0.05$ as a significant difference. The values of MDA, electrolyte leakage and total chlorophyll are mean \pm SE of three biological replicate samples. Each replicate sample was a composite of leaves from nine seedlings.

Conclusion

In summary, we isolated the pepper DHN gene *CaDHN1* from the leaves of pepper cv P70. *CaDHN1* transcription differed in different tissues, and *CaDHN1* expression was strongly induced by high salt, osmotic and cold stress. Moreover, *CaDHN1* expression was significantly upregulated by SA treatment but not by ABA. Virus-induced gene silencing (VIGS) revealed that mutant plants lacking *CaDHN1* were more susceptible to low temperature, high salt and mannitol treatment. Overall, these results suggest that *CaDHN1* plays an important role in resistance to abiotic stresses in pepper plants. Genetic transformation studies are needed to further explore the function of *CaDHN1*.

Author contribution statement RGC, HJ, WLG, and ZHG conceived and designed the experiments; RGC, WLG, HJ and FM performed the experiments; SBW, BGP and WPD analyzed the data; ZHG contributed reagents/materials/analysis tools; RGC wrote the paper. All authors read and approved the final manuscript.

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Table 1 Primer sequences used for RT-qPCR analysis

Gene	Primer	Sequence (5'–3')
<i>CaUbi3</i>	Q <i>CaUbi3</i> -F	TGTCATCTGCTCTCTGTTG
	Q <i>CaUbi3</i> -R	CACCCAAGCACAATAAGAC
<i>CaDHN1</i>	Q <i>CaDHN1</i> -F	AGTGATCATTCTTTGCTTTATTC
	Q <i>CaDHN1</i> -R	TTAACTTTCTCACAAACTCAGA
<i>Mn-SOD</i>	Q <i>Mn-SOD</i> -F	CTCTGCCATAGACACCAACTT
	Q <i>Mn-SOD</i> -R	CCAAGTTCGGTCCTTTAATAA
<i>POD</i>	Q <i>POD</i> -F	GCAGCATTCTCCTCCTACT
	Q <i>POD</i> -R	ATTTCTTTGCCTTGTGTTG
<i>KINI</i>	Q <i>KINI</i> -F	AAATGTCAGAGACCAACAAGAA
	Q <i>KINI</i> -R	CTACTTGTTTCAGGCCGGTCTT

