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High Temperature Stress Resistance of *Escherichia coli* Induced by a Tobacco Class I Low Molecular Weight Heat-Shock Protein

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TLHS1 is a class I low molecular weight heat-shock protein (LMW HSP) of tobacco (*Nicotiana tabacum*). For a functional study of TLHS1, a recombinant DNA coding for TLHS1 with a hexahistidine tag at the amino-terminus was constructed and expressed in *Escherichia coli*. An expressed fusion protein, H_6TLHS1, was purified using a Ni^{2+} affinity column and a Sephacryl S400 HR column. A polyclonal antibody against H_6TLHS1 was produced to follow the fate of H_6TLHS1 in *E. coli*. The fusion protein in *E. coli* maintained its solubility at a temperature of up to 90°C and most of the proteins in the *E. coli* cell lysate with H_6TLHS1 were prevented from thermally induced aggregation at up to 90°C. We compared the viability of *E. coli* cells expressing H_6TLHS1 to the *E. coli* cells without H_6TLHS1 at a temperature of 50°C. After 8 h of high temperature treatment, *E. coli* cells with H_6TLHS1 survived about three thousand times more than the bacterial cells without H_6TLHS1. These results showed that a plant class I LMW HSP, TLHS1, can protect proteins of *E. coli* from heat denaturation, which could lead to a higher survival rate of the bacterial cells at high temperature.

Keywords: Cell Viability; *Escherichia coli*; Low Molecular Weight Heat-Shock Protein; Molecular Chaperone; Tobacco.

Introduction

Under heat-shock conditions, all organisms synthesize several families of evolutionally conserved heat-shock proteins (HSPs) for which fundamental functions in cell physiology and development in addition to stress-resistance are now increasingly documented. Based on the amino acid sequence homology and molecular weight, HSPs are grouped into families of HSP100/C1p (Nieto-Sotelo *et al.*, 1999; Schirmer *et al.*, 1996), HSP90 (Mogelsvang and Simpson, 1998), HSP70 (Miernyk, 1997), chaperonins (Siegler *et al.*, 1998), and the low molecular weight (LMW) HSPs (Waters *et al.*, 1996). LMW HSPs range from about 15 to 30 kDa and, unlike high molecular weight HSPs, LMW HSPs appear to be involved primarily in stress response. LMW HSPs also seem to be involved in various developmental processes, but these functions are usually linked to stress response (Arrigo, 1998). Thus, LMW HSPs are attracting much attention in stress physiology. Plants respond to stresses by synthesizing many LMW HSPs that can be up to 1% of the total cellular protein (DeRocher *et al.*, 1991; Waters *et al.*, 1996). Unlike yeast and mammalian cells that synthesize only one or a few members of LMW HSPs, the abundance and diversity of LMW HSPs are unique to plants. This implies the particularly important function of LMW HSPs in protecting plants from stresses.

Evidence is accumulating in plants for the function of LMW HSPs in heat-resistance. It has been shown that a chloroplast LMW HSP of tomato protects photosystem II electron transport during heat stress (Harndahl *et al.*, 1999; Heckathorn *et al.*, 1998). *In vitro* chaperone activity of plant LMW HSPs has also been reported (Downs and Heckathorn, 1998; Jinn *et al.*, 1995; Lee and Vierling, 2000; Lee *et al.*, 1995). Reports showing strong correlations between the presence of LMW HSPs and heat resistance in plants are also accumulating, which support the function of LMW HSPs in high temperature stress resistance in plants (Banzet *et al.*, 1998; DeRocher *et al.*, 1991; Lund *et al.*, 1998; Miernyk, 1999; Visioli *et al.*, 1997). However, considering the diversity of LMW HSPs in plants, the work done to decipher the function of LMW HSPs in plants can be considered

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fragmentary. Especially, direct evidence for *in vivo* molecular chaperone function of plant LMW HSPs is very limited.

