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Full Length Research Paper

Cloning and expression of a small heat shock protein gene *CaHSP24* from pepper under abiotic stress

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The sequence of a small heat shock protein (sHSP) gene *CaHSP24* was obtained by homology-based candidate gene method and rapid amplification of cDNA ends (RACE). The cDNA sequence of this gene is 920 bp in size (GenBank: HM132040) and contains an open reading frame (ORF) of 636 bp, which was predicted to encode a protein with 211 amino acid residues. The phylogenetic tree showed that *CaHSP24* was quite similar to mitochondrial sHSPs from other plants but was distantly related to sHSPs of pepper cytoplasm and chloroplast. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) showed that *CaHSP24* was hardly detectable at 32°C, but accumulated markedly at 40°C. The gene was expressed 0.5 h after exposure to heat stress and the expression reached a peak in 1.5 h; the expression level in heat-resistant cultivar B35 was higher than that in heat-sensitive cultivar B6. The gene was also expressed weakly under salinity, heavy metal, low temperature and oxidative stresses; the expression levels under these conditions were remarkably lower than those under heat stress. Cell viability experiments showed that the heterologous expression of *CaHSP24* could enhance the viability of *Escherichia coli* under heat stress. To sum up, *CaHSP24* may play an important role for response to heat stress condition.

Key words: Pepper, *CaHSP24*, heat stress, gene expression.

INTRODUCTION

Heat stress because of global warming is a serious challenge for crop production. *Capsicum annuum* L., which originates from the tropical zone of Latin America, is a typical warm-season crop, but it is very sensitive to high temperature. The appropriate growth temperature of pepper is 20 to 30°C; the plant's growth stops when the temperature increases above 40°C and it is adversely affected if this high temperature persists. Small heat shock proteins are a kind of main synthetically HSPs in plants under heat stress and they play an important role in heat resistance in plants (Sun et al., 2002).sHSPs are smaller than 30 kD in size. Plant sHSPs have been divided into 5 classes on the basis of sequence similarity and cellular localization (Vierling et al., 1991; Waters et al., 1996; Sun and MacRae, 2005). One class of proteins localizes to the chloroplast, one to the endoplasmic reticulum, one to the mitochondria, and two to the cytosol (cytosolic I and II) (Waters et al., 1996; Waters, 1995; Lenne et al., 1995). The sHSPs function as molecular chaperones and can bind partially denatured proteins, thereby preventing irreversible protein aggregation during stress conditions. They play a crucial role in protecting plants against abiotic stresses (Sun et al., 2002; Wang et al., 2004) and prevent thermal denaturation of glucose oxidase and citrate synthase, which are the 2 critical enzymes in oxidative phosphorylation (Jakob et al., 1993). Liu and Shono (1999) cloned the sHSP gene *LeHSP23.8*, located in the mitochondrion, from tomato. *LeHSP23.8* mRNA was hardly detectable at about 36°C, but marked

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Abbreviations: ddH_2O, Double distilled water; **sHSPs,** small heat shock proteins; **IPTG,** isopropylthio-β-D-galactoside; **kD,** kilodalton; **ORF,** open reading frame; **RACE,** rapid amplification of cDNA ends; **RT-PCR,** reverse transcription polymerase chain reaction; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis.

#The authors contributed equally to this work.

accumulation was noted at 40℃ and recombinant *LeHSP23.8* could protect citrate synthase from thermal inactivation and enhance the renaturation of chemically denatured citrate synthase. *LeHSP23.8* also enhances heat tolerance of transgenic tobacco plants and can protect the mitochondria (Liu and Shono, 1999, 2001). Another mitochondrial sHSP gene *ZmHSP22* from *Zea mays* could enhance the heat stress tolerance of transgenic *Arabidopsis thaliana* (Rhoads et al., 2005). Guo et al. (2005, 2006) cloned a cytoplasmic (class I) sHSP *CaHSP1*8 and a chloroplast sHSP *CaHSP26* from sweet pepper and found that the heterologous expression of these 2 sHSPs could enhance the viability of *Escherichia coli* under heat and cold stress. sHSP is also associated with cold tolerance in plants. Both LeHSP23.8 and CaHSP18 could enhance cold tolerance in transgenic tomato plants (Wang et al., 2008; Guo et al., 2008). In the absence of environmental stresses, the synthesis of sHSPs in plants is restricted to certain stages of development, such as embryogenesis, germination, pollen development and fruit maturation; this restriction indicate the special role of sHSPs in the development of plants (Sun et al, 2002, 2005).

C. annuum L. is very sensitive to high temperature, but its responses to high temperature, especially with regard to sHSPs, have seldom been studied. In this study, we cloned a sHSP gene *CaHSP24* from pepper and analyzed its expression patterns under heat stress and other abiotic stresses like salinity, heavy metal, cold and oxidative stresses. We also constructed a prokaryotic expression vector for *CaHSP24* to study its effect on the viability of *E. coli* under heat stress and to determine the relationship between CaHSP24 and heat resistance in pepper.

