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Expression of a gene encoding a 16.9-kDa heat-shock protein, Oshsp16.9, in *Escherichia coli* enhances thermotolerance

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ABSTRACT **A gene encoding the rice 16.9-kDa class I low-molecular-mass (LMM) heat-shock protein (HSP), Oshsp16.9, was introduced into *Escherichia coli* using the pGEX-2T expression vector to analyze the possible function of this LMM HSP under heat stress. It is known that *E. coli* does not normally produce class I LMM HSPs. We compared the survivability of *E. coli* XL1-Blue cells transformed with a recombinant plasmid containing a glutathione *S*-transferase (GST)–Oshsp16.9 fusion protein (pGST-FL cells) with the control *E. coli* cells transformed with the pGEX-2T vector (pGST cells) under heat-shock (HS) after isopropyl β-D-thiogalactopyranoside induction. The pGST-FL cells demonstrated thermotolerance at 47.5°C, a treatment that was lethal to the pGST cells. When the cell lysates from these two *E. coli* transformants were heated at 55°C, the amount of protein denatured in the pGST-FL cells was 50% less than that of the pGST cells. Similar results as pGST-FL cells were obtained in pGST-N78 cells (cells produced a fusion protein with only the N-terminal 78 aa in the Oshsp16.9 portion) but not in pGST-C108 cells (cells produced a fusion protein with C-terminal 108 aa in the Oshsp16.9 portion). The acquired thermotolerant pGST-FL cells synthesized three types of HSPs, including the 76-, 73-, and 64-kDa proteins according to their abundance at a lethal temperature of 47.5°C. This finding indicates that a plant class I LMM HSP, when effectively expressed in transformed prokaryotic cells that do not normally synthesize this class of LMM HSPs, may directly or indirectly increase thermotolerance.**

All organisms respond to heat stress by inducing the synthesis of a group of proteins called heat-shock proteins (HSPs). HSPs can be induced at various temperatures in a wide variety of cells and organisms. The induction of HSP synthesis correlates with tolerance to extreme heat. The roles of individual HSPs in stress protection have been shown to be different, and different organisms rely on different sets of HSPs to establish tolerance to similar levels of heat stress (1). For example, tolerance to extreme stress depends largely upon Hsp104 in yeast, Hsp70 in *Drosophila*, and Hsp70, Hsp27, and perhaps Hsp110 in mammals (2–5).

The HSP families in plants are more complex than in other organisms. All plants synthesize multiple families of low-molecular-mass (LMM) HSPs, ranging in size from ≈15 to 30 kDa (6). The LMM HSPs are classified into six multi-gene families that encode the most abundant proteins induced by heat stress in many plant species. They comprise a gene superfamily encoding proteins specific to different cellular compartments (7, 8). Compared with higher plants, other eukaryotes contain far fewer LMM HSP genes. For example, four LMM HSP genes are found in *Drosophila*, and only one LMM HSP gene has been identified in yeast and mammals (9). No homologous LMM HSPs have been found in prokaryotes except in *Mycobacterium* (10). In *Escherichia coli*, there are several high-molecular-mass (HMM) HSPs including GroE (Hsp60), DnaK (Hsp70), HtpG (Hsp90), Clp protease (Hsp100), and Lon protease, but there are no homologous LMM HSPs.

So far, at least two general roles of HSPs have been suggested for helping cells to cope with stress-induced damage to proteins (1). Some HSPs can promote degradation of abnormal proteins, whereas others can reactivate stress-damaged proteins and function as "molecular chaperones" to prevent the aggregation or promote the proper refolding of denatured proteins (1). However, little is known about the function of LMM HSPs. Recently, it has been demonstrated that mammalian LMM HSPs and the related α-crystallin lens proteins possess molecular chaperone activity *in vitro* (11–14). LMM HSPs were able to promote refolding of chemically denatured proteins in an ATP-independent manner, in contrast to the ATP-dependent molecular chaperones of the Hsp60 and Hsp70 classes (15). Other recent data have suggested that mammalian LMM HSPs may regulate actin filament dynamics (16–18).