Plant LMW HSPs share conserved sequences mostly toward the carboxy terminus and are found in high-molecular mass complexes which range in size from approximately 200 to 300 kDa (Chen *et al*., 1994; Jinn *et al*., 1995; Lee *et al*., 1995; Suzuki *et al*., 1998). Plant LMW HSPs have been grouped into several classes based on sequence homology, immunological cross-reactivity, and localization in different cellular compartments. There are two classes of cytosolic LMW HSPs (class I and II), as well as distinct types of LMW HSPs localized to the ER, the mitochondria, and the chloroplast (Waters *et al*., 1996). Previously, we reported a cDNA clone, *TLHS1*, coding for a class I LMW HSP of tobacco (Park and Hong, 1998). The *in vivo* molecular chaperone activity of TLHS1 will be presented in the present report. Overexpression of histidine tagged TLHS1, H_6TLHS1, in *Escherichia coli* protected most of the *E. coli* proteins from aggregation at high temperature. In turn, *E. coli* cells overexpressing H_6TLHS1, and otherwise lethal, showed much enhanced viability under high temperature.

Materials and Methods

Chemicals and bacterial strains *Taq* DNA polymerase and DNA ligase were from Promega (USA). Chelating sepharose fast flow and sephacryl S400 HR were from Pharmacia (Sweden). The enhance chemiluminescence kit and sequencing kit were from Amersham (USA). Freund's adjuvant and other chemical reagents were from Sigma (USA). *E. coli* strain DH5α was used for recombinant DNA processes, and strain MC1061 was used for the expression of recombinant proteins.

Preparation of the H_6TLHS1 expression construct The open reading frame of *TLHS1* was amplified by the polymerase chain reaction with primers covering both termini of the coding region of TLHS1 cDNA which encodes a full length 159 amino acid polypeptide. The 5′ primer was 5′ATATGAGCTCATGTCTCTGATTCCAAGC3′ and the 3′ primer was 5′TATAGCATGCTTAACCAGAGATGTCAATG3′. *Sac*I and *Sph*I restriction sites, respectively underlined, were introduced into the primers. After heating to 95°C for 5 min, PCR was performed for 30 cycles of (95°C, 30 s; 50°C, 30 s; and 72°C, 1 min) using a thermal cycler (Perkin-Elmer Co., USA). The amplified product was digested with *Sac*I and *Sph*I, and ligated into the pBADNH expression vector at the *Sac*I and *Sph*I sites to produce H_6TLHS1, i.e. wild type TLHS1 fused to an N-terminal hexahistidine tag. The sequence of the cloned coding region was confirmed by dideoxy chain termination sequencing (Sanger *et al*., 1977) using Sequenase version 2.0 (Progema, USA).

Expression and purification of H_6TLHS1, and polyclonal antibody production against H_6TLHS1 The expression construct was transformed into *E. coli* strain MC1061. Exponentially growing cells were induced with 0.125% (w/v) arabinose for 4 h and harvested by centrifugation at $3{,}000 \times g$ for 10 min. After the pellet was resuspended in buffer (20 mM Tris-HCl, 250 mM KCl, 2.8 mM β-mercaptoethanol, pH 8.0), it was treated with lysozyme for 3 min at 37°C, freezing-thawed, and centrifuged at $20{,}000 \times g$ for 30 min. The resulting soluble extract was precipitated with 80% ammonium sulfate followed by a centrifugation at $15{,}000 \times g$ for 15 min. Then, the precipitate was dialyzed against buffer A (25 mM Tris-HCl, 100 mM KCl, 10% glycerol, 2.8 mM β-mercaptoethanol, pH 8.0), and H_6TLHS1 was partially purified on a Ni^{2+} affinity column by 20 mM imidazole washing and 250 mM imidazole elution in buffer A. Fractions containing the eluted H_6TLHS1 were pooled and further purified on a Sephacryl S400 HR in buffer A. H_6TLHS1 was eluted shortly after the void column volume. Purified protein was used to produce a polyclonal antibody against H_6TLHS1. One hundred μg of H_6TLHS1 in Freund's complete adjuvant was injected into a New Zealand female white rabbit and boosted three times at 3-week intervals using the same amount of H_6TLHS1 in Freund's incomplete adjuvant. Immune serum was taken 5 days after the fourth injection.

Thermal stability of proteins in the *E. coli* cell lysates *E. coli* cell lysates with (from pBAD-TLHS1) or without (from pBADNH only) H_6TLHS1 were prepared in the same way as described above for protein purification except for the step of ammonium sulfate precipitation. Protein in the cell lysate was diluted to 3 mg/ml in buffer A. Diluted cell lysates of 100 μl were covered with mineral oil and incubated at a temperature from 30°C to 100°C for 15 min in a circulating water bath (Polyscience Co., USA). Cell lysates were then allowed to cool to room temperature, the mineral oil was removed, and the samples were centrifuged at $15{,}000 \times g$ for 10 min to remove the denatured proteins. The supernatant was analyzed on a 15% SDS-polyacrylamide gel (Sambrook *et al*., 1989).