MATERIALS AND METHODS

cDNA cloning

Cultivars B35 and B6 are 2 typical pepper cultivars with contrasting characteristics of heat-resistance (Jia et al., 2010). A pre-experiment showed that at 40℃, the germination percentage and pollen viability of B35 were significantly higher than those of B6 and the chlorophyll content decreased much more in B6 than in B35. Moreover, B35 had higher proline content and lower malonaldehyde content at 40℃ than B6. This finding confirmed that B35 is heat-resistant, while B6 is heat-sensitive.

Pepper plants (heat-resistant cultivar B35) were grown in a green house until 6 to 8 true leaves stage. Total RNA was extracted from freshly harvested pepper leaves treated at 40℃ for 2 h and cDNA was obtained using the M-MLV reverse transcriptase kit (Promega, Madison, America). The sequence of *CaHSP24* was partly obtained by homology-based candidate gene method from the published expressed sequence tags of pepper, and the *LeHSP23.8* (AB017134) sequence was used as an information probe. The sequence of the 3′ region of *CaHSP24* was determined by 3′ RACE.

The primers *CaHSP24*-F(5′-GATTGGTAGCAGAAATGGCAAC-3′) and *CaHSP24*-R(5′-CTTCTCAGTAATTTAACTAAACAGACTC-3′) were designed on the basis of these sequences. Subsequently, we obtained the entire cDNA sequence and then aligned it with the amino acid sequences of other sHSP from GenBank.

Semi-quantitative RT-PCR

The expression of *CaHSP24* was analyzed by semi-quantitative RT-PCR. Different temperature points (36, 40 and 44℃) were used to determine the optimal stress-inducing temperature and the heat-resistant cultivar B35 and heat-sensitive cultivar B6 were treated at this optimal temperature for different time periods (0.5, 1, 1.5, 2, 2.5, 3, 4, and 5 h) to determine the peak expression time of *CaHSP24*. In order to examine the expression of *CaHSP24* under other abiotic stresses, B35 was subjected to salinity stress (100 mmol/l NaCl, 200 mmol/l NaCl and 300 mmol/l NaCl), heavy metal stress (0.1 mmol/l $CuSO_4$ and 1 mmol/l $CuSO_4$), oxidative stress (1 mmol/l H_2O_2), and cold stress (4℃) for 2 h.

The following primers were used for semi-quantitative RT-PCR:
CaHSP24-F(5′-GATTGGTAGCAGAAATGGCAAC-3′);
CaHSP24-R(5′-CTTCTCAGTAATTTAACTAAACAGACTC-3′);
EFI-α-F(5′-CCACCAATCTTGTAACATCC-3′);
EFI-α-R(5′-AGACCACTAAGTACTACTGCAC-3′).

About 25 µl of reaction mixture (cDNA template, 1 µl; 10× PCR buffer, 2.5 µl; 5 U/µl Taq DNA polymerase, 0.2 µl; 10 mmol/l dNTP Mixture, 0.5 µl; ddH2O, 16.8 µl; and CaHSP24-F, CaHSP24-R, EFI-α-F, and EFI-α-R, 1 µl each) was used for PCR. Amplification consisted of 30 cycles of 45 s at 94℃ (denaturation), 60 s at 51℃ (primer annealing) and 40 s at 72℃ (extension) (Giorno et al., 2010).The optical density of the electrophoresis bands of CaHSP24 and EFI-α was detected by the gel-imaging analysis system (Syngene, Cambridge, Britain). The relative expression level of *CaHSP24* was represented as the ratio of the optical density of *CaHSP24* to that of *EFI*-α. Each treatment was done 3 times. The data was analyzed using the SAS statistical software.

Prokaryotic expression of *CaHSP24*

CaHSP24-PMD19-T and prokaryotic expression vector PET28a were double-digested with the restriction enzymes *BamHI* and *Sal*I and *CaHSP24* was then subcloned into PET28a. The recombinant plasmid was then transformed into *E. coli* (BL21) cells. Protein expression was induced by 1 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG) at 37℃ for 0, 0.5, 1, 2, 3, 4 and 5 h. The expression of the recombinant protein was analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie brilliant blue.

Cell viability experiments

For heat shock experiments, the cell cultures were grown at 37℃ until they reached an absorbance at 600 nm (A_{600}) of 1.0; they were then diluted once with fresh Luria-Bertani medium supplemented with kanamycin at 100 mg/ml and IPTG at 1 mmol/l. Two hours after induction, the cultures were diluted to 6×10^6 cells/ml and 1 ml samples were shifted to 50℃. Aliquots (100 µl) were taken 0, 0.5, 1 and 2 h later and serial dilutions were plated in triplicate onto Luria-Bertani plus kanamycin plates. After the plates were incubated overnight at 37℃, cell viability was estimated by counting the number of colony-forming units (Soto et al., 1999). For heat shock treatments, the means of the 3 experiments were determined from 2 independent transformants. The data were analyzed using the SAS statistical software.

