

A cytosolic class I small heat shock protein, RcHSP17.8, of *Rosa chinensis* confers resistance to a variety of stresses to *Escherichia coli*, yeast and *Arabidopsis thaliana*

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ABSTRACT

Among the heat shock proteins (HSPs) of higher plants, those belonging to the small HSP (sHSP) family remain the least characterized in functional terms. To improve our understanding of sHSPs, we have characterized *RcHSP17.8* from *Rosa chinensis*. Sequence alignments and phylogenetic analysis reveal this to be a cytosolic class I sHSP. *RcHSP17.8* expression in *R. chinensis* was induced by heat, cold, salt, drought, osmotic and oxidative stresses. Recombinant RcHSP17.8 was overexpressed in *Escherichia coli* and yeast to study its possible function under stress conditions. The recombinant *E. coli* and yeast cells that accumulated RcHSP17.8 showed improved viability under thermal, salt and oxidative stress conditions compared with control cultures. We also produced transgenic *Arabidopsis thaliana* that constitutively expressed RcHSP17.8. These plants exhibited increased tolerance to heat, salt, osmotic and drought stresses. These results suggest that *R. chinensis* cytosolic class I sHSP (RcHSP17.8) has the ability to confer stress resistance not only to *E. coli* and yeast but also to plants grown under a wide variety of unfavorable environmental conditions.

Key-words: drought stress; osmotic stress; oxidative stress; salt stress; stress-induced; thermotolerance.

INTRODUCTION

The heat shock protein (HSP) superfamily is one of the most ubiquitous and evolutionarily conserved groups of molecular chaperones across all species. The main function of HSPs is their chaperone activity, which protects proteins from being irrevocably denatured, misfolded or aggregating under various exogenous environmental abiotic stress conditions (Queitsch *et al.* 2000; Frydman 2001; Bosl, Griminger & Walter 2006; Goloubinoff & De Los Rios 2007).

The HSP family has been classified on the basis of their molecular weight into five groups (Wang *et al.* 2004): HSP100s; HSP90s; HSP70s; HSP60s; and small HSPs

(sHSPs; 12–40 kDa). sHSPs, which are by far the most complex group of HSPs in plants, play important roles in plant stress tolerance because of their abundance and diversity. The sHSP family can be further classified by intracellular location and sequence similarity into cytosolic class I, cytosolic class II, chloroplast, mitochondria, endoplasmic reticulum and peroxisome sHSPs (Low *et al.* 2000; Ma *et al.* 2006; Kotak *et al.* 2007a; Mamedov & Shono 2008; Siddique *et al.* 2008).

In the last two decades, studies have shown that the expression and accumulation of sHSPs in plants can be triggered by various abiotic stress conditions, such as extreme temperatures (Malik *et al.* 1999; Lopez-Matas *et al.* 2004; Sanmiya *et al.* 2004; Charng *et al.* 2006), salinity (Hamilton & Heckathorn 2001), drought (Sato & Yokoya 2008), osmotic (Sun *et al.* 2001) and oxidative stress (Lee *et al.* 2000; Neta-Sharir *et al.* 2005; Volkov *et al.* 2006). Aside from environmental stresses, the expression of sHSPs is also regulated by plant developmental signals, such as embryogenesis, germination, pollen development and fruit maturation (Ramakrishna *et al.* 2003; Volkov, Panchuk & Schoffl 2005; Kotak *et al.* 2007b). Heterologous expression of sHSPs *in vivo* can also enhance stress tolerance of the transgenic organisms. Enhanced thermotolerance was reported in recombinant *Escherichia coli* cells expressing a glutathione *S*-transferase/rice HSP16.9 fusion protein (Yeh *et al.* 1997), and also in *E. coli* cells overexpressing chestnut CsHSP17.5 (Soto *et al.* 1999). Transgenic *Arabidopsis thaliana* plants constitutively expressing the chloroplast sHSP, HSP21, were more resistant to heat and high-light stresses than the wild type (Harndahl *et al.* 1999). In addition, introduction of the carrot HSP17.7 into potato (*Solanum tuberosum* L.) could enhance cellular membrane stability and tuberization *in vitro* (Ahn & Zimmerman 2006). This made the function of sHSPs in plant more diversified. However, the important roles of many plant sHSPs remain to be discovered.

Rosa chinensis, a species of *Rosa*, is one of the most important groups of horticultural and ornamental plants throughout the world (Krussmann 1981). It not only has commercial value but also has pharmaceutical applications (Gao & Chen 2000). However, the growth of most *R. chinensis* varieties is limited by unfavorable environmental

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conditions such as high temperature, high salt concentration and drought. Under stress conditions, these varieties do not blossom but enter dormancy directly, thus research to enhance the plant's tolerance to these stresses is of interest.

In our previous work (Jiang *et al.* 2006), we found that there was a low molecular weight HSP expressed in the leaves of a *R. chinensis* variety that exhibits strong tolerance to high temperature under 38 °C heat treatment for 3 h. In this study, we identified this novel gene whose expression was strongly induced under high temperature. Analyses of the nucleotide and amino acid sequences showed that it is a cytosolic class I sHSP-encoding gene, *RcHSP17.8*. We also found that this gene is induced under other abiotic stresses, like salt, drought, osmotic and oxidative stress. Analyses in both *E. coli* and yeast cells also suggested a role for this sHSP protein in helping resist various abiotic stresses, and the results were consistent with the studies in transgenic *A. thaliana* plants.

MATERIALS AND METHODS

Plant materials and growth conditions

The heat-resistant *R. chinensis*, *Sohloss Mannieim* (SM) was grown in the field of the Shanghai Botanical Garden under natural conditions, and propagated *in vitro* on Murashige and Skoog (MS) differentiation medium containing 4.4 g L⁻¹ MS salts, 1 mg L⁻¹ 6-benzyl aminopurine, 0.2 mg L⁻¹ IAA, 3% sucrose and 0.7% agar, pH 6.0, and MS rooting medium containing 2.2 g L⁻¹ MS salts, 0.5 mg L⁻¹ IAA, 3% sucrose and 0.7% agar, pH 6.0. Seedlings were grown in soil in the greenhouse (16 h light/8 h dark, 60% relative humidity, 22–25 °C).

Standard *A. thaliana* ecotype Columbia plants were grown in soil in the greenhouse (16 h light/8 h dark, 60% relative humidity, 22–25 °C) or *in vitro* on MS medium containing 4.4 g L⁻¹ MS salts, 1% sucrose and 0.8% agar, pH 5.7.

RcHSP17.8 cDNA cloning

To obtain open reading frame (ORF) of *RcHSP17.8*, degenerate primers were designed based on the conserved sequence (GenBank accession number: AF161179) of the sHSP of another *Malus* species of *Rosaceae*: 5'-GAYTGGARGGAGACMCCT-3' as the sense primer and 5'-ATGGARAATGGWGTKYT-3' as the antisense primer. Total RNA was extracted from leaves of *R. chinensis* grown under 38 °C for 3 h using the RNAout kit (TianDZ, China). The PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China) was used for the first-strand cDNA synthesis. RT-PCR was performed using the two primers mentioned earlier. For PCR, standard parameters and an appropriate number of cycles were used. The PCR products were purified and cloned into pGEM-T vector (Promega, Beijing, China) for sequencing.

To obtain the genomic DNA sequence of *RcHSP17.8*, genomic DNA was extracted using the cetyl trimethyl

ammonium bromide (CTAB) method, and then digested with *Eco*R I and self-ligated to generate circular molecules. The molecules were used as templates for nested amplifications using the following primers that are deduced from the peptide sequences that already known in our previous work (Jiang *et al.* 2006):

- 1 IN1L: 5'-AGGACAAGAACGACAAGTGG-3';
- 2 IN1R: 5'-GTCATCTTCAATCTCAACCT-3';
- 3 IN2L: 5'-CGAGAGAAGCAGCGGCAAGT-3';
- 4 IN2R: 5'-CCTCTTCTTTCTTCAGCCCC-3';
- 5 IN3L: 5'-AGGCTGCTATGGAGAATGGA-3'; and
- 6 IN3R: 5'-ACACATGGGCTTCTGGGGTC-3'.

PCR was performed using the program: 4 min at 94 °C; 10 s at 98 °C; 15 min at 65 °C for 30 cycles; and 10 min at 72 °C. First round of PCR was performed with primers IN1L/IN1R and the products were diluted 100-fold for the second round of amplification with primers IN2L/IN2R, and the products were diluted to 100-fold as template for the third round amplification with IN3L/IN3R primers. The final PCR products were purified and cloned into pGEM-T vector for sequencing.

To obtain 3'-UTR of *RcHSP17.8*, 3'-RACE was performed using a 3'-Full RACE Core Set (TaKaRa). 3'-RACE nested PCR was performed using three nested primers above IN1F, IN2F, IN3F in combination with AUAP (5'-GGCCACGCGTCGACTAGTAC-3') using the following programs: 4 min at 94 °C; 30 cycles of 30 s at 94 °C; 40 s at 55 °C; 1 min at 72 °C; and 10 min at 72 °C. PCR was performed successively with IN1F, IN2F and IN3F using a 10-fold dilution of the PCR products in each round. The final PCR products were purified and cloned into pGEM-T vector for sequencing.

After comparing the incomplete ORF, 3'-UTR with the genomic DNA sequence and knowing the whole cDNA sequence of *RcHSP17.8*, the complete ORF of *RcHSP17.8* was obtained by RT-PCR using two primers, 5'-ATGT CGCTTATCCCAAATTT-3' and 5'-TTACCCAGAAAT TCAATGG-3', under the following amplification conditions: 4 min at 94 °C; 30 cycles of 30 s at 94 °C; 40 s at 55 °C; 1 min at 72 °C; and 10 min at 72 °C. The PCR products were purified and cloned into pGEM-T vector for sequencing.

Sequence alignment and phylogenetic analysis

The deduced amino acid sequence of RcHSP17.8, along with other representative cytosolic class I sHSPs genes, were aligned using the computer program clustalX (Higgins & Sharp 1988).