Immunoblot analysis An equal amount of soluble protein was separated in each lane on a 15% SDS-polyacrylamide gel. Following electrophoresis, proteins on a polyacrylamide gel were electroblotted onto a nitrocellulose membrane with a glycine electrode buffer and reacted with anti-H_6TLHS1 antiserum at a 1:2,000 dilution (Sambrook *et al*., 1989). Protein bands crossreacting with antibodies were identified by reaction with horseradish peroxidase conjugated goat anti-rabbit IgG and visualized with an enhanced chemiluminescence kit as recommended by the manufacturer (Amersham).

Cell viability For the measurement of cell viability under high temperature stress conditions, exponentially growing *E. coli* cells transformed with pBAD-TLHS1 or pBADNH were cultured with 0.125% (w/v) arabinose for 2 h and then transferred to 50°C for heat treatment. Culture aliquots of 100 μl were taken at 2, 4 and 8 h of heat treatment, and serial dilutions were plated on a LB agar plate containing ampicillin. Cell viability was determined by counting colony forming units (CFU) after incubation of the plates overnight at 37°C. Cell viability was plotted as the percentage of CFUs formed after heat treatment relative to the number of CFUs formed in each culture without heat treatment.

Results

A cDNA clone, *TLHS1*, coding a tobacco LMW HSP was isolated from a cDNA library of heat-shocked tobacco plants, and this cDNA clone was characterized to code for a protein belonging to the class I LMW HSP family by comparison of the deduced amino acid sequences and hydropathy plots with other known LMW HSPs (Park and Hong, 1998). *TLHS1* showed a typical expression pattern of the heat-shock protein gene under high temperature stress conditions (Park and Hong, 1998; Park *et al.*, 1994).

For a functional analysis and easier purification of TLHS1, the coding region of *TLHS1* was inserted into an *E. coli* expression vector, pBADNH, to produce histidine tagged TLHS1, H_6TLHS1 (Fig. 1). *E. coli* cells containing pBADNH with the coding region of *TLHS1* were grown to mid-log phase (A_{600} = 0.3), and induced to produce the fusion protein by adding arabinose. Cells were harvested, and cell lysates on a 15% SDS-polyacrylamide gel showed overexpression of H_6TLHS1 in *E. coli* as a soluble protein with a size of about 21 kDa (Fig. 2, lane 2). H_6TLHS1 was purified on a Ni^{2+} chelate affinity column and a Sephacryl S400 HR column. The purity of the purified H_6TLHS1 was over 95% (Fig. 2, lane 3).

The molecular chaperone activity of class I LMW HSPs *in vitro* at high temperature has been reported from various sources (Ehrnsperger *et al.*, 1997; Kantorow and Piatigorsky, 1994; Leroux *et al.*, 1997; Muchowski and Clark, 1998; Muchowski *et al.*, 1997; Spiess *et al.*, 1999; Yang *et al.*, 1999). Plant class I LMW HSP has also been shown to protect model proteins, like malate dehydrogenase, from heat denaturation and aggregation *in vitro* (Lee *et al.*, 1995, 1997). When the crude extract from *E. coli* was heated and spun down to remove heat-denatured and aggregated proteins, a significant decrease in soluble proteins in *E. coli* could be observed from 50°C, the temperature that usually induces bacterial cell lysis (Fig. 3A). On the other hand, in the crude extract from *E. coli* which overproduced H_6TLHS1, we could not observe significant heat-denaturation of cellular proteins at a temperature of up to 90°C. Over 70% of the proteins in the cell lysate with H_6TLHS1 were protected from aggregation at 90°C (Fig. 3B). Incubation of the crude extract at 100°C resulted in the precipitation of most *E. coli* cellular proteins and H_6TLHS1 (Fig. 3B). H_6TLHS1 in the *E. coli* cell-free extract was confirmed by protein blot analysis with a polyclonal antibody against H_6TLHS1 (Fig. 3C).