RESULTS

Isolation and characterization of a sHSP-encoding gene from pepper

The gene that we determined on the basis of the sequence

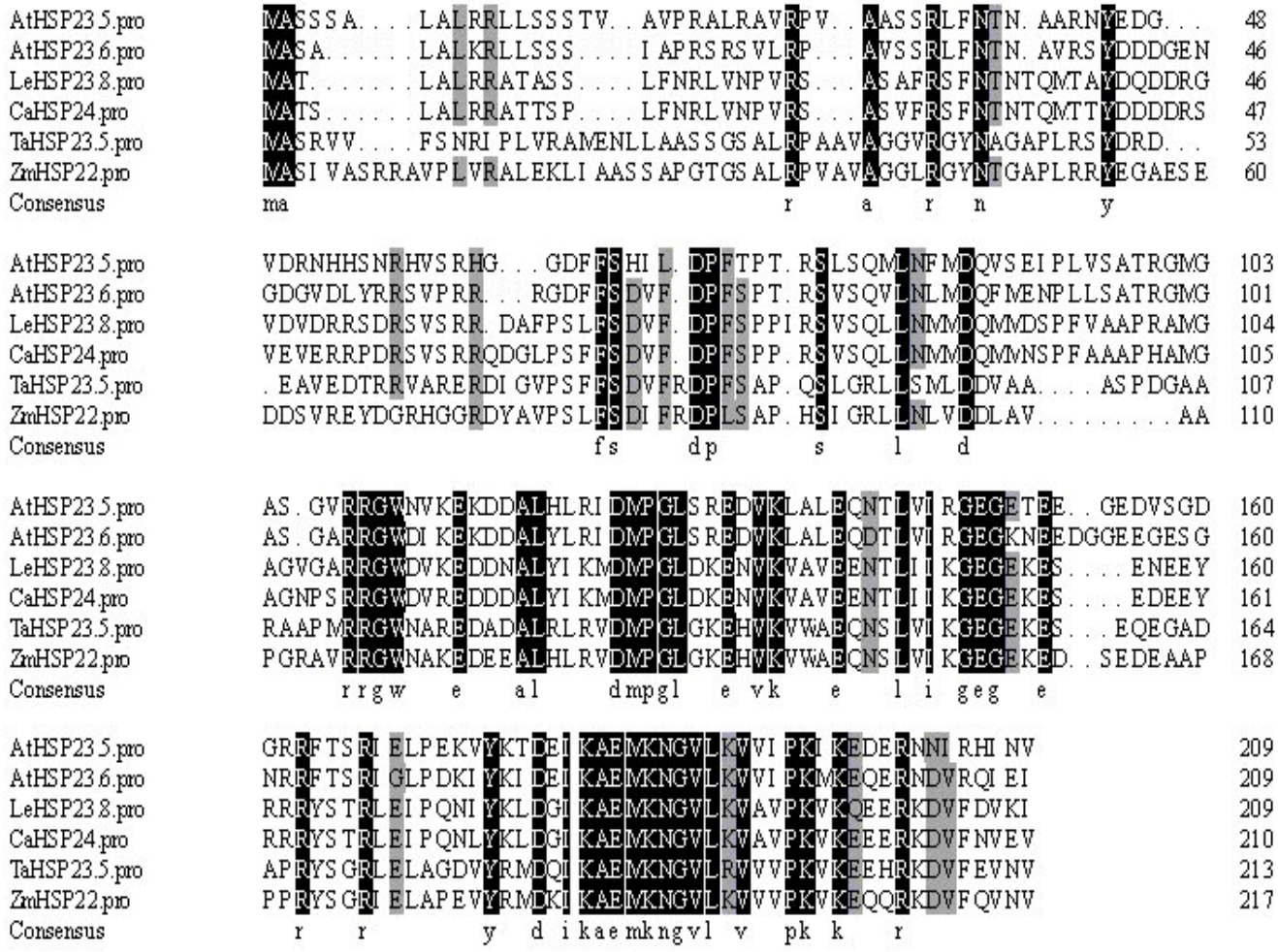

Figure 1. Alignment of amino acid sequences of CaHSP24 and mitochondrial sHSPs from other plants. The sources of the sHSPs and their accession numbers in GenBank are as follows: *A. thaliana*, AtHSP23.6 (EU289286); *C. annuum* L., CaHSP24 (HM132040); *S. lycopersicum,* LeHSP23.8 (AB017134); *A. thaliana,* AtHSP23.5 (Q9FGM9); *Z. mays,* ZmHSP22 (AY758275); *T. aestivum,* TaHSP23.5 (AF104107).