A phylogenetic tree was constructed using the neighbour-joining method provided by the computer program Mega 3 (Kumar, Tamura & Nei 2004). Bootstrapping (1000 replicates) was performed to quantify the relative support for branches of the inferred phylogenetic tree.

RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted from different tissues of *R. chinensis* using RNAout kit (TianDZ, Mianyang, China). The

PrimeScript™ RT Reagent Kit (TaKaRa) was used for the first-strand cDNA synthesis. This cDNA served as template in PCR carried out as previously described, using *RcHSP17.8* gene-specific primers 5'-ATGTCGCTTATCCCAAATTT-3' and 5'-TTACCCAGAAATTTCAATGG-3'. The primers of *R. chinensis* actin-F: 5'-TCTGGCATCATACCTTCTACA-3' and actin-R: 5'-GGATGGCTGGAAGAGGAC-3' were used for control amplification of actin.

For *A. thaliana*, total RNA was extracted from leaves of 4-week-old wild-type and T3 generation transgenic *RcHSP17.8 Arabidopsis* with different treatment using a Redzol kit (Saibaisheng-SBS, Beijing, China). The PrimeScript™ RT Reagent Kit (TaKaRa) was used for the first-strand cDNA synthesis. This cDNA served as template in PCR carried out as previously described, using *RcHSP17.8* gene-specific primers: 5'-TCCGACGAAACAGCG-3' and 5'-CAGCACCTGTTCATT-3'. The primers of *A. thaliana* actin-F: 5'-AGGTAATCAGTAAGGTCACGG-3' and actin-R: 5'-GGATGGCTGGAAGAGGAC-3' were used for control amplification of actin.

Semi-quantitative RT-PCR was performed using a PrimeScript™ RT Reagent Kit (TaKaRa) according to the manufacturer's instruction. For PCR, the following amplification conditions were used for all genes: 4 min at 94 °C; 30 cycles of 30 s at 94 °C; 40 s at 55 °C; 1 min at 72 °C; and at last 10 min at 72 °C. PCR products were analysed by 0.8% agarose gel electrophoresis.

Constitutive expression of *RcHSP17.8* cDNA in *E. coli*

Sac I and *Sal* I restriction sites were added to 5' and 3' ends of the *RcHSP17.8* cDNA ORF by PCR using the primers 5'-CGGAGCTCATGTGCTTATCCCAAATTT-3' and 5'-CGGTCGACCCAGAAATTTCAATGGC-3', respectively. The *Sac* I and *Sal* I sites were used to ligate the fragment into the *Sac* I/*Sal* I-digested pET32a, a prokaryotic expression vector, and transformed into *E. coli* strain BL21 by the freeze-thaw method. The resulting *RcHSP17.8* expression construct was sequenced to confirm its identity and designated as pET32a-*RcHSP17.8*. The transformants were used to characterize the effects of *RcHSP17.8* on the bacterial cell viability and stress tolerance. *E. coli* cells carrying pET32a vector alone were used as a negative control.

For thermotolerance assays, *E. coli* cell cultures were grown at 37 °C to optical density at 600 nm (OD₆₀₀) of 1.0 and then diluted once with fresh Luria-Bertani (LB) medium supplemented with ampicillin (100 mg mL⁻¹) and isopropyl-beta-D-thiogalactopyranoside (IPTG; 1 mmol). Cultures were shifted to 50 °C after 2 h of IPTG induction. One milliliter of the culture was taken at 0, 0.5, 1, 2, 3, 4 and 5 h, respectively, serial dilutions were plated in triplicate and cell viability was measured by counting colony-forming units in triplicate plates. For cold treatments, aliquots from IPTG-induced cultures were plated and kept at 4 °C for 0, 2, 4, 6, 8, 12 d and cell viability was measured as described

earlier. For both treatments (heat and cold), the means of two experiments were determined from two parallel transformants with pET32a-*RcHSP17.8* and pET32a.

For other stress treatments, aliquots from IPTG-induced cultures were streaked on LB medium with different stress substances – NaCl (500 mmol, 800 mmol, 1 mol, 1.2 mol) and H₂O₂ (0.1, 0.2, 0.4, 0.6 mmol) – and photos were taken after 12 h of cultivating at 37 °C.

Constitutive expression of *RcHSP17.8* cDNA in yeast

The ORF sequence of *RcHSP17.8* cDNA with *Sac* I restriction site at 5' end and *Not* I site at 3' end was obtained by PCR as previously described. *Sac* I-*Not* I fragments were introduced into pET32a plasmid to produce pET32a-*RcHSP17.8*. The fragment from this new construct digested by *Bam*H I and *Not* I was then introduced into eukaryotic expression vector pPIC3.5K as pPIC3.5K-*RcHSP17.8* for yeast heterologous expression. The pPIC3.5K-*RcHSP17.8* was transformed into *Pichia pastoris* strain SMD1168 by electroporation and integrated in the SMD1168 genome by homologous recombination. After phenotypic expression, the transformants were selected on yeast extract peptone dextrose (YEPD) medium plates containing G418 at a concentration of 200 mg L⁻¹. Positive colonies were identified after 2 d of incubation at 28 °C. Verification of positive colonies was accomplished by genomic DNA PCR. SMD1168 cells carrying pPIC3.5K vector alone was taken as negative control.

SMD1168 cells were pre-cultivated aerobically for 12–14 h in buffered glycerol complex medium (BMGY; 28 °C, 250 r.p.m.) to OD₆₀₀ of 1.0–1.8. The cultures were centrifuged and washed once with 1 × phosphate-buffered solution (PBS) and then inoculated in buffered methanol complex medium (BMMY) of which the initial OD₆₀₀ was adjusted to 0.05. Cultures were cultivated aerobically with a speed of 250 r.p.m. at 28 °C. When the OD₆₀₀ was up to 1.4 (at 12 h), cultures were shifted to 50 °C for 0.5 h heat shock treatment. One milliliter of the cultures was taken at the end of the first hour. The samples were centrifuged and washed once with 1 × PBS. Five series of 10-fold dilutions were taken until the cell numbers were 1000 cells mL⁻¹. Aliquots (5 μL) from each dilution were spotted in triplicate onto YEPD medium plates and then incubated at 28 °C for 2–3 d. For cold treatment, cultures cultivated until OD₆₀₀ was up to 1.4 (at 12 h) were plated on YEPD medium and shifted to 4 °C for a number of weeks (2, 4, 6, 8 or 10) before being incubated at 28 °C for 2–3 d for recovery.

To assess other stress tolerances of SMD1168 recombinant cells, various stress substances at different concentrations were added to YEPD medium: NaCl (500 mmol, 800 mmol, 1 mol, 1.2 mol), H₂O₂ (3, 6, 9, 12 mmol).

In all yeast experiments, cell viability was calculated by observing the growth after incubated at 28 °C for 2–3 d.

Plasmid construction and *Arabidopsis* transformation

The *RcHSP17.8* cDNA ORF with *Xba* I restriction site at 5' end and *Sac* I site at 3' end was obtained by PCR as previously described. *Xba* I-*Sac* I fragment was inserted into the *Xba* I/*Sac* I site of pCAMBIA2301 under the control of a cauliflower mosaic virus (CaMV) 35S RNA promoter. The pCAMBIA2301-35S-RcHSP17.8 recombinant vector was subsequently transformed into *Agrobacterium* GV3101 by the freeze-thaw method.

Arabidopsis transformation was carried out by the floral dip method. Transgenic plants were obtained by screening successive generations on hygromycin.

Stress treatments of transgenic *Arabidopsis* plants

For heat stress experiment, two lines of T3 generation transgenic *Arabidopsis* and a wild-type control line were germinated on MS medium for 10 d, and then shifted to 45 °C for 2 h before transferred to normal conditions for 3 d of recovery. For salt stress treatment, 10 d germinated seedlings were shifted to two new MS medium plates plus 100, 150, 200, 250, 300 mmol NaCl, respectively, and MS medium plate without NaCl as a control. For osmotic stress treatment, 9 d germinated seedlings were shifted to two new MS medium plates with 350, 450, 550 mmol mannitol, respectively, and MS medium plate without mannitol as a control. The percentage of survivors (ratio of survived to total plants) and their root length were measured and relative root length was calculated. For drought stress treatment, drought stress was inflicted on 2-week-old plants by withholding water for 15 d, and then transferring them to normal conditions for 14 d of recovery.

In the hypocotyl thermotolerance assay, wild-type and transgenic plants were germinated on the same MS medium plates. Seedlings were grown in the dark for 3 d and then heat-shocked at 45 °C for 2 h, seedlings were allowed to recover for 3 d at 22 °C, and the extent of hypocotyl elongation after recovery was measured.

Superoxide dismutase (SOD) activity assay, proline content estimation and electrolyte leakage measurement

SOD activity was assayed by the inhibition of photochemical reduction of nitroblue tetrazolium as described (Dhindsa, Plumb-Dhindsa & Thorpe 1981). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of reduction of nitroblue tetrazolium measured at 560 nm. Average values and SD were calculated from three or more independent experiments.

Proline content was determined by the method described by Bates *et al.* (Bates, Waldren & Teare 1973) with minor modifications. Rosette leaves (0.5 g) were excised into 5 mL 3% aqueous sulfosalicylic acid and incubated at 100 °C for 10 min. The homogenate was centrifuged at 12 000 g for

10 min. The reaction mixture containing 2 mL of supernatant, 2 mL glacial acetic acid and 2 mL ninhydrin reagent (2.5% ninhydrin in 60% phosphoric acid) was incubated at 100 °C for 30 min and terminated by cooling the tubes on ice. The absorbance was determined at 520 nm.

Electrolyte leakage of rosette leaves were measured at room temperature using a conductance meter (DDS-307, Shanghai Precision and Scientific Instrument Co., Ltd., Shanghai, China) as described previously (Sharom, Willemot & Thompson 1994). Three independent experiments were done.