In soybean (*Glycine max*), under heat-shock (HS) conditions the accumulation of the LMM HSPs is increased, yielding final concentrations up to 1% of total cellular protein (19). Thermostabilization of soluble proteins can be demonstrated *in vitro* by an HSP-enriched fraction (20); such HSP-enriched fractions are exchangeable among soybean, mung bean (*Vigna radiata* L.), and rice (*Oryza sativa* L.) in providing protein thermostability (21). In soybean and pea (*Pisum sativum* L.), the class I and class II LMM HSPs form multimeric complexes under native conditions (22, 23), and such complexes function as molecular chaperones in thermostabilization by interacting with soluble proteins *in vitro* after heating (22, 23).

We have previously shown that a recombinant rice 16.9-kDa LMM HSP can form a complex in *E. coli* cells and provide thermoprotection to proteins *in vitro* (24). In an attempt to understand the possible structure–function relationship of the plant class I LMM HSPs, we introduced a rice cDNA encoding a 16.9-kDa class I LMM HSP into *E. coli*, an organism that does not synthesize homologous LMM HSPs in nature, and studied its function in thermotolerance of the transformed *E. coli* cells. We suggest here that the transgenic expression of a rice 16.9-kDa LMM HSP apparently can result in a thermoprotection of *E. coli* cells to a lethal treatment *in vivo*.

MATERIALS AND METHODS

Construction of *E. coli* Strains Expressing Glutathione *S*-Transferase (GST) and GST–Oshsp16.9 Fusion Proteins.

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Abbreviations: GST, glutathione *S*-transferase; HMM, high-molecular-mass; HS, heat shock; HSP (Hsp, hsp), HS protein; IPTG, isopropyl β-D-thiogalactopyranoside; LMM, low-molecular-mass; LB, Luria–Bertani.

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The expression vector (pGEX-2T) used for gene fusion construction was the GST Gene Fusion System (Pharmacia). The *Oshsp16.9* (reported as "*pTS1*" in ref. 25) coding region (designated as "FL", full-length) of about 0.45 kb was prepared by PCR with the 5′ end primer (5′-GCGGATCCATGTCGCTGGTG-3′) to add a *Bam*HI site 1 base upstream from the start codon ATG (nt 118) and with the 3′ end primer (5′-CCGAATTCCTTAACCGGAGATCTC-3′) to add an *Eco*RI site after the stop codon TAA (nt 568) using the PCR kit (Perkin–Elmer/Centus). Two deletion constructs to encode the N-terminal 78 aa, designated as "N78," and the C-terminal 108 aa, designated as "C108," of the Oshsp16.9 were also prepared by PCR using primer sets of 5′-GCGGATCCATGTCGCTGGTG-3′/5′-GTGAATTCCAGCACGTTGCCTT-3′ and 5′-AATGGATCCGCCCGCATCGACTGG-3′/5′-CCGAATTCCTTAACCGGAGATCTC-3′, respectively, to introduce a *Bam*HI site (5′ end) and a *Eco*RI site (3′ end) to each construct. The PCR was 40 sec at 94°C, 40 sec at 44°C (50°C for "C108" construct), 40 sec at 72°C for 30 cycles followed by 10 min at 72°C. The PCR products were gel-purified and then ligated into the pGEX-2T plasmid at *Bam*HI and *Eco*RI sites. The resultant recombinant plasmids can produce fusion proteins in which the N terminus is the GST and the C terminus is the FL, N78, or C108 of Oshsp16.9 with two extra amino acids in-between. *E. coli* strain XL1-Blue was transformed with the vector alone or the three recombinant plasmid DNA to produce pGST cells, pGST-FL cells, pGST-N78 cells, or pGST-C108 cells following the standard protocol (26).