sequence of the tomato mitochondrial sHSP gene *LeHSP23.8* has been designated as *CaHSP24* (GenBank accession number, HM132040). The gene consists of a single ORF of 636 bp flanked by 5′- and 3′-noncoding sequences of 17 and 267 nucleotides, respectively and it encodes a protein consisting of 211 amino acids with a predicted molecular mass of 24 kD and isoelectric point (pI) of 5.22. Alignment of the deduced amino acid sequence revealed strong homologies with those of other known eukaryotic mitochondrial sHSPs from the GenBank database (Figure 1). Phylogenic analysis of amino acid sequences of sHSPs from *C. annuum* L. and some other species showed that CaHSP24 was quite similar to mitochondrial sHSPs from other plants, such as LeHSP23.8 from *Solanum lycopersicum*, AtHSP23.6 from *A. thaliana*, TaHSP23.5 from *Triticum aestivum* and ZmHSP22 from *Z. mays*, but was distantly related to sHSPs of the cytoplasm (CaHSP18) and chloroplast (CaHSP26) from *C. annuum* L. (Figure 2).

The expression of *CaHSP24* under heat stress and other abiotic stresses

The results of semi-quantitative RT-PCR showed that *CaHSP24* was expressed at different stress temperatures in cultivars B35 and B6; the expression in B35 was higher than that in B6 and the optimal stress-inducing temperature was 40℃ (Figure 3). *CaHSP24* in both B35 and B6 was expressed 0.5 h after heat stress treatment (40℃) and its level dropped remarkably 2 h later. The expression peaks in B35 and B6 were noted 1.5 and 1 h, respectively, after stress treatment. On the whole, the expression level of *CaHSP24* in B35 was significantly higher than that in B6 at different stress times (Figure 4).

Other than heat stress, sHSPs can also be induced by other stresses, such as drought, salinity, pathogens, oxidative stress, chilling and wounding stress (Sun et al., 2002; Waters et al., 1996; Sun and MacRae, 2005; Wang et al., 2004). In our study, B35 was exposed to salinity

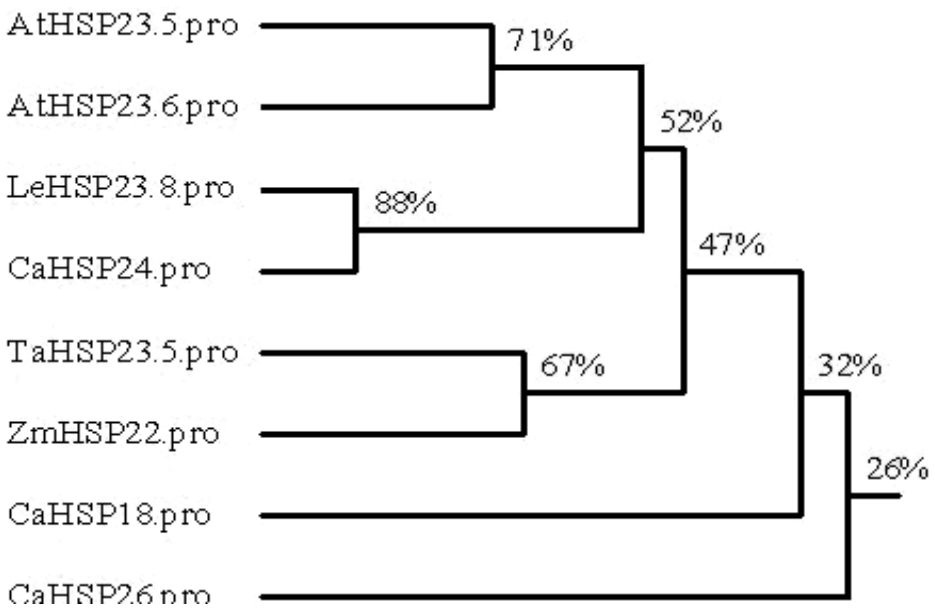

Figure 2. Phylogenic analysis of amino acid sequences of sHSPs from *C. annuum* L. and some other species. The sources of the sHSPs and their accession numbers in GenBank are as follows: *A. thaliana,* ATHSP23.6 (EU289286); *A. thaliana,* ATHSP23.5 (Q9FGM9); *C. annuum* L., CaHSP24 (HM132040); *S. lycopersicum,* LeHSP23.8 (AB017134); *Z. mays*, ZmHSP22 (AY758275); *T. aestivum,* TaHSP23.5 (AF104107); *C. annuum* L., CaHSP18 (AY284925); *C. annuum* L., CaHSP26 (AY224603).