Protein identification and Western blot analysis

Proteins were extracted by homogenizing ground material in extraction buffer [15 mL 1 mol Tris-HCl (pH 8.0), 25 mL glycerol, 2 g insoluble PVP, water up to 100 mL], followed by shaking on a shaker for 3–4 h. The extract was then centrifuged at 10 000 g for 20 min at 4 °C. Supernatants were mixed with loading buffer and incubated at 100 °C for 5 min. Debris was removed by centrifugation at 10 000 g for 3 min at room temperature. Protein concentrations were measured using the Bradford method.

For Western blotting, 10 µg of protein samples in each lane were separated on 15% polyacrylamide-sodium dodecyl sulphate gel and transferred to nitrocellulose membranes for antibody probing. A monoclonal antibody against RcHSP17.8 [anti-His-tag and anti-green fluorescent protein (anti-GFP) antibody] was purchased from ImmunoGen (Suzhou, China). After incubation with the primary antibody (dilution at 1:1000), the protein blot was probed with rat anti-rabbit IgG-conjugated horseradish peroxidase (dilution at 1:1000; ImmunoGen). The protein-antibody complex was detected using the substrate solution bought from Shenergy Biocolor Bioscience Company (Shanghai, China).

RESULTS

RcHSP17.8 is a member of plant cytosolic class I sHSP gene family

A full-length cDNA containing a 465 bp ORF was isolated from *R. chinensis* and showed significant homology to sHSP genes of various species using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). It was predicted to encode a protein of 154 amino acids with a molecular weight of 17.8 kDa. It was designated as *RcHSP17.8* (GenBank accession number EF053229). The deduced amino acid sequence of RcHSP17.8 was aligned with other representative cytosolic class I sHSPs and demonstrated significant homology to sHSPs of other plants (Fig. 1). RcHSP17.8 contains two sHSP conserved domains (Waters, Lee & Vierling 1996; Scharf, Siddique & Vierling 2001): +72 to +97; and +121 to +148. Within these regions, the identity of RcHSP17.8 with sHSPs of other plant species was between 75 and 92% at the amino acid level.

A phylogenetic tree (Fig. 2) separated the plant sHSP gene family into six clades. Consistent with the alignment,

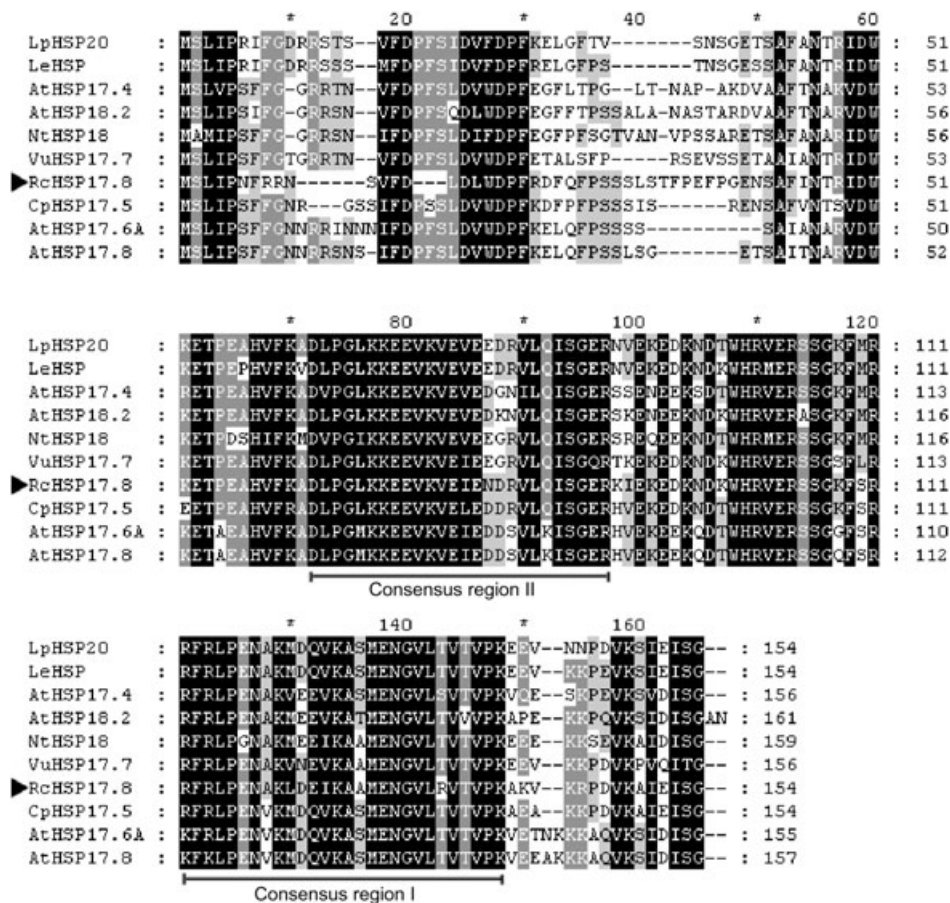
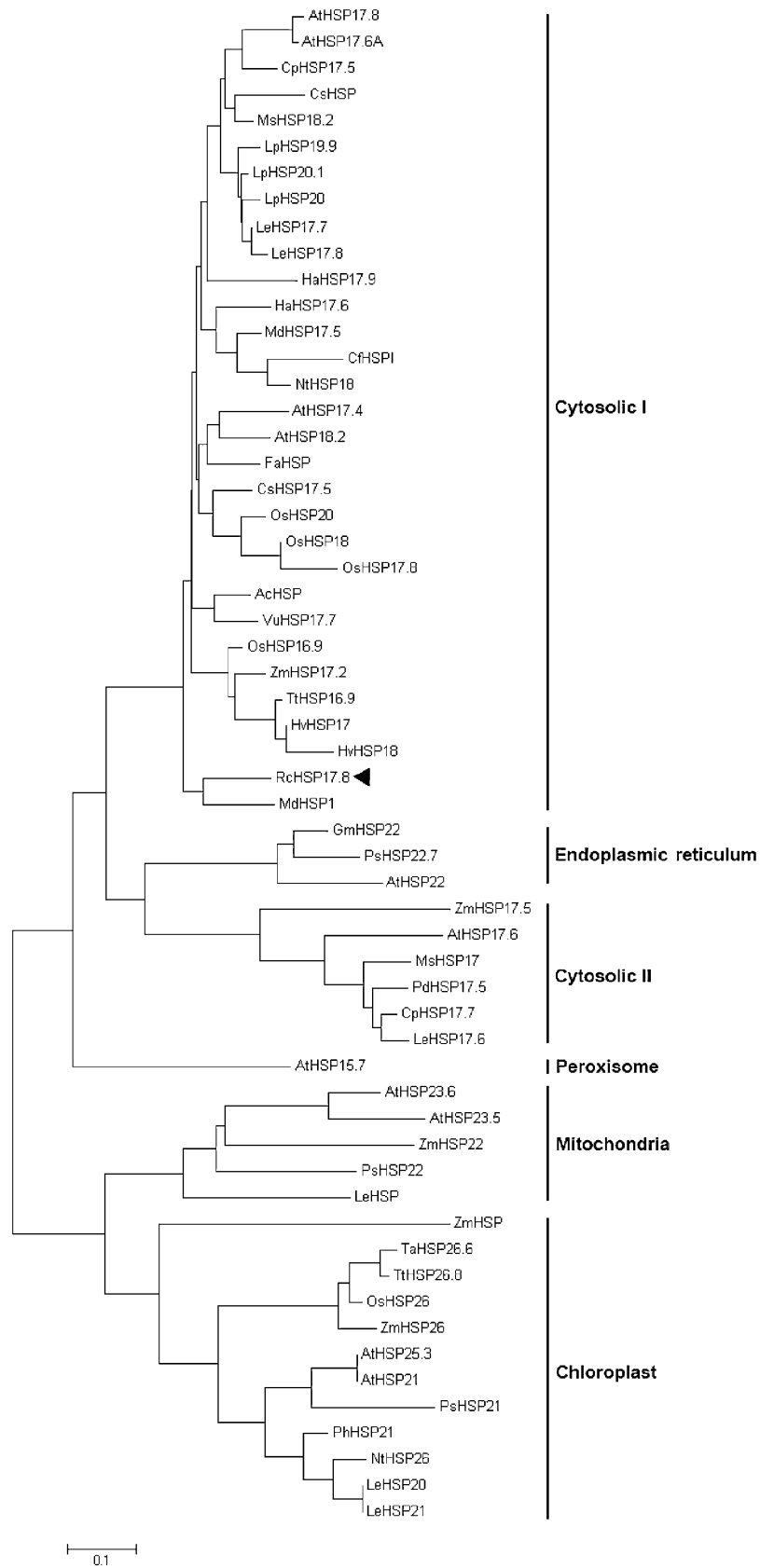


Figure 1. Alignment of the deduced amino acid sequences of RcHSP17.8 with other plant sHSPs. The two conserved regions in sHSPs are underlined and identical amino acid residues are highlighted. Their GenBank accession numbers are: RcHSP17.8 of *Rosa chinensis* (EF053229); AtHSP17.6A (*Arabidopsis thaliana*) (NM_104679); AtHSP17.4 (NM_114492); AtHSP17.8 (NM_100614); AtHSP18.2 (NM_125364); NtHSP18 (*Nicotiana tabacum*) (X70688); VuHSP17.7 (*Vigna unguiculata*) (EF514500); CpHSP17.5 (*Carica papaya*) (AY387588); LpHSP20 (*Lycopersicon peruvianum*) (AJ225048); LeHSP (*Lycopersicon esculentum*) (X56138). RcHSP17.8 is indicated with a solid triangle at the left.

RcHSP17.8 is closer to cytosolic class I sHSP than to other sHSP classes and shares greatest similarity to a sHSP (MdHSP1) from another *Malus* species of *Rosaceae* – *Malus domestica*. Southern blot result revealed that

RcHSP17.8 is encoded by a member of a small multigene family in *R. chinensis* (data not shown). Taken together, these results support the view that RcHSP17.8 is a member of plant cytosolic class I sHSP gene family.