To investigate the molecular chaperone function of H_6TLHS1 *in vivo*, the attenuation in lethality at 50°C was checked for the *E. coli* cells overproducing H_6TLHS1. The viability of *E. coli* cells expressing H_6TLHS1 was compared with that of *E. coli* cells carrying the vector only. Heating at 50°C for 2 h caused pBADNH cells to survive at a level of only 0.4%, whereas more than 30% pBAD-TLHS1 cells survived. The difference in survival rate under heat stress became larger as the length of heat stress increased. After 8 h, the viability of pBAD-TLHS1 cells was about three thousand fold higher than that of pBADNH cells (Fig. 4).

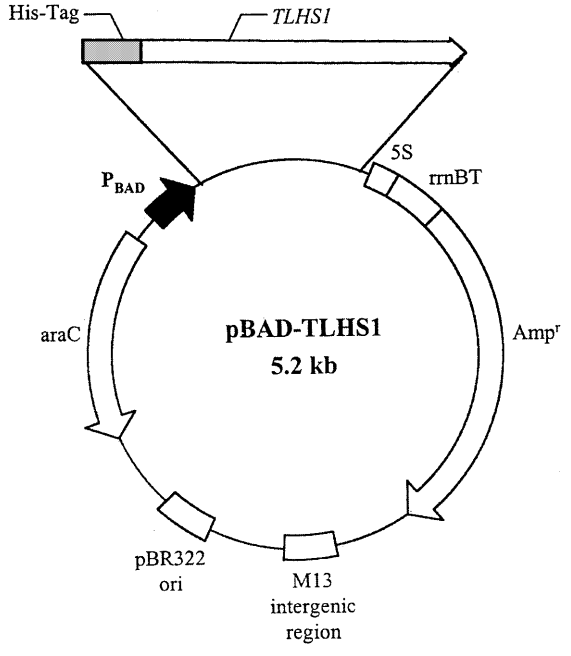

Fig. 1. Map of the plasmid constructed to overexpress H_6TLHS1 in *E. coli*.

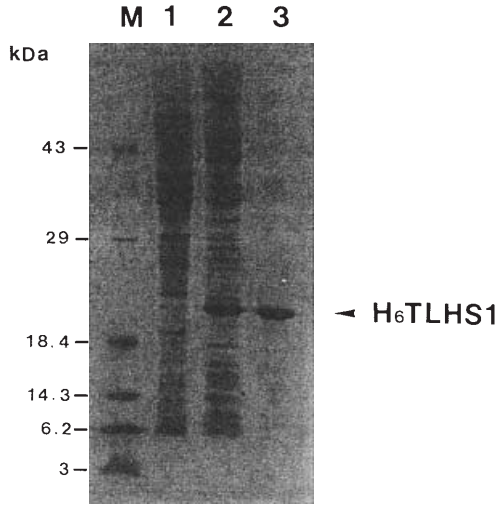

Fig. 2. Overexpression of H_6TLHS1 in *E. coli* and purification of H_6TLHS1. Proteins were analyzed on 15% SDS-PAGE and stained with coomassie brilliant blue. Lane 1, lysate of *E. coli* cultured without arabinose; lane 2, lysate of *E. coli* cultured with 0.125% (w/v) arabinose; lane 3, H_6TLHS1 purified on a Ni^{2+} affinity column and a Sephacryl S400 HR column. M, protein size marker.