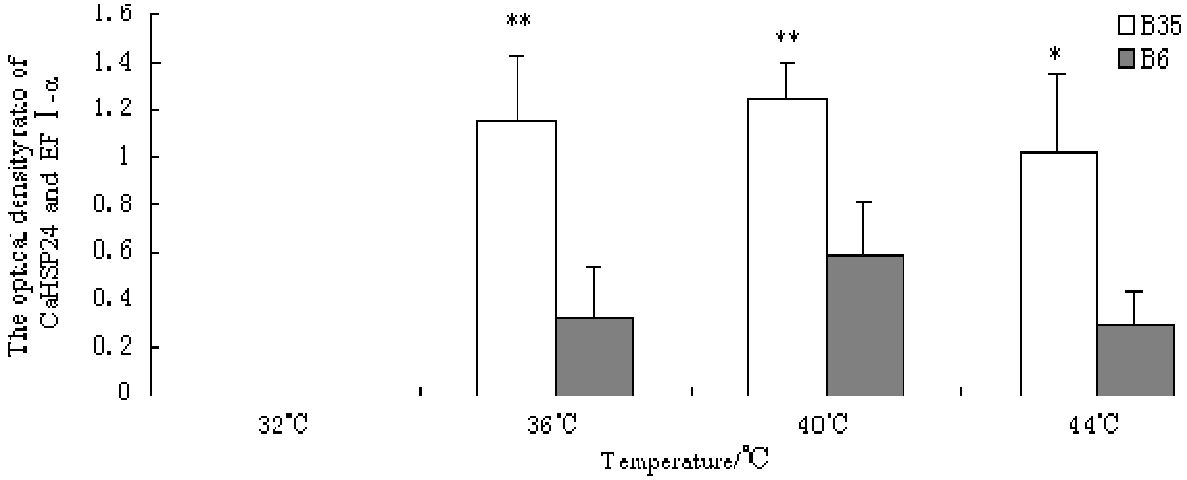

Figure 3. The expression levels of *CaHSP24* at different stress temperatures. The significant levels of difference between expressions in cultivar B35 and cultivar B6 are shown by asterisks. *, $P < 0.05$ and **, $P < 0.01$.

stress (100 mmol/l NaCl, 200 mmol/l NaCl and 300 mmol/l NaCl), heavy metal stress (0.1 mmol/l $CuSO_4$ and 1 mmol/l $CuSO_4$), oxidative stress (1 mmol/l H_2O_2) and cold stress (4℃ for 2 h). The result of semi-quantitative RT-PCR revealed that *CaHSP24* could be weakly expressed under all the earlier mentioned abiotic stress treatments (except 300 mmol/l NaCl) and the expression levels of *CaHSP24* when under these stresses were markedly lower than those under heat stress (Figure 5).

Heterologous expression in *E. coli* cells

To analyze the functions of *CaHSP24 in vivo* under stress conditions, the complete coding sequence for *CaHSP24* was introduced into *E. coli* cells by using the PET28a expression vector. The empty vector was introduced into *E. coli* cells, which were then used as controls. Under normal culture conditions, similar growth rates were observed for both types of recombinant cells (PET28a-HSP

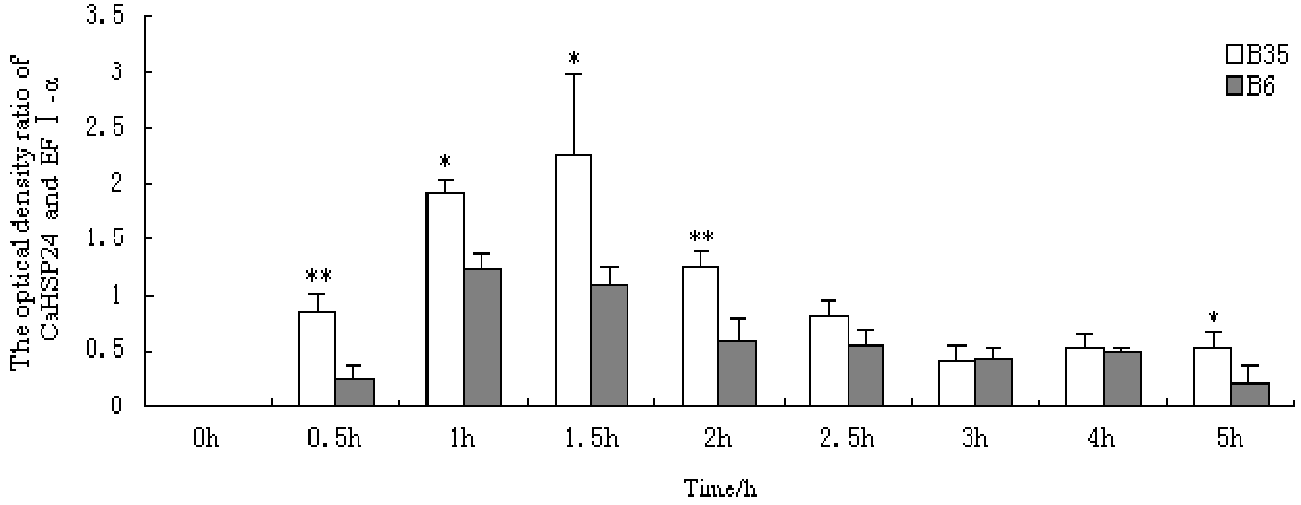

Figure 4. The expression levels of *CaHSP24* at different stress times. The significant levels of difference between expressions in cultivar B35 and cultivar B6 are shown by asterisks. *, P < 0.05 and **, P < 0.01.