F forward primer, R reverse primer

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Allagulova CR, Gimalov FR, Shakirova FM, Vakhitov VA (2003) The plant dehydrins: structure and putative functions. *Biochemistry* 68(9):945–951
- Amara I, Odena A, Oliveira E, Moreno A, Masmoudi K, Pages M, Goday A (2012) Insights into maize LEA proteins: from proteomics to functional approaches. *Plant Cell Physiol* 53:312–329
- Arkus KAJ, Cahoon EB, Jez JM (2005) Mechanistic analysis of wheat chlorophyllase. *Arch Biochem Biophys* 438:146–155
- Artlip TS, Callahan AM, Bassett CL, Wisniewski ME (1997) Seasonal expression of a dehydrin gene in sibling deciduous and evergreen genotypes of peach (*Prunus persica*). *Plant Mol Biol* 33(1):61–70
- Borovskii GB, Stupnikova IV, Antipina AI, Vladimirova SV, Voinikov VK (2002) Accumulation of dehydrin-like proteins in the mitochondria of cereals in response to cold, freezing, drought and ABA treatment. *BMC Plant Biol*. doi:10.1186/1471-2229-2-5
- Brini F, Hanin M, Lumbreras V, Amara I, Khoudi H, Hassairi A, Pages M, Masmoudi K (2007) Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*. *Plant Cell Rep* 26:2017–2026
- Campbell SA, Close TJ (1997) Dehydrins: genes, proteins, and associations with phenotypic traits. *New Phytol* 137:61–74
- Chen RG, Guo WL, Yin YX, Gong ZH (2014) A novel F-Box protein CaF-Box is involved in responses to plant hormones and abiotic stress in pepper (*Capsicum annuum* L.). *Int J Mol Sci* 15:2413–2430
- Chini A, Grant JJ, Seki M, Shinozaki K, Loake GJ (2004) Drought tolerance established by enhanced expression of the CC-NBS-LRR gene, *ADRL1*, requires salicylic acid, EDS1 and ABI1. *Plant J* 38:810–822
- Choi DW, Close TJ (2000) A newly identified barley gene, *Dhn12*, encoding an YSK2 DHN, is located on chromosome 6H and has embryo-specific expression. *Theor Appl Genet* 100:1274–1278
- Choudhury S, Panda P, Sahoo L, Panda SK (2013) Reactive oxygen species signaling in plants under abiotic stress. *Plant Signal Behav* 8(4):e23681. doi:10.4161/psb.23681
- Chung E, Kim SY, Yi SY, Choi D (2003) *Capsicum annuum* dehydrin, an osmotic-stress gene in hot pepper plants. *Mol Cells* 15(3):327–332
- Chung E, Seong E, Kim YC, Chung EJ, Oh SK, Lee S, Park JM, Joung YH, Choi D (2004) A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L.cv.Bukang). *Mol Cells* 17:377–380
- Close TJ (1997) Dehydrins: a commonality in the response of plants to dehydration and low temperature. *Physiol Plant* 100:291–296
- Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. *Annu Rev Plant Biol* 61:651–679
- Dhanaraj AL, Slovin JP, Rowland LJ (2005) Isolation of a cDNA clone and characterization of expression of the highly abundant, cold acclimation-associated 14 kDa dehydrin of blueberry. *Plant Sci* 168:949–957
- Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J Exp Bot* 32:93–101
- Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. *Plant Sci* 135:1–9
- Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K (2011) ABA-mediated transcriptional regulation in response to osmotic stress in plants. *J Plant Res* 124:509–525
- Guo WL, Chen RG, Gong ZH, Yin YX, Li DW (2013) Suppression subtractive hybridization analysis of genes regulated by application of exogenous abscisic acid in pepper plant (*Capsicum annuum* L.) leaves under chilling stress. *PLoS ONE* 8(6):e66667. doi:10.1371/journal.pone.0066667
- Hara M, Terashima S, Fukaya T, Kuboi T (2003) Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco. *Planta* 217:290–298
- Hara M, Fujinaga M, Kuboi T (2004) Radical scavenging activity and oxidative modification of citrus dehydrin. *Plant Physiol Biochem* 42:657–662
- Hara M, Fujinaga M, Kuboi T (2005) Metal binding by citrus dehydrin with histidine-rich domains. *J Exp Bot* 56:2695–2703
- Hirayama T, Shinozaki K (2010) Research on plant abiotic stress responses in the post-genome era: past, present and future. *Plant J* 61:1041–1052
- Koag MC, Fenton RD, Wilkens S, Close TJ (2003) The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity. *Plant Physiol* 131:309–316
- Kosova K, Vitamvas P, Prasil IT (2007) The role of dehydrins in plant response to cold. *Biol Plantarum* 51:601–617
- Kovacs D, Agoston B, Tompa P (2008) Disordered plant LEA proteins as molecular chaperones. *Plant Signal Behav* 3:710–713
- Kurkela S, Franck M (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol Biol* 15:137–144
- Li ZG, Zhao LX, Kai GY, Yu SW, Cao YF, Pang YZ, Sun XF, Tang KX (2004) Cloning and expression analysis of a water stress-induced gene from *Brassica oleracea*. *Plant Physiol Biochem* 42:789–794
- Li Q, Yu B, Gao Y, Dai AH (2011) Cinnamic acid pretreatment mitigates chilling stress of cucumber leaves through altering antioxidant enzyme activity. *Plant Physiol* 168:927–934
- Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. *Plant J* 31:777–786
- Liu H, Yu CY, Li HX, Ouyang B, Wang TT, Zhang JH, Wang X, Ye ZB (2015) Overexpression of *ShDHN*, a dehydrin gene from *Solanum habrochaites* enhances tolerance to multiple abiotic stresses in tomato. *Plant Sci* 231:198–211
- Nguyen HT, Leipner J, Stamp P, Guerra-Peraza O (2009) Low temperature stress in maize (*Zea mays* L.) induces genes involved in photosynthesis and signal transduction as studied by suppression subtractive hybridization. *Plant Physiol Biochem* 47:116–122
- Ochoa-Alfaro AE, Rodriguez-Kessler M, Perez-Morales M, Delgado-Sanchez BP, Cuevas-Velazquez CL, Gomez-Anduro G, Jimenez-Bremont JF (2012) Functional characterization of an acidic SK3 dehydrin isolated from an *Opuntia streptacantha* cDNA library. *Planta* 235:565–578
- Qiu HL, Zhang LH, Liu C, Li H, Wang AY, Liu HH, Zhu JB (2014) Cloning and characterization of a novel dehydrin gene, *SiDhn2*, from *Saussurea involucreata* Kar. et Kir. *Plant Mol Biol* 84:707–718
- Rorat T (2006) Plant dehydrins-tissue location, structure and function. *Cell Mol Biol Lett* 11:536–556