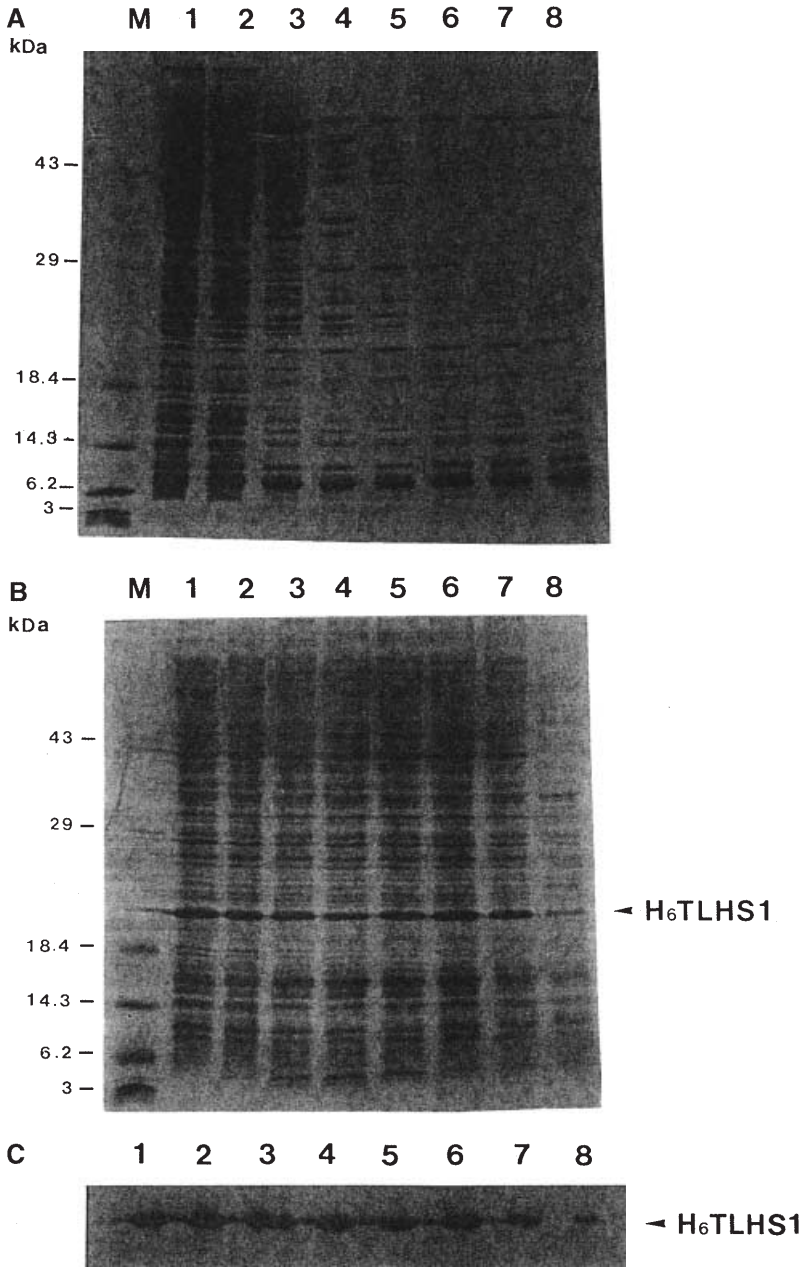

Fig. 3. Effect of H_6TLHS1 on the thermal stability of proteins in an *E. coli* cell lysate. The lysate with (from pBAD-TLHS1) or without (from pBADNH only) H_6TLHS1 was incubated at 30°C (1), 40°C (2), 50°C (3), 60°C (4), 70°C (5), 80°C (6), 90°C (7) and 100°C (8) for 15 min, and then soluble proteins were analyzed on 15% SDS-PAGE. **A**. SDS-PAGE analysis of an *E. coli* cell lysate without H_6TLHS1. **B**. SDS-PAGE analysis of an *E. coli* cell lysate with H_6TLHS1. **C**. Protein blot hybridization of the gel in (**B**) with a polyclonal antibody against H_6TLHS1. M, protein size marker.

Discussion

All plants synthesize abundant multiple LMW HSPs encoded by superfamily genes; on the other hand animals and yeast have only a few LMW HSP members. Plant LMW HSPs are grouped into six gene families: cytosolic class I and II, mitochondria, chloroplast, endoplasmic reticulum and membrane LMW HSPs. A single plant species synthesizes several LMW HSPs of the same class that show high homology in amino acid sequence (Waters *et al.*, 1996).

Evidence is accumulating in plants for the function of LMW HSPs in heat-resistance. It has been shown that a chloroplast LMW HSP of tomato protects photosystem II electron transport during heat stress (Harndahl *et al.*, 1999; Heckathorn *et al.*, 1998). The *in vitro* chaperone activity of plant LMW HSPs also has been reported (Downs and Heckathorn, 1998; Jinn *et al.*, 1995; Lee