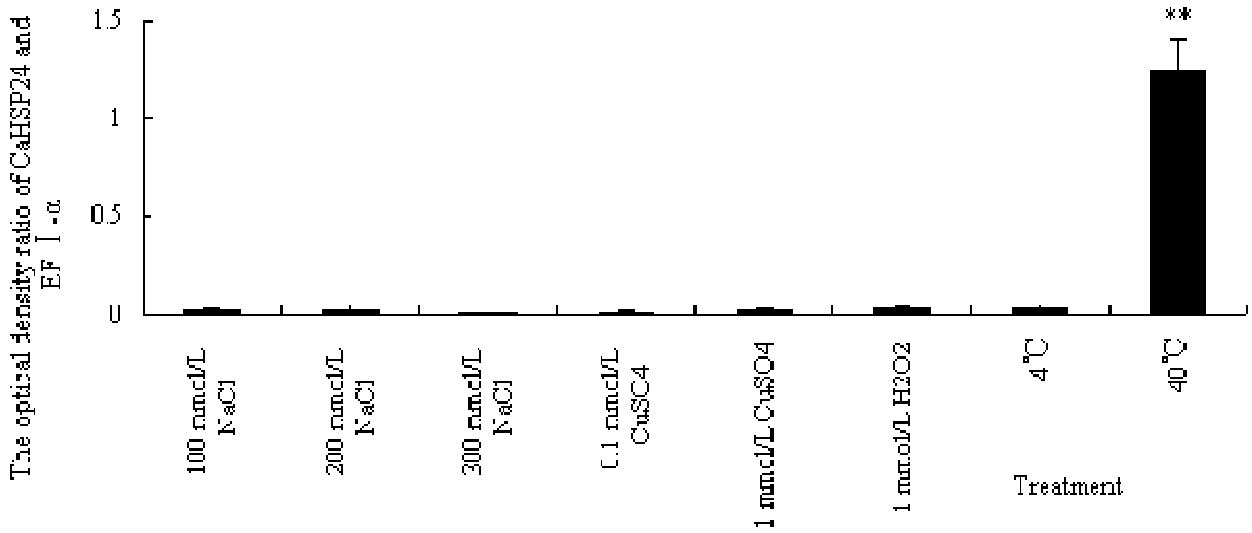

Figure 5. The expression levels of *CaHSP24* in cultivar B35 under different abiotic stress treatments. The significant level of difference between treatments is shown by asterisks. **, P < 0.01.

and PET28a) and for untransformed wild-type cells (Figure 6). SDS-PAGE showed that recombinant CaHSP24 (27.5 kD) was expressed in PET28a-HSP cells 0.5 h after IPTG-mediated induction, and had the same apparent size as CaHSP24; maximal expression was noted 2 to 5 h after induction (Figure 7).

The viability of PET28a-HSP and PET28a declined quickly upon exposure to heat stress, and the measured survival rates were significantly higher in cells over-expressing *CaHSP24*. Two hours after exposure to heat stress, the survival rate of the PET28a-*CaHSP24* cells was 39%, while that of the PET28a cells was only 12% (Figure 8).

DISCUSSION

In this study, we cloned the sHSP gene *CaHSP24* from pepper and obtained its sequence by homology-based candidate gene method and 3′ RACE; the sequence was obtained on the basis of the published sequence of the sHSP gene *LeHSP23.8* from tomato.

The amino acid sequence of CaHSP24 is quite similar to that of mitochondrial sHSPs from other plants and the similarity between CaHSP24 and LeHSP23.8 is as high as 88.07%. However, the sequence was distantly related to the published sequence of sHSP CaHSP18 (cytoplasmic sHSP) and CaHSP26 (chloroplast sHSP) from *C. annuum*