Figure 2. Phylogenetic tree of the deduced amino acid sequences of RcHSP17.8 and other small sHSPs. Amino acid sequences used in the phylogenetic analysis were retrieved from GenBank, their GenBank/EMBL/DBJ database accession numbers are as follows: RcHSP17.8 (*Rosa chinensis*) (EF053229); AtHSP17.6A (*Arabidopsis thaliana*) (NM_104679); AtHSP17.4 (NM_114492); AtHSP17.8 (NM_100614); AtHSP18.2 (NM_125364); AtHSP22 (ATU11501); AtHSP25.3 (DQ446875); AtHSP15.7 (DQ403190); AtHSP17.6 (NM_121240); AtHSP23.6 (NM_118652); AtHSP21 (NM_118906); AtHSP23.5 (NM_124523); AchSP (*Ananas comosus*) (AY098528); CfhSP (*Capsicum frutescens*) (AY284925); CpHSP17.5 (*Carica papaya*) (AY387588); CpHSP17.7 (AY242075); CsHSP17.5 (*Castanea sativa*) (AJ582679); CsHSP (*Camellia sinensis*) (EU727315); FaHSP (*Fragaria × ananassa*) (U63631); GmHSP22 (*Glycine max*) (X63198); HaHSP17.9 (*Helianthus annuus*) (AJ237596); HaHSP17.6 (X59701); HvHSP17 (*Hordeum vulgare* ssp. *vulgare*) (Y07844); HvHSP18 (X64561); LeHSP17.6 (*Lycopersicon esculentum*) (LEU72396); LeHSP17.7 (AF123255); LeHSP20 (SLU59917); LeHSP17.8 (AF123256); LeHSP21 (LEU66300); LeHSP (AB017134); LpHSP20 (*Lycopersicon peruvianum*) (AJ225048); LpHSP19.9 (AJ225047); LpHSP20.1 (AJ225046); MdHSP17.5 (*Malus × domestica*) (EU636239); MdHSP1 (AF161179); MshSP18.2 (*Medicago sativa*) (X58711); MshSP17 (X98617); NtHSP18 (*Nicotiana tabacum*) (X70688); NtHSP26 (D88584); OsHSP26 (*Oryza sativa*) (AB020973); OsHSP18 (FJ383169); OsHSP17.8 (X75616); OsHSP16.9 (X60820); OsHSP20 (EU325986); PdHSP17.5 (*Prunus dulcis*) (AF159562); PhHSP21 (*Petunia × hybrida*) (X54103); PshSP22 (*Pisum sativum*) (X86222); PshSP21 (X07187); PshSP22.7 (M33898); TahSP26.6 (*Triticum aestivum*) (AF097659); TihSP16.9 (*Triticum turgidum* ssp. *dicoccon*) (AM709752); TihSP26.8 (AJ971372); VuHSP17.7 (*Vigna unguiculata*) (EF514500); ZmHSP26 (*Zea mays*) (L28712); ZmHSP22 (AY758275); ZmHSP17.2 (X65725); ZmHSP (EU966283); ZmHSP17.5 (EU970990). RcHSP17.8 is indicated with a solid triangle.



Expression of *RcHSP17.8* in *R. chinensis* is induced not only by heat shock, but also by other abiotic stresses

To study the expression of *RcHSP17.8* in *R. chinensis*, the effects of various abiotic stress conditions on sHSP expression in leaf were analysed by semi-quantitative RT-PCR using gene-specific oligonucleotide primers. The results indicated that *RcHSP17.8* was not expressed in leaves (as well as other tissues like flower, stem and root) of plants in the absence of stress (under room temperature 25 °C) (Fig. 3b), but was induced upon 3 h heat treatment (38 °C) or 6 h cold stress condition (2 °C), and with the highest expression after 30 min at 50 °C (Fig. 3a). We then tested expression when plantlets of the same age were subjected to different abiotic stress conditions including salt stress (NaCl), polyethylene glycol (PEG)-induced drought stress, osmotic stress (mannitol) and oxidative stress (H₂O₂). Increased transcript abundance was observed in all plant leaves analysed, but transcript accumulation was slower and less abundant than during heat shock (Fig. 3c).

In order to study the tissue specific expression pattern of *RcHSP17.8* in *R. chinensis*, mRNAs were isolated from different tissues of the same heat-treated plant (38 °C for 3 h).

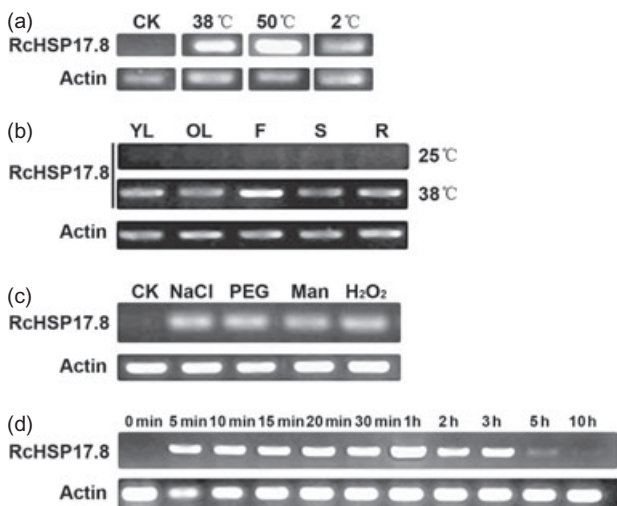


Figure 3. Semi-quantitative RT-PCR of expression of *RcHSP17.8* in *Rosa chinensis*. (a) mRNAs were extracted from leaves of *Rosa chinensis* under different temperatures. CK, no treatment as control (25 °C); 38 °C heat stress temperature (38 °C, 3 h); 50 °C heat shock temperature (50 °C, 0.5 h); 2 °C cold stress temperature (2 °C, 6 h). (b) mRNAs were extracted from different tissues from *Rosa chinensis* grown under 38 °C for 3 h and 25 °C (as control). (c) mRNAs were extracted from leaves of *Rosa chinensis* under different treatments (0.3 M NaCl, 10% polyethylene glycol, 0.4 M mannitol, 0.03% H₂O₂) for 3 h. (d) RT-PCR of temporal expression pattern of *RcHSP17.8*. mRNAs were extracted from leaves of *Rosa chinensis* grown under 38 °C for different treatment time. YL, young leaves (2 d from emergence); OL, old leaves (2 weeks from emergence); F, flower (petals) (3 d from flowering time); S, stem from 2-week-old seedling; R, root (whole roots) from 2-week-old seedling.

The accumulation of *RcHSP17.8* mRNA was higher in the flower relative to other tissues (leaf, stem and root) (Fig. 3b). We then investigated transcript accumulation for different lengths of 38 °C heat shock. Increased transcript abundance was observed after 5 min of treatment. An mRNA maxima was reached at 10 min of treatment, and began to decline after 3 h of treatment, totally disappearing 5 h later (Fig. 3d).

Overexpression of *RcHSP17.8* enhance the viability of *E. coli* and yeast recombinant cells under thermal, salt and oxidative stress conditions

To analyse the possible function of *RcHSP17.8* *in vivo* and under stress conditions, the *RcHSP17.8* coding sequence was expressed in *E. coli* using the expression vector pET32a. Western blot analysis with an anti-His-tag antibody demonstrated the presence of the recombinant protein in the *E. coli* cells (Fig. 4a). We also expressed *RcHSP17.8* in *P. pastoris* using the pPIC3.5K expression vector. Western blot analysis with an anti-GFP antibody also demonstrated the presence of the recombinant protein in the *P. pastoris* cells (Fig. 4b). Empty vectors were introduced into the two species as controls. Similar growth rates were observed for test and control recombinant *E. coli* and *Pichia* cells under normal conditions (data not shown).

The effects of high and low temperature on survival of recombinant *E. coli* cells was measured in cultures subjected to heat and cold stresses before shifting to normal conditions for recovery. Cell survival was measured by counting colony-forming units. Fig. 4c shows that cell viability decreased rapidly in cultures grown under heat shock (50 °C) and cold stress (4 °C) conditions for different time, but *E. coli* expressing *RcHSP17.8* exhibited higher survival rates than cells without *RcHSP17.8*. Following a 1 h heat shock, the survival rate of cells without *RcHSP17.8* was 15% in contrast to a 70% survival rate of cells expressing *RcHSP17.8*. Furthermore, following a 4 h heat shock, all cells without *RcHSP17.8* were dead, whereas 10% of cells expressing *RcHSP17.8* were still viable. Similarly, after 6 d of cold treatment the survival rate of cells expressing *RcHSP17.8* was twice that of the controls. Under low temperature stress, half of the cells without *RcHSP17.8* died within 4 d at 4 °C, and after 10 d only approximately a third of the cells remained alive; in contrast, approximately 65% of the cells expressing *RcHSP17.8* survived after the same period.