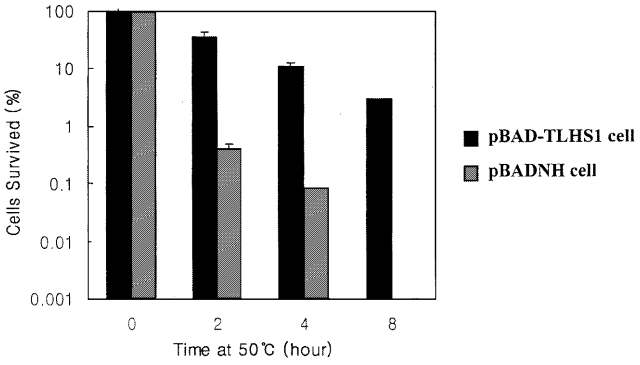

Fig. 4. Influence of H_6TLHS1 on *E. coli* cell viability at 50°C. *E. coli* cells carrying pBAD-TLHS1 or pBADNH were heat-treated at 50°C for 0, 2, 4 and 8 h. Cell viability was plotted as the percentage of colony forming units relative to the number of colonies without heat treatment from 6 replicates. Error bars represent the standard deviation.

and Vierling, 2000; Lee *et al.*, 1995). Reports showing strong correlations between the presence of LMW HSPs and heat resistance in plants that support the function of LMW HSPs in high temperature stress resistance are also accumulating (Banzet *et al.*, 1998; DeRocher *et al.*, 1991; Lund *et al.*, 1998; Miernyk, 1999; Visioli *et al.*, 1997). However, direct evidence showing a molecular chaperone function of plant LMW HSP *in vivo* has only very recently been reported in a single case. LMW HSP was purified from the chestnut cotyledon, and a cDNA for the protein was cloned. The cDNA clone, *CsHSP17.5*, belonging to cytosolic class I LMW HSP, was introduced into *E. coli* and was overexpressed. *E. coli* cells overexpressing CsHSP17.5 showed a two fold higher survival rate at the temperature of 50°C for 1 h (Soto *et al.*, 1999). Considering the diversity of LMW HSPs in plants, the result from CsHSP17.5 cannot be a proof to generalize the molecular chaperone function of LMW HSPs *in vivo*. The fact that the recalcitrant seeds of chestnut, the place where CsHSP17.5 was abundantly expressed, have one of the highest moisture contents known at shedding again raised the necessity to analyze the molecular chaperone activity of another plant LMW HSP in *E. coli*, an intermediate step toward the *in vivo* assay of plants.

Molecular chaperones are proteins that bind to other proteins in a non-native structural state. It has been reported that LMW HSPs can selectively bind to non-native proteins and prevent their aggregation. HSP18.1, a dodecameric LMW HSP from pea, prevents the aggregation of malate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase heated to 45°C (Lee *et al.*, 1995). Mammalian LMW HSPs and α-crystallins can function as molecular chaperones by preventing thermal aggregation of other proteins (for a recent review, see Arrigo, 1998). It is generally agreed that LMW HSPs can form a complex with partially denatured substrate polypeptides, and the hydrophobic region in LMW HSPs is important for binding with substrate polypeptides (Ehrnsperger *et al.*, 1997). Proteins in the cell lysate with H_6TLHS1 were maintained soluble at a temperature as high as 90°C (Fig. 3B), whereas those without H_6TLHS1 started to precipitate at 50°C (Fig. 3A). Over 70% of proteins in the cell lysate with H_6TLHS1 were protected from aggregation at 90°C. This result showed that H_6TLHS1 acts as a molecular chaperone with little substrate specificity that could protect various proteins from thermally induced aggregation *in vivo*. Since *E. coli* does not have a homolog of plant LMW HSPs (Buchner, 1996), it is not likely that *E. coli* cells have specific systems interacting with plant LMW HSPs. The result shown in Fig. 3 can be a strong explanation for the *E. coli* cells with pBAD-TLHS1 exhibiting strong heat-resistance. Broad range protection of proteins from heat denaturation by TLHS1 can certainly be a lot of help for *E. coli* cells under high temperature stress.

There seemed to be a big difference between CsHSP17.5, the chestnut class I LMW HSP (Soto *et al.*, 1999), and TLHS1 in this report on the survival rate of *E. coli* cells under the high temperature stress of 50°C, i.e. a two fold increase from CsHSP17.5 after 1 h of heat-treatment and about a seventy fold increase from TLHS1 after 2 h of heat-treatment, which is certainly one important point for further investigation. Since the system used in both work was basically identical, the difference must be mainly from the difference of the plant LMW HSPs in both studies, for example they show about 25% difference in amino acid residues. Protection of most *E. coli* proteins from heat aggregation at a temperature as high as 90°C by TLHS1 is another important point for further investigation. Whatever the mechanism for this strong protection of *E. coli* cells and proteins from high temperature stress is, it must reside in the molecular characteristics of TLHS1.

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