Growth of *E. coli* Cells and Expression of GST–Oshsp16.9 Fusion Protein in *E. coli*. Wild-type and transformed *E. coli* cells were grown in Luria–Bertani (LB) broth containing 100 μg/ml of ampicillin at 37°C overnight. The overnight cultures were diluted 1,000-fold using fresh LB broth plus ampicillin, and incubation continued at 37°C. For growth curve determination, samples were taken every 30 min to measure the optical density at 600 nm. To test the induction conditions for fusion protein expression, the diluted overnight cultures were grown at 37°C until mid-log phase (3–4 h, or OD_{600} = ≈0.6). Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM, and incubation was continued at 37°C for 1–6 h. During the incubation time (total about 10 h), the growth curves of IPTG-induced cells were also determined by measuring the optical density at 600 nm every 30 min. After IPTG induction, the cultures were harvested and cell pellets were obtained by centrifugation. Pellets were resuspended in 100 μl of Laemmli sample buffer (27). Thirty micrograms of protein samples, determined by the Bradford assay (28), was subjected to SDS/PAGE.

SDS/PAGE and Western Blot Analysis. One-dimensional SDS/PAGE was performed according to Laemmli (27) using 12.5% (wt/vol) polyacrylamide gels. For immunoblotting, proteins were transferred from polyacrylamide gels to Immobilon polyvinylidene difluoride membranes (Millipore) with Tris/glycine electroblotting buffer according to Towbin *et al.* (29). Protein bands cross-reacting with the rice Oshsp16.9 antibody (24), *E. coli* GroEL antibody (a gift from M.-Y. Cheng, National Yang-Ming University, Taipei, Taiwan), *E. coli* DnaK, DnaJ, and GroES antibodies (StressGen Biotechnologies, Victoria, BC, Canada) were identified by reaction with horseradish peroxidase conjugated with goat anti-rabbit (anti-mouse for DnaK) IgG (Bio-Rad). The conditions of immunoreactions were according to the manufacturer's specification (Bio-Rad).

Thermotolerance Experiments with Transformed *E. coli* Cells. For thermotolerance experiments, cell cultures were grown as above. IPTG was added to mid-log phase cultures (≈0.6 OD_{600}) to a final concentration of 1 mM, and incubation was continued at 37°C for 2 h. After IPTG induction, the cultures were diluted to 5.0×10^6 cells·ml^{-1} and 1 ml of the culture was transferred to 47.5°C. Samples (100 μl) were taken at 0, 5, 15, 30, 45, and 60 min after 47.5°C treatment, and serial dilutions were plated in triplicate onto LB plus ampicillin plates. The plates were incubated overnight at 37°C prior to scoring colony formation to determine the percentage of survivors.

Protein Labeling. Aliquots of 1 ml diluted cells (as described above) were incubated at 37°C or 47.5°C for 15 min; cells were then labeled with 7.6×10^6 cpm of [^{35}S]methionine (Amersham; 1,000 Ci mmol^{-1}; 1 Ci = 37 GBq) for 15 min. After labeling, cells were spun down at 8,000 × *g* for 5 min, washed five times with PBS (150 mM NaCl/16 mM Na_2HPO_4/4 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.8) containing 1 mM cold methionine, and resuspended in 30 μl of Laemmli sample buffer. Samples (2 μl) were assayed for radioactivity and 15 μl samples were subjected to SDS/PAGE (12.5% acrylamide) followed by autoradiography.

Assay for Thermostability of Proteins in Transformed *E. coli* Cells. Transformed *E. coli* cells with or without IPTG treatment as described above were used to determine the amount of denatured *E. coli*-soluble proteins during the heat treatments. Cells were spun down at 8,000 × *g* for 10 min and washed with PBS twice. The cells were then broken by sonication in PBS and spun at 16,000 × *g* for 10 min. The supernatant was further centrifuged at 300,000 × *g* for 1.5 h, using a Beckman TL-100 Ultracentrifuge with TLA 100.3 rotor, to obtain the soluble protein fraction. Proteins were quantified by the Bradford assay (28). For the thermostability assay, protein samples were diluted to 4 mg·ml^{-1}, heated at 55°C for 30 min, and the denatured proteins were spun down at 16,000 × *g* for 10 min. The amount of protein in the pellet and supernatant fractions was determined.