The effects of high and low temperature on the viability of recombinant *Pichia* cells was evaluated by observing the growth of 10-fold serial dilutions of cultures spotted on the YEPD medium plate. The YEPD plate spotted with five serial dilutions were grown at 50 °C for 1 h before being incubated at 28 °C for 2 d or at 4 °C for 6 weeks before being shifted to 28 °C for 2 d of recovery. The result showed that there were obvious differences in yeast cells expressing *RcHSP17.8* compared with controls (Fig. 4d). These results showed that *RcHSP17.8* could enhance *E. coli* transformant

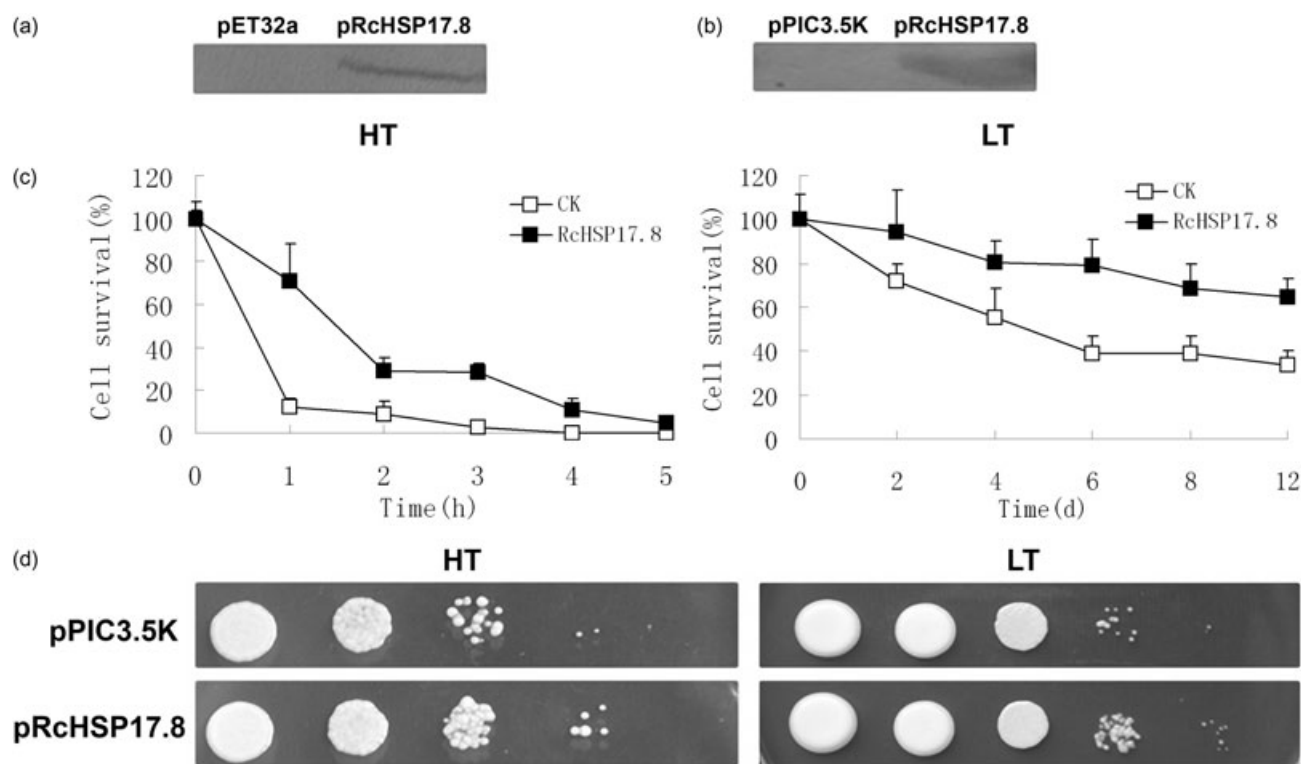


Figure 4. Heterogeneous expression of RcHSP17.8 in *Escherichia coli* strain BL21 and yeast *Pichia pastoris* strain SMD1168 enhances the host's thermal tolerance. (a) Western blot analysis with an anti-His-tag antibody demonstrated the presence of the recombinant protein in the *E. coli* cells. Proteins were extracted from 1 h isopropyl-beta-D-thiogalactopyranoside-induced *E. coli* cell cultures. pET32a, *E. coli* cells carrying empty vector; pRcHSP17.8, *E. coli* cells carrying vector pET32a-RcHSP17.8. (b) Western blot analysis with an anti-green fluorescent protein antibody demonstrated the presence of the recombinant protein in the *Pichia pastoris* cells. Proteins were extracted from *P. pastoris* cell cultures that were cultivated in buffered methanol complex medium until the optical density at 600 nm (OD_{600}) was up to 1.4. pPIC3.5K, SMD1168 cells carrying empty vector; pRcHSP17.8, SMD1168 cells carrying vector pPIC3.5K-RcHSP17.8. (c) Cell survival of *E. coli* BL21 wild type (CK) and transformants (RcHSP17.8) grown under high temperature (HT; 50 °C) and low temperature (LT; 4 °C) for different times (x-axis) before shifted to normal conditions for recovery. (d) Growth of *Pichia pastoris* SMD1168 transformants after HT (50 °C) and LT (4 °C). For the heat shock treatment, after the initial OD_{600} reached 1.4 (at 12 h), cultures were shifted to 50 °C for 1 h. The samples were centrifuged and washed once with $1 \times$ PBS. Cultures grown in yeast extract peptone dextrose (YEPD) medium were diluted 10-fold in five series, and 5 μ L samples were spotted onto the YEPD plates, and then incubated at 28 °C. Cell viability was estimated by counting the colony-forming units after 2 d. For cold treatment, cultures of OD_{600} up to 1.4 (at 12 h) were plated on YEPD medium and shifted to 4 °C for 4 weeks (2, 3, 4, 5, 6) before incubated at 28 °C for 2–3 d of recovery. The spots in part (d) refer to colony-forming units. pPIC3.5K, SMD1168 cells carrying empty vector; pRcHSP17.8, SMD1168 cells carrying vector pPIC3.5k-RcHSP17.8.

cells' viability under heat and cold stress and also alter *Pichia* survival.

To examine the influence of the introduced RcHSP17.8 on other abiotic stresses, cultures of wild type, empty vector and vector carrying pET32a-RcHSP17.8 strains were streaked on LB medium plates with different stress substances; salt stress (NaCl) and oxidative stress (H_2O_2). As shown in Fig. 5a, cell survival of two *E. coli* strains expressing RcHSP17.8 (H1 and H2) was higher than strains of wild type and empty vector grown at 800 mmol NaCl or 200 μ mol H_2O_2 . Similarly, the growth of *Pichia* cells expressing RcHSP17.8 was better than cells without RcHSP17.8 under the same of stresses (Fig. 5b). It is especially evident on the YEPD plate with 6 mmol H_2O_2 ; cells expressing RcHSP17.8 were still present in the third dilution whereas cells without RcHSP17.8 only grew from the second dilution. All the results mentioned earlier revealed that

RcHSP17.8 could increase the tolerance to thermal, salt and oxidative stresses of both *E. coli* and *Pichia* cells.

Constitutive expression of *RcHSP17.8* in *A. thaliana* confers higher thermotolerance and resistance to salt, drought and osmotic stresses

T3 plants for two of the transgenic *Arabidopsis* lines, L1 and L3, were obtained by screening successive generations on hygromycin of $35S::RcHSP17.8$ transformants. RT-PCR and Western blot analyses of the 4-week-old transformants confirmed that *RcHSP17.8* was constitutively expressed in the two transgenic *Arabidopsis* lines (Fig. 6).

To identify if the transgenic plants exhibited improved thermotolerance, we developed a quantitative assay for thermotolerance based on the measurement of hypocotyl

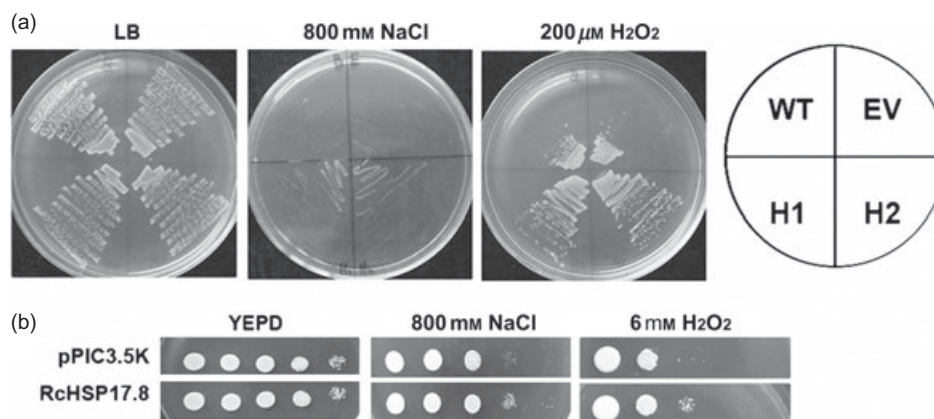


Figure 5. Heterogeneous expression of RcHSP17.8 in *Escherichia coli* strain BL21 and yeast *Pichia pastoris* strain SMD1168 enhances the host's stress tolerance. (a) Cell viability of *E. coli* BL21 WT and transformants grown on LB medium with 800 mmol NaCl or 200 μmol H₂O₂. Scale bar = 1 cm. (b) Growth of *Pichia pastoris* SMD1168 cells on yeast extract peptone dextrose medium with 800 mmol NaCl or 6 mmol H₂O₂. EV, transformants with empty vector; H1, H2, two positive RcHSP17.8 clones; WT, wild type.

elongation (Hong & Vierling 2000). Wild-type and 35S::RcHSP17.8 plants were germinated on MS medium, and then transferred to new MS plates for vertical growth. Seedlings were grown in the dark for 3 d at 22 °C and then heat-shocked at 45 °C for 2 h before recovering for 3 d at 22 °C. The extent of hypocotyl elongation after recovery was measured. As shown in Fig. 7b, in transgenic lines, L1 and L3, the relative hypocotyl length was longer than the wild type following heat shock. To further examine the thermotolerance acquired by the transgenic plants, soil-cultivated 10 d seedlings were shifted from normal temperature (22 °C) to non-permissive temperature (45 °C) for 2 h heat stress, and photos were taken after 3 d of recovery. Two lines of transgenic seedlings grew better than the wild type after being subjected to the non-permissive temperature (45 °C) whereas all seedlings grew equally at normal temperature (22 °C). The survival rate of L3 transgenic seedlings was nearly twice of that of wild-type seedlings, whereas the survival rate of L1 transgenic seedlings was 1.5 times higher than the wild-type seedlings (Fig. 7a).