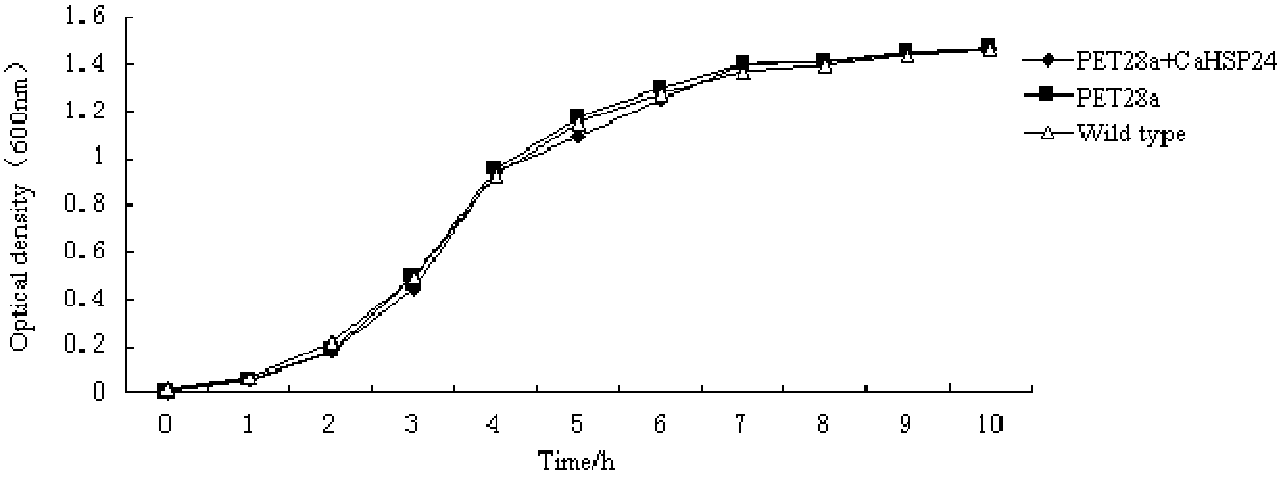

Figure 6. The growth curve of wild type, empty vector-containing and transgenic *E. coli* strains.

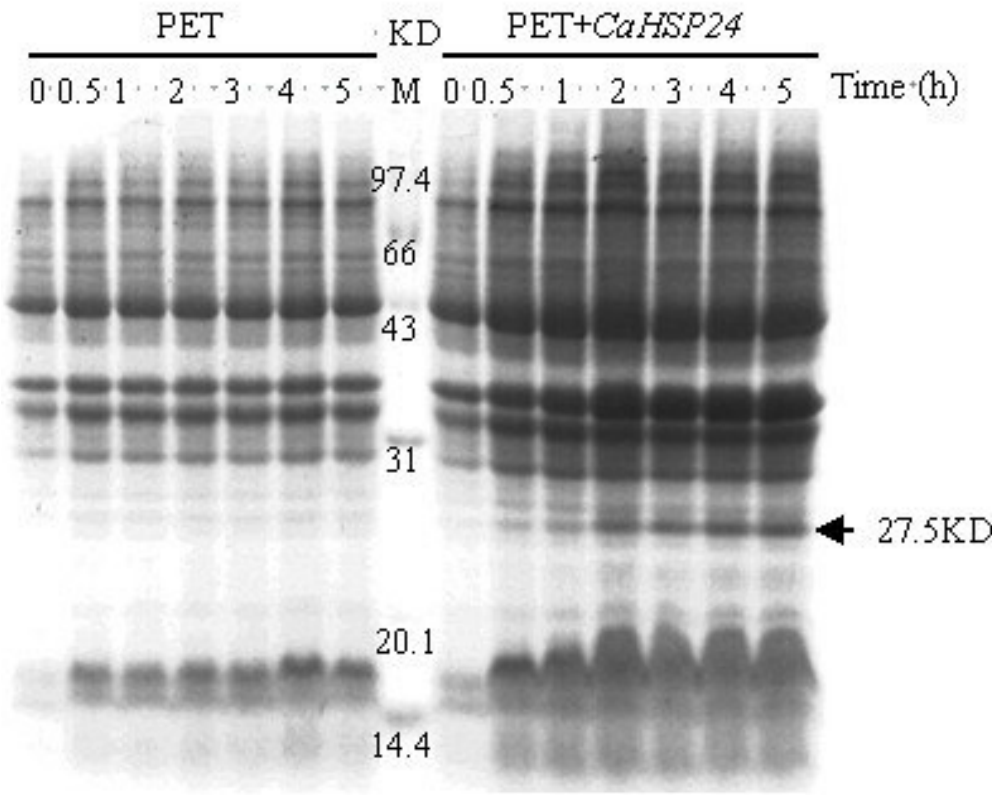

Figure 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the bacterial soluble protein CaHSP24 after IPTG-mediated induction for different time periods. M: marker; PET: soluble protein of cells transformed with PET28a empty vector; PET + *CaHSP24*: soluble protein of cells transformed with recombinant plasmid of PET28a + *CaHSP24*.

L. (Figure 2). It has been reported that heat shock protein is one of the most conserved protein, HSPs derived from the same organelle share very high similarities in different species (Feder and Hofmann, 1999; Sun et al., 2002; Sørensen et al., 2003; Vierling, 1991; Waters et al., 1996). On the basis of this information, we predicted that the sHSP obtained in our study might also be located in the mitochondria, but this hypothesis needs further verification.