RESULTS

Induction of Fusion Protein in Transformed *E. coli* Cells. A 16.9-kDa rice class I LMM HSP gene was introduced into *E. coli* XL1-Blue cells to analyze the possible function of this LMM HSP under heat stress. It is known that *E. coli* does not normally produce class I LMM HSPs. The *Oshsp16.9* coding region for a 16.9-kDa class I LMM HSP was cloned into the pGEX-2T expression vector for fusion protein production in *E. coli*. The pGEX-2T vector alone was also introduced into *E. coli* as a control. The pGST cells (cells transformed with vector alone) and pGST-FL cells (cells transformed with the recombinant plasmid containing the full-length Oshsp16.9) had similar growth rates when compared with the wild-type XL1-Blue *E. coli* cells (Fig. 1). After IPTG (1 mM) induction, similar growth rate of the wild-type XL1-Blue cells was observed; however, the growth of both pGST and pGST-FL cells ceased as shown in Fig. 1. The number of remaining pGST-FL cells was similar to that of pGST cells. This growth may be due to the adverse effect of overproduced foreign proteins in *E. coli*. The fusion protein was induced in transformed cells by 1 mM IPTG for up to 6 h. The pGST cells produced GST, whereas the pGST-FL cells produced a GST–Oshsp16.9 fusion protein. Two different fusion proteins with shorter sequences of Oshsp16.9 were also produced in transformed cells for further analysis. The pGST-N78 cells produced a fusion protein with only the N-terminal 78 aa in the Oshsp16.9 portion: these 78 aa residues (1–78) include the consensus II region of known plant LMM HSPs (30). The pGST-C108 cells produced a fusion protein with C-terminal 108 aa in the Oshsp16.9 portion: these 108 aa residues (43–150) include both the consensus I and consensus II regions of known plant LMM HSPs (30) without the N-terminal 42 aa residues. The growth curves of the pGST-N78 and pGST-C108 cells were similar to that of the pGST and pGST-FL cells with or without IPTG treatment (data not shown). Fig. 2*A* shows by silver staining the fusion protein produced in transformed cells

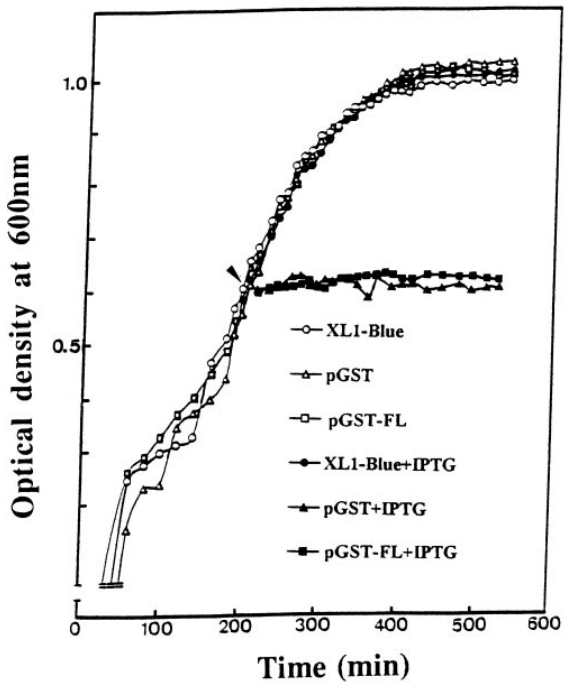

FIG. 1. Growth of wild-type and transformed *E. coli.* XL1-Blue cells at 37°C with or without IPTG. Cultures of XL1-Blue *E. coli* cells (circles), pGST cells (triangles), and pGST-FL cells (squares) were grown at 37°C (open symbols) or subjected to 1 mM of IPTG (filled symbols) as described. Samples were taken at the time indicated and the optical density at 600 nm was determined (1 OD_{600} = 6 × 10^7 cells·ml^{-1}). Arrowhead indicates the addition of IPTG.