SOD has been suggested to be an important enzyme in protecting cells against oxidative stress that may be caused

by heat stress. Four-week-old transgenic *Arabidopsis* seedlings and the wild type grown in soil were heat-shocked at 45 °C for 2, 4, and 6 h. The rosette leaves were taken for SOD activity assays. We found that the SOD activity ascended faster in leaves of the transgenic *Arabidopsis* compared with the wild-type plants under 45 °C for 6 h (Fig. 7c), but the SOD activity values of plants under 45 °C for 4 h were not significant different and resembled the values of the 2 h treatment (data not shown). We then measured electrolyte leakage, which is thought to reflect stress caused impairment-induced lipase activity, leading to lipid phase changes and membrane leakiness (Sharom *et al.* 1994). As shown in Fig. 7d, transgenic *Arabidopsis* plant leaves had a lower electrolyte ion leakage than wild-type leaves when plants were under 45 °C for 2 h, but there were no significant differences between two plants with 4 and 6 h of heat treatment (data not shown). Therefore, the results suggested that transgenic RcHSP17.8 *Arabidopsis* plants exhibit higher tolerance and lower level of impairment compared with wild-type plants under heat shock stress.

To test salt stress tolerance in 35S::RcHSP17.8 *Arabidopsis* plants, a quantitative root elongation assay was performed. We measured the elongation of roots of vertically grown seedlings, but no significant difference was observed among the L1, L3 and wild-type plants (data not shown), all seedlings struggled under high concentrations of NaCl (250 mmol). However, using the proline content estimation assay, we observed that the transgenic plants had a higher proline content than the wild-type plants (Fig. 8b). Proline accumulation is another characteristic feature in protecting cells against different stresses, so this suggested that transgenic RcHSP17.8 *Arabidopsis* plants exhibit higher salt stress resistance than wild-type plants.

We also employed the root elongation assay to determine whether RcHSP17.8 enhances tolerance to mannitol-induced osmotic stress. Wild-type and 35S::RcHSP17.8 plants were germinated on MS medium for 9 d before transfer to an MS medium plate supplemented with different

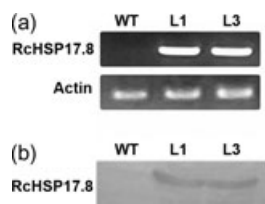


Figure 6. RT-PCR and Western blot analysis of transgenic RcHSP17.8 *Arabidopsis* lines. (a) RT-PCR analysis. mRNAs were extracted from rosette leaves of 4-week-old WT and T3 generation transgenic RcHSP17.8 *Arabidopsis*. (b) Western analysis. Proteins were extracted from rosette leaves of 4-week-old WT and T3 generation transgenic RcHSP17.8 *Arabidopsis*. WT, wild type; L1, L3, two transgenic *Arabidopsis* lines.

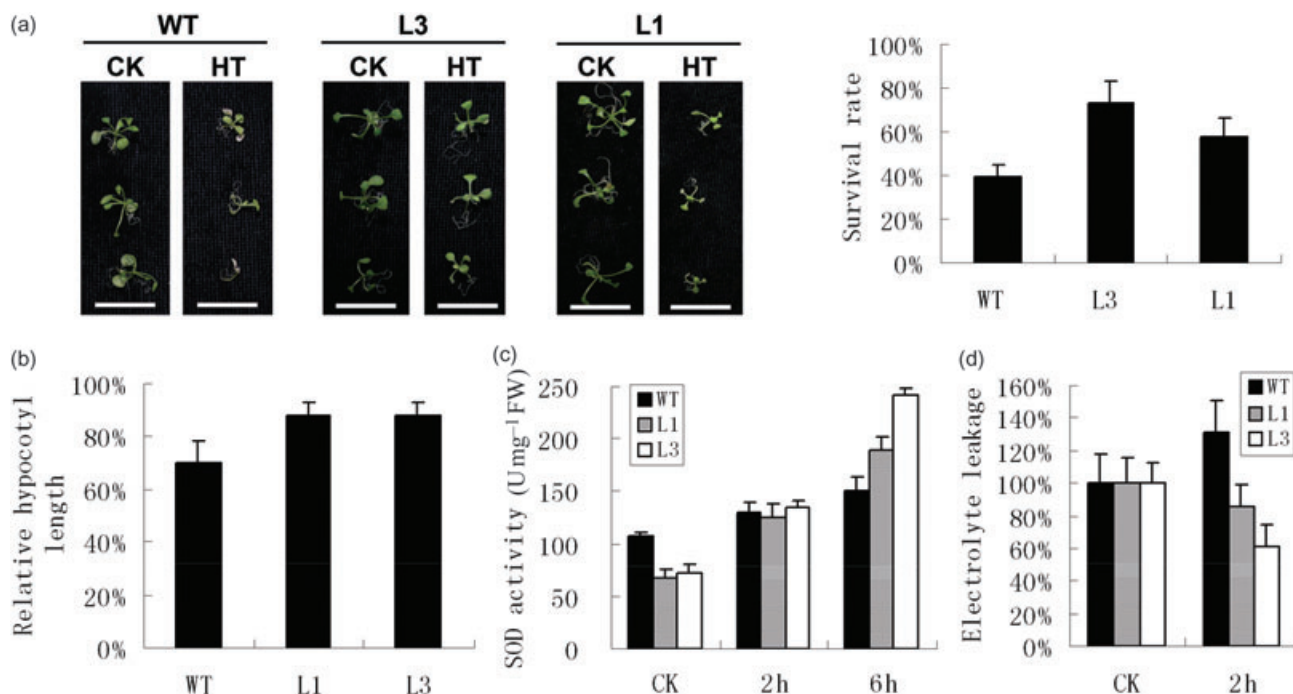


Figure 7. Ectopic expression of RcHSP17.8 in *Arabidopsis* enhances the plants' thermotolerance. (a) Soil-cultivated 10 d seedlings were shifted from normal temperature (22 °C) to non-permissive temperature (45 °C) for 2 h heat stress, and photos were taken after 3 d of recovery (22 °C). The percentage of survivors (ratio of survived to total plants) calculated. Bars show means \pm standard deviation (SD). Scale bar = 1 cm. (b) Hypocotyl elongation experiment of transgenic *Arabidopsis* seedlings and WT. Plants were germinated on Murashige and Skoog (MS) medium plates, and then transferred to a new MS plate. Seedlings were grown in the dark for 3 d and then heat-shocked at 45 °C for 2 h before recovering for 3 d at 22 °C and then photographed, and the extent of hypocotyl elongation after recovery was measured and relative hypocotyls length was calculated by HT (high temperature 45 °C)/CK (normal temperature). Bars show means \pm SD. (c) Superoxide dismutase (SOD) activity assays of transgenic *Arabidopsis* and the WT. Four-week-old seedlings grown in soil were heat-shocked at 45 °C for 2, 4, 6 h, and the rosette leaves were taken for SOD activity assays. Results were from three independent experiments. Bars show means \pm SD. (d) Comparison of leaf electrolyte leakage between transgenic *Arabidopsis* and the WT. Four-week-old seedlings grown in soil were heat-shocked at 45 °C for 2, 4, and 6 h, and the rosette leaves were taken for electrolyte leakage measurements. Results were from three independent experiments. Bars show means \pm SD. CK, seedlings under normal conditions for control; HT, seedlings under non-permissive temperature (45 °C); L1, L3, two transgenic *Arabidopsis* lines; WT, wild type.

concentrations (350, 450, 550 mmol) of mannitol for 7 d. As shown in Fig. 8a, quantitative relative root length revealed that transgenic plants grew better than wild-type plants. This conclusion was also validated in the proline content estimation assay (Fig. 8b).

For drought stress treatment, water was withheld from 2-week-old plants for 15 d, plants were transferred to normal conditions for 14 d of recovery before being photographed. As shown in Fig. 9a and b, the two lines of transgenic *Arabidopsis* plants recovered better after drought stress treatment than the wild-type plants. We also calculated the relative silique number, whole plant fresh weight and rosette leaf fresh weight, as shown in Fig. 9c–e, respectively. All these results demonstrated that the expression of *RcHSP17.8* in *Arabidopsis* enhances the transformed plants' drought tolerance.

It is well established that in comparison with seedlings or germinating seeds, transgenic *Arabidopsis* plants have a higher level of basal tolerance to high temperature and other stress conditions. Taken together, the constitutive expression of *RcHSP17.8* in *Arabidopsis* confers on the

transformed plants higher thermotolerance and resistance to salt, drought and osmotic stresses.

DISCUSSION

There are many studies focusing on the function of sHSPs. Nevertheless, their biological functions have still not been fully understood. In this study, we isolated and characterized a full-length cDNA clone that encodes RcHSP17.8 from *R. chinensis*. The highest DNA sequence identity found in GenBank was to cytosolic class I sHSPs, and this was validated by the results of alignment and phylogenetic analysis with sHSP sequences of other plants (Figs 1 & 2). However, though the middle and C-terminal amino acids of RcHSP17.8 are homologous to other sHSPs listed in Fig. 1, the N-terminal region of RcHSP17.8 is much different from others. This difference may partially explain why RcHSP17.8 could increase resistance to a broader array of stressors than other sHSPs that have been reported.

Numerous studies have previously documented a correlation between sHSPs induction and adaptation to various

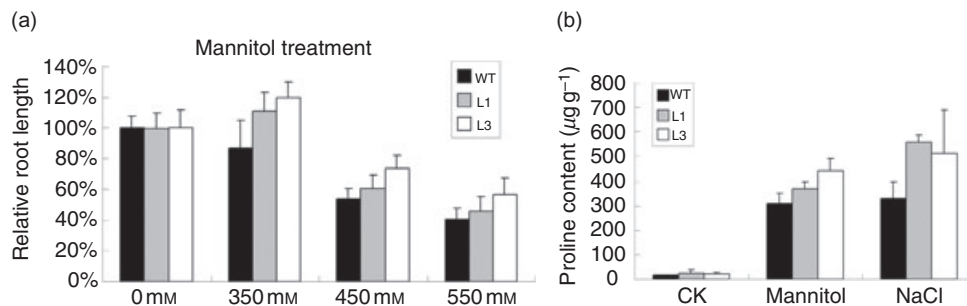


Figure 8. Ectopic expression of RcHSP17.8 in *Arabidopsis* enhances the plants' resistance to salt and osmotic stresses. (a) WT and transgenic plants were germinated on Murashige and Skoog (MS) medium plates for 9 d before transferred to a new MS medium plate supplemented with different concentrations (350, 450, 550 mmol) of mannitol for 7 d. Their root length was measured and relative root length was calculated. Bars show means \pm standard deviation (SD). (b) Proline content estimation of transgenic *Arabidopsis* and the WT. Four-week-old seedlings grown in soil were irrigated with 200 mmol of NaCl or 350 mmol of mannitol for 5 d, and the rosette leaves were taken for proline content estimation. Bars show means \pm SD. L1, L3, two transgenic *Arabidopsis* lines WT, wild type.