Semi-quantitative RT-PCR showed that the optimal

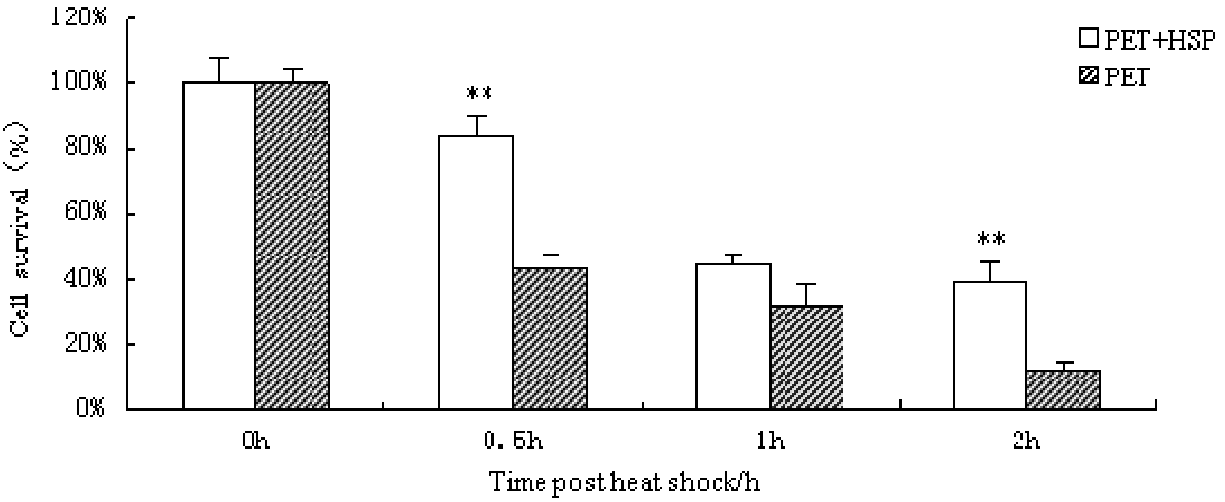

Figure 8. Effect of recombinant CaHSP24 on cell viability at 50°C. The significant levels of difference between treatments are shown by asterisks. **, $P < 0.01$.

stress-inducing temperatures in both B35 and B6 were the same, but the peak expression times of *CaHSP24* differed (Figures 3 and 4). In the heat-resistant cultivar B35, the expression level reached a peak 1.5 h after the cultivar was subjected to heat stress; then, the expression decreased and ever could be detected 5 h after stress. In the heat-sensitive cultivar B6, the peak expression time was 1 h after exposure to heat stress. This result was in accordance with the results published by Liu and Shonos (2001), who reported that in tomato, the expression peak was reached earlier in heat-sensitive varieties than in the heat-resistant ones. Differences in peak expression time may be because of the different expression patterns of heat-resistant and heat-sensitive varieties. Although the expression peak appeared earlier in B6, the expression level of *CaHSP24* was higher in B35 than B6 during heat stress. These results indicated that the expression level of *CaHSP24* might be related to heat resistance in pepper.

Besides heat stress, HSPs can be induced by other abiotic stresses, including salinity, chilling and heavy metal stress (Sun et al., 2002; Waters et al., 1996; Sun and MacRae, 2005; Wang et al., 2004). In our study, we found that *CaHSP24* could be weakly induced by the other abiotic stress treatments, except by treatment with 300 mmol/l NaCl. The expression levels of *CaHSP24* noted under these stress conditions were remarkably lower than those noted under heat treatment. This showed that *CaHSP24* in particular could be induced by high temperature and might play an important role in providing heat resistance in pepper. Guo et al. (2005, 2006) found that the cytoplasmic sHSP gene *CaHSP18* could be expressed in sweet pepper leaves by subjecting the plant to cold stress (4°C), but chloroplast sHSP gene *CaHSP26* could not be induced by cold stress. In our study, we found that *CaHSP24* could be induced by heat stress and also weakly expressed by cold stress. These differences in findings may be because of the different response mechanisms to temperature stress in different classes of sHSP.

We constructed a prokaryotic expression vector for *CaHSP24* and transformed the recombinant plasmid PET28a-*CaHSP24* into *E. coli* (BL21) cells. We found that after exposure to heat stress (50°C), the survival rate of cells transformed with PET28a-*CaHSP24* was higher than that of cells transformed with empty vector PET28a. This result indicated that the heterologous expression of *CaHSP24* could enhance the viability of *E. coli* under heat stress.

Taken together, our results indicated that the expression of *CaHSP24* in pepper was induced by heat stress and the expression level was markedly higher in the heat-resistant cultivar than in the heat-sensitive cultivar. The gene could also enhance the viability of *E. coli* under heat stress. It is concluded that *CaHSP24* might be a stress-related gene in *Capsicum* under abiotic stress conditions. Further investigation is needed to gain more information about the function and regulatory mechanism of *CaHSP24* in plant-stress responses.

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