during a 2 h induction period with IPTG, in which the amount of accumulated fusion proteins reached a peak (data not shown). This condition was used for the rest of the experiments in this study. The fusion proteins produced in the pGST-FL, pGST-N78, and pGST-C108 cells were of molecular mass of about 45 kDa, 37 kDa, and 40 kDa as predicted, respectively. These fusion proteins, either containing full or partial sequences of Oshsp16.9, were all recognized by the polyclonal antiserum against Oshsp16.9 (Fig. 2*B*). In pGST-FL and pGST-C108 cells, some LMM proteins that were found to bind

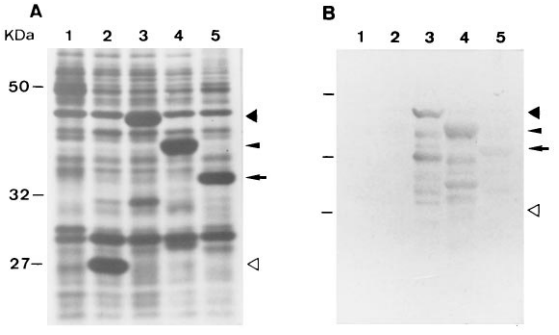

FIG. 2. Induction of fusion proteins by IPTG in transformed cells. Transformed cell cultures were induced by IPTG to produce fusion proteins as described. Samples (30 μg protein) were taken after 2 h of IPTG induction and subjected to SDS/PAGE (12.5% acrylamide) and subsequently immunoblotting using anti-Oshsp16.9 antiserum. The silver stained gel (*A*) and immunoblot (*B*) are shown. Samples (30 μg protein) shown in lanes 1, 2, 3, 4, and 5 were from cell cultures of wild-type, pGST, pGST-FL, pGST-C108, and pGST-N78, respectively. The open triangle indicates the GST (27.5 kDa) protein, the filled triangle indicates the GST–Oshsp16.9 (≈45 kDa) fusion protein, the arrow and arrowheads indicate the fusion proteins produced in the pGST-N78 (≈37 kDa) and pGST-C108 (≈40 kDa) cells, respectively.

the GST-affinity column (data not shown) reacted also with the antiserum (Fig. 2*B*). These proteins represented premature translation termination products of pGST-FL as we have shown in our previous study (24).

Thermotolerance of *E. coli* Cells Producing GST–Oshsp16.9 Fusion Protein. Cells producing the GST–Oshsp16.9 protein demonstrated more thermotolerance than cells producing only the GST protein. As shown in Fig. 3, after exposing cells to 47.5°C for 15 min, the survival rate of pGST cells dropped to about 1%, whereas that of the pGST-FL cells was more than 70% (the difference in survival rates was greater than 50-fold). Even after exposure to this high temperature for 1 h, the pGST-FL cells still had a survival rate of more than 10%, a 40-fold higher survivability compared with the pGST cells. Expression of pGST-N78 and pGST-C108 in *E. coli* had different effects on thermotolerance. The survival rate of pGST-N78 cells (more than 70% and 30% at 15 min and 1 h treatments, respectively) was somewhat higher than that of the pGST-FL cells, whereas the survival rate of pGST-C108 cells was only slightly higher than that of the pGST cells (Fig. 3) although the wild-type cells survived at 50°C with or without IPTG in the medium (data not shown). This finding is not due to the effect of IPTG but maybe due to the accumulation of foreign proteins leading to physiological problems for *E. coli* survival at higher temperatures.

Proteins from *E. coli* Cells Producing GST–Oshsp16.9 Show Higher Thermostability. To understand if Oshsp16.9 can protect *E. coli* proteins from precipitation during heat stress, we analyzed the stability of *E. coli* proteins isolated from pGST cells and pGST-FL cells following a heat treatment at 55°C for 30 min according to our previous study (24). The precipitated proteins were pelleted after heat treatment. The amount of protein in the pellets and supernatants was measured to