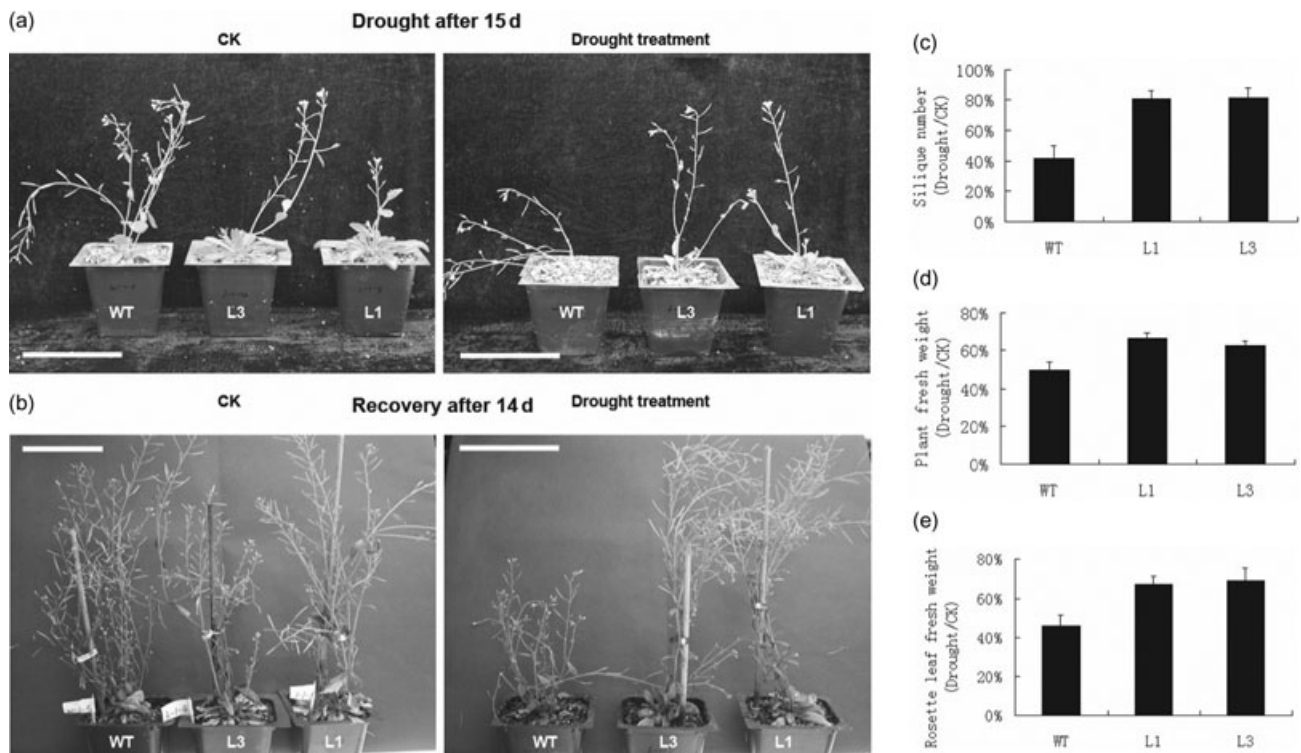


Figure 9. Ectopic expression of RcHSP17.8 in *Arabidopsis* enhances the plants drought stress resistance. (a) Drought stress was inflicted on 4-week-old plants by withholding water for 15 d. Scale bar = 5 cm. (b) Drought treated plants were transferred to normal conditions for 14 d for water recovery. Scale bar = 5 cm. (c) Relative silique number calculation of transgenic *Arabidopsis* (L1 and L3) and wild type (WT) after drought treatment. Siliques of 14 d recovering plants after drought treated were counted and relative numbers were calculated. Bars show means \pm standard deviation (SD). (d) Whole plant fresh weight measurement of transgenic *Arabidopsis* (L1 and L3) and WT after drought treatment. All 14 d recovering plants after drought treated were wholly weighed and relative plant fresh weight was calculated. Bars show means \pm SD. (e) Rosette leaf fresh weight measurement of transgenic *Arabidopsis* (L1 and L3) and WT after drought treatment. Rosette leaves of 14 d recovering plants after drought treated were weighed and relative rosette leaf fresh weight was calculated. Bars show means \pm SD. CK, plants grown under normal conditions; drought treatment, plants grown by withholding water.

stresses in plants. The tomato chloroplast small sHSP, HSP21, is induced by heat treatment in leaves (Neta-Sharir *et al.* 2005). Cortical parenchyma cells of mulberry trees accumulate endoplasmic reticulum sHSPs as a result of seasonal cold acclimation (Ukaji *et al.* 1999). A rice chloroplast-localized sHSP (Oshsp26) is induced by heat and oxidative stress (Lee *et al.* 2000). However, few of these works fixed a correlation between one sHSP and most of these abiotic stresses. In this work we demonstrated that the expression of *RcHSP17.8* is rapidly induced in leaves of *R. chinensis* under different abiotic stresses, including heat (38 and 50 °C), cold (2 °C), salt (NaCl), PEG-induced drought stress, osmotic (mannitol) and oxidative stress (H₂O₂) (Fig. 3a,c). In Fig. 3b, *RcHSP17.8* is more strongly expressed in the flowers of *R. chinensis* under heat stress. This might represent a specific mechanism for RcHSP17.8 to protect flower development correlative proteins and strengthen their adaptability in response to environmental changes that might hamper the normal blossoming of plants. It is a little different from the rose (*Rosa hybrida*) sHSP17.5-CI, which increases dramatically during flower development, and accumulates in closed bud petals and leaves only in response to heat stress (Dafny-Yelin *et al.* 2008), because there was no detectable expression of *RcHSP17.8* in any tissues, including flower and leaf, of *R. chinensis* under normal condition (Fig. 3b), whereas sHSP17.5-CI had a low-level constitute expression in petal and leaf. Besides, RcHSP17.8 could increase resistance to a broader array of stressors than sHSP17.5-CI. The increased transcript abundance of *RcHSP17.8* in the leaf of *R. chinensis* reached an expression peak in the first half hour under 38 °C heat stress (Fig. 3d), suggesting that the time of signal transduction from detecting the heat signal to regulation of sHSP expression, is instantaneous and transient. This supports the view that the expression of *RcHSP17.8*, like other sHSPs, is one of the mechanisms for plant rapid adaptation to heat stress.

Many previous works on plant sHSP function introduced their coding sequences into *E. coli* for investigating their possible *in vivo* functions. Heterologous expression of a *Quercus suber* L. class-CI sHsp protected *E. coli* from oxidative and high-temperature stress (Jofre, Molinas & Pla 2003), and expression of a chestnut (*Castanea sativa*) CsHSP17.5-CI enhanced *E. coli* viability under heat and cold stress (Soto *et al.* 1999). In this work, we introduced the RcHSP17.8 coding sequence not only into *E. coli* strain BL21, but also into *P. pastoris* strain SMD1168. As shown in Fig. 4a, we found that overexpression of RcHSP17.8 in *E. coli* was correlated with maintenance of viability under heat and cold stress conditions, and also significantly protected the *E. coli* cells from salt and oxidative stress (Fig. 5a). The results of experiments on transformant SMD1168 cells (Figs 4d & 5b) matched the *E. coli* expression experiments except the degree of visible growth difference between cells containing RcHSP17.8 and vector only. The protective effects of RcHSP17.8 seem more efficient in prokaryotic *E. coli* cells than in eukaryotic yeast cells under these stress conditions. It is known that both prokaryotic *E. coli* and eukaryotic yeast could synthesize sHSPs in response to heat

stress (Tilly *et al.* 1983; Praekelt & Meacock 1990), but sHSPs in yeast may be more homologous to plant cytosolic class I sHSPs than in *E. coli*. Consequently, the effects of overexpression of RcHSP17.8 in eukaryotic yeast cells can be masked by endogenous sHSP genes, whereas in prokaryotic *E. coli* cells, the effect of RcHSP17.8 expression is likely to be more pronounced.

Many studies have demonstrated constitutive overexpression of sHSP in plants (Malik *et al.* 1999; Sun *et al.* 2001; Wang *et al.* 2005; Zhao *et al.* 2007; Sato & Yokoya 2008). In this study, the RcHSP17.8 coding sequence was introduced into *A. thaliana* by Agrobacterium-mediated transformation and overexpressed under the control of a CaMV 35S promoter. Using the survival rate calculation, hypocotyl elongation measurement, SOD activity assay and electrolyte leakage measurement, we established that, in comparison with seedlings or germinating seeds, transgenic *Arabidopsis* plants have a higher level of basal tolerance to high temperature (Fig. 7). We also established that salt, osmotic and drought stress tolerance were enhanced in transgenic *Arabidopsis* plants as measured by the relative root length and proline content estimation (Figs 8 & 9), as demonstrated of cytosolic class II sHSP AtHSP17.6A in *Arabidopsis* (Sun *et al.* 2001). In summary, constitutive expression of *RcHSP17.8* in *Arabidopsis* confers the transformant higher thermotolerance and resistance to salt, drought and osmotic stresses.