- Rorat T, Szabala BM, Grygorowicz WJ, Wojtowicz B, Yin Z, Rey P (2006) Expression of SK3-type dehydrin in transporting organs is associated with cold acclimation in *Solanum* species. *Planta* 224:205–221
- Ruibal C, Salamó IP, Carballo V, Castro A, Bentancor M, Borsani O, Szabados L, Vidal S (2012) Differential contribution of individual dehydrin genes from *Physcomitrella patens* to salt and osmotic stress tolerance. *Plant Sci* 190:89–102
- Santos AB, Mazzafera P (2012) Dehydrins are highly expressed in water-stressed plants of two coffee species. *Tropical Plant Biol* 5:218–232
- Sun X, Lin HH (2010) Role of plant dehydrins in antioxidation mechanisms. *Biologia* 65:755–759
- Svenson J, Ismail AM, Palva ET, Close TJ (2002) Dehydrins. In: Storey KB, Storey JM (eds) Sensing, signalling and cell adaptation. Elsevier Science, BV, pp 155–171
- Szabala BM, Fudali S, Rorat T (2014) Accumulation of acidic SK3 dehydrins in phloem cells of cold- and drought-stressed plants of the Solanaceae. *Planta* 239:847–863
- Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* 50:571–599
- Wan HJ, Yuan W, Ruan M, Ye Q, Wang R, Li Z, Zhou G, Yao Z, Zhao J, Liu S, Yang Y (2011) Identification of reference genes for reverse transcription quantitative real-time PCR normalization in pepper (*Capsicum annuum* L.). *Biochem Biophys Res Co* 416:24–30
- Wang JE, Liu KK, Li DW, Zhang YL, Zhao Q, He YM, Gong ZH (2013) A novel peroxidase *CanPOD* gene of pepper is involved in defense responses to *Phytophthora capsici* infection as well as abiotic stress tolerance. *Int J Mol Sci* 14:3158–3177
- Welin BV, Olson A, Nylander M, Palva ET (1994) Characterization and differential expression of *dhn/lea/rab*-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*. *Plant Mol Biol* 26:131–144
- Wisniewski ME, Bassett CL, Renaut J, Farrell R, Tworzoski T, Artlip TS (2006) Differential regulation of two dehydrin genes from peach (*Prunus persica*) by photoperiod, low temperature and water deficit. *Tree Physiol* 26:575–584
- Xu J, Zhang YX, Wei W, Han L, Guan ZQ, Wang Z, Chai TY (2008) *BjDHNs* confer heavy-metal tolerance in plants. *Mol Biotechnol* 38:91–98
- Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance, to dehydration and cold stresses. *Annu Rev Plant Biol* 57:781–803
- Zhang YX, Li JM, Yu F, Cong L, Wang LY, Burkard G, Chai TY (2006) Cloning and expression analysis of SKn-type dehydrin gene from bean in response to heavy metals. *Mol Biotechnol* 32:205–218