To the best of our knowledge, there are few works demonstrating that overexpressing a cytosolic class I sHSP gene could simultaneously enhance thermotolerance, oxidative and osmotic stress tolerance of transformants. The expression of *R. chinensis* cytosolic class I sHSP *RcHSP17.8* correlates with various abiotic stresses associated with growth under unfavorable environmental conditions. Analysis of the function on this gene suggests that this gene is able to function in both eukaryotic yeast cells and prokaryotic *E. coli* cells. Furthermore, it has the ability to confer stress resistance not only to *E. coli* and yeast, but also to plants grown under a wide variety of unfavorable environmental conditions.

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REFERENCES

- Ahn Y.J. & Zimmerman J.L. (2006) Introduction of the carrot hsp17.7 into potato (*Solanum tuberosum* L.) Enhances cellular

- membrane stability and tuberization *in vitro*. *Plant, Cell & Environment* **29**, 95–104.
- Bates L.S., Waldren R.P. & Teare I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205–207.
- Bosl B., Grimminger V. & Walter S. (2006) The molecular chaperone Hsp104 – a molecular machine for protein disaggregation. *Journal of Structural Biology* **156**, 139–148.
- Chang Y.Y., Liu H.C., Liu N.Y., Hsu F.C. & Ko S.S. (2006) *Arabidopsis* Hsa32, a novel heat shock protein, is essential for acquired thermotolerance during long recovery after acclimation. *Plant Physiology* **140**, 1297–1305.
- Dafny-Yelin M., Tzfira T., Vainstein A. & Adam Z. (2008) Non-redundant functions of sHSP-CIs in acquired thermotolerance and their role in early seed development in *Arabidopsis*. *Plant Molecular Biology* **67**, 363–373.
- Dhindsa R.A., Plumb-Dhindsa P. & Thorpe T.A. (1981) Leaf Senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *Journal of Experimental Botany* **126**, 93–101.
- Frydman J. (2001) Folding of newly translated proteins *in vivo*: the role of molecular chaperones. *Annual Review of Biochemistry* **70**, 603–647.
- Gao Y. & Chen Y. (2000) [Advances in the study of the effects of danggui buxue decoction and its modified recipes on immunity and hematopoietic function]. *Zhong Yao Cai* **23**, 177–180 (in Chinese).
- Goloubinoff P. & De Los Rios P. (2007) The mechanism of Hsp70 chaperones: (entropic) pulling the models together. *Trends in Biochemical Sciences* **32**, 372–380.
- Hamilton E.W. 3rd & Heckathorn S.A. (2001) Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine. *Plant Physiology* **126**, 1266–1274.
- Harndahl U., Hall R.B., Osteryoung K.W., Vierling E., Bornman J.F. & Sundby C. (1999) The chloroplast small heat shock protein undergoes oxidation-dependent conformational changes and may protect plants from oxidative stress. *Cell Stress and Chaperones* **4**, 129–138.
- Higgins D.G. & Sharp P.M. (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237–244.
- Hong S.W. & Vierling E. (2000) Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 4392–4397.
- Jiang R., Hu Y.H., Jiang C.H., Zhao H.W., Hu S.L. & Ming F. (2006) 2D-PAGE analysis of Chinese rose leaf protein under heat shock stress. *China Biotechnology* **26**, 91–94 (in Chinese).
- Jofre A., Molinas M. & Pla M. (2003) A 10-kDa class-CI sHsp protects *E. coli* from oxidative and high-temperature stress. *Planta* **217**, 813–819.
- Kotak S., Larkindale J., Lee U., von Koskull-Doring P., Vierling E. & Scharf K.D. (2007a) Complexity of the heat stress response in plants. *Current Opinion in Plant Biology* **10**, 310–316.
- Kotak S., Vierling E., Baumlein H. & von Koskull-Doring P. (2007b) A novel transcriptional cascade regulating expression of heat stress proteins during seed development of *Arabidopsis*. *The Plant Cell* **19**, 182–195.
- Krussmann G. (1981) The history of the modern garden rose. In *The Complete Book of Roses*, pp. 67–105. Timber Press, Portland.
- Kumar S., Tamura K. & Nei M. (2004) MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinformatics* **5**, 150–163.
- Lee B.H., Won S.H., Lee H.S., Miyao M., Chung W.I., Kim I.J. & Jo J. (2000) Expression of the chloroplast-localized small heat shock protein by oxidative stress in rice. *Gene* **245**, 283–290.
- Lopez-Matas M.A., Nunez P., Soto A., Allona I., Casado R., Collada C., Guevara M.A., Aragoncillo C. & Gomez L. (2004) Protein cryoprotective activity of a cytosolic small heat shock protein that accumulates constitutively in chestnut stems and is up-regulated by low and high temperatures. *Plant Physiology* **134**, 1708–1717.
- Low D., Brandle K., Nover L. & Forreiter C. (2000) Cytosolic heat-stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones *in vivo*. *Planta* **211**, 575–582.
- Ma C., Haslbeck M., Babujee L., Jahn O. & Reumann S. (2006) Identification and characterization of a stress-inducible and a constitutive small heat-shock protein targeted to the matrix of plant peroxisomes. *Plant Physiology* **141**, 47–60.
- Malik M.K., Slovin J.P., Hwang C.H. & Zimmerman J.L. (1999) Modified expression of a carrot small heat shock protein gene, hsp17.7, results in increased or decreased thermotolerance double danger. *The Plant Journal* **20**, 89–99.
- Mamedov T.G. & Shono M. (2008) Molecular chaperone activity of tomato (*Lycopersicon esculentum*) endoplasmic reticulum-located small heat shock protein. *Journal of Plant Research* **121**, 235–243.
- Neta-Sharir I., Isaacson T., Lurie S. & Weiss D. (2005) Dual role for tomato heat shock protein 21: protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. *The Plant Cell* **17**, 1829–1838.
- Praekelt U.M. & Meacock P.A. (1990) HSP12, a new small heat shock gene of *Saccharomyces cerevisiae*: analysis of structure, regulation and function. *Molecular and General Genetics* **223**, 97–106.
- Queitsch C., Hong S.W., Vierling E. & Lindquist S. (2000) Heat shock protein 101 plays a crucial role in thermotolerance in *Arabidopsis*. *The Plant Cell* **12**, 479–492.
- Ramakrishna W., Deng Z., Ding C.K., Handa A.K. & Ozminkowski R.H., Jr (2003) A novel small heat shock protein gene, vis1, contributes to pectin depolymerization and juice viscosity in tomato fruit. *Plant Physiology* **131**, 725–735.
- Sanmiya K., Suzuki K., Egawa Y. & Shono M. (2004) Mitochondrial small heat-shock protein enhances thermotolerance in tobacco plants. *FEBS Letters* **557**, 265–268.
- Sato Y. & Yokoya S. (2008) Enhanced tolerance to drought stress in transgenic rice plants overexpressing a small heat-shock protein, sHSP17.7. *Plant Cell Reports* **27**, 329–334.
- Scharf K.D., Siddique M. & Vierling E. (2001) The expanding family of *Arabidopsis thaliana* small heat stress proteins and a new family of proteins containing alpha-crystallin domains (Acid proteins). *Cell Stress and Chaperones* **6**, 225–237.
- Sharom M., Willemot C. & Thompson J.E. (1994) Chilling injury induces lipid phase changes in membranes of tomato fruit. *Plant Physiology* **105**, 305–308.
- Siddique M., Gernhard S., von Koskull-Doring P., Vierling E. & Scharf K.D. (2008) The plant sHSP superfamily: five new members in *Arabidopsis thaliana* with unexpected properties. *Cell Stress and Chaperones* **13**, 183–197.
- Soto A., Allona I., Collada C., Guevara M.A., Casado R., Rodriguez-Cerezo E., Aragoncillo C. & Gomez L. (1999) Heterologous expression of a plant small heat-shock protein enhances *Escherichia coli* viability under heat and cold stress. *Plant Physiology* **120**, 521–528.
- Sun W., Bernard C., van de Cotte B., Van Montagu M. & Verbruggen N. (2001) At-HSP17.6A, encoding a small heat-shock protein in *Arabidopsis*, can enhance osmotolerance upon over-expression. *The Plant Journal* **27**, 407–415.

- Tilly K., McKittrick N., Zylicz M. & Georgopoulos C. (1983) The dnaK protein modulates the heat-shock response of *Escherichia coli*. *Cell* **34**, 641–646.
- Ukaji N., Kuwabara C., Takezawa D., Arakawa K., Yoshida S. & Fujikawa S. (1999) Accumulation of small heat-shock protein homologs in the endoplasmic reticulum of cortical parenchyma cells in mulberry in association with seasonal cold acclimation. *Plant Physiology* **120**, 481–490.
- Volkov R.A., Panchuk I.I. & Schoffl F. (2005) Small heat shock proteins are differentially regulated during pollen development and following heat stress in tobacco. *Plant Molecular Biology* **57**, 487–502.
- Volkov R.A., Panchuk I.I., Mullineaux P.M. & Schoffl F. (2006) Heat stress-induced H₂O₂ is required for effective expression of heat shock genes in *Arabidopsis*. *Plant Molecular Biology* **61**, 733–746.
- Wang L., Zhao C.M., Wang Y. & Liu J. (2005) Overexpression of chloroplast-localized small molecular heat-shock protein enhances chilling tolerance in tomato plant. *Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao* **31**, 167–174 (in Chinese).
- Wang W., Vinocur B., Shoseyov O. & Altman A. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science* **9**, 244–252.
- Waters E.R., Lee G.J. & Vierling E. (1996) Evolution, structure and function of the small heat shock proteins in plants. *Journal of Experimental Botany* **47**, 325–338.
- Yeh C.H., Chang P.F., Yeh K.W., Lin W.C., Chen Y.M. & Lin C.Y. (1997) Expression of a gene encoding a 16.9-kDa heat-shock protein, Oshsp16.9, in *Escherichia coli* enhances thermotolerance. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10967–10972.
- Zhao C., Shono M., Sun A., Yi S., Li M. & Liu J. (2007) Constitutive expression of an endoplasmic reticulum small heat shock protein alleviates endoplasmic reticulum stress in transgenic tomato. *Journal of Plant Physiology* **164**, 835–841.

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