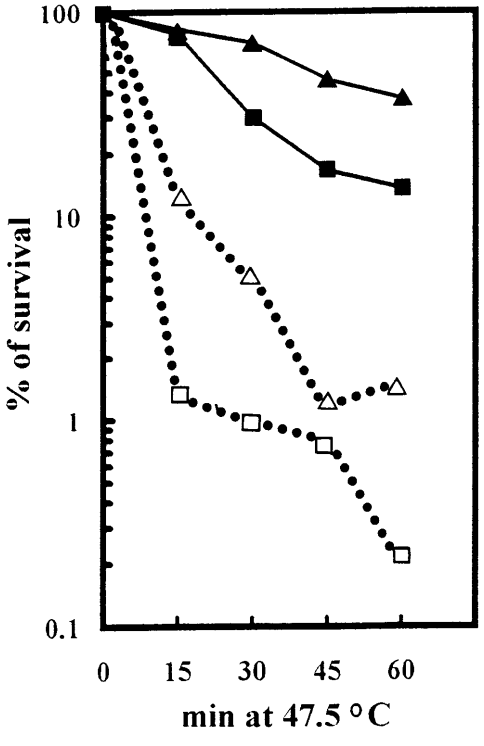

FIG. 3. Survival versus time at 47.5°C. Four independent *E. coli* transformants for each of the pGST, pGST-FL, pGST-C108, and pGST-N78 constructs were cultured as described and subjected to 47.5°C treatment for up to 60 min. After heat treatment, samples were taken at the time indicated, diluted, and immediately plated on LB plus ampicillin plates. Survival (%) of pGST cells (□), pGST-FL cells (■), pGST-C108 cells (△), and pGST-N78 cells (▲) were shown. Mean survival is expressed as a percentage of the survival obtained in cultures (SD was less than 5%).

determine the percentage of thermostabilized and precipitated proteins. As shown in Fig. 4, ≈63% of the proteins from pGST cells (and wild-type cells; data not shown) either with or without IPTG treatment and pGST-FL cells without IPTG treatment was precipitated after heating, whereas only about 32% of the protein was precipitated in IPTG-treated pGST-FL cells. The majority of GST–Oshsp16.9 protein remained in the supernatant (data not shown). IPTG treatment or the induction of GST *per se* did not contribute to the thermoprotection of transformed *E. coli* cells because from the *in vitro* protein precipitation test of *E. coli* lysates, we observed the same amount of protein precipitated at 55°C for wild-type and pGST cells either with or without IPTG.

Synthesis of the *E. coli* HSPs Was Enhanced During Heat Stress in Transformed *E. coli* Cells Producing GST–Oshsp16.9 Protein. For further analysis of proteins synthesized during heat stress in pGST-FL cells, the ^{35}S-labeled proteins were separated by SDS/PAGE followed by autoradiography. As shown in Fig. 5*A*, a number of *E. coli* HSPs ranging from 60 to 90 kDa were synthesized in abundance in the pGST-FL cells (lane 6) but much less in the pGST cells (lane 5) at 47.5°C treatments. These proteins were HSPs of 76, 73, and 64 kDa according to the previous reports (31, 32); note that equivalent protein bands were newly synthesized at elevated temperatures in wild-type cells at 47.5°C but not at 37°C (Fig. 5*A*, lanes 4 and 1, respectively). In pGST cells, little of the [^{35}S]methionine was incorporated into GST and *E. coli* HSPs (Fig. 5*A*, lane 5). The protein profiles for IPTG-treated wild-type, pGST, and pGST-FL cells at 37°C were similar except for the presence of the GST and GST–Oshsp16.9, although the incorporation of [^{35}S]methionine in the wild-type cells was slightly higher (Fig. 5*A*, lanes 1 vs. 2 and 3). In addition to the GST or fusion proteins, incorporation into some LMM (about 27–35 kDa) proteins was greater in pGST-FL cells than in pGST cells at 37°C treatments (Fig. 5*A*, lanes 3 and 2, respectively). These proteins represented premature translation termination products of pGST-FL, as we have shown in Fig. 2*B* by Western blot analysis. Although the pGST-FL cells synthesized *E. coli* HSPs in abundance at 47.5°C, whereas the pGST cells did not, transformation and expression of pGST and pGST-FL in *E. coli* cells did not change the levels of pre-existed HSPs. In wild-type, pGST, and pGST-FL cells, with or without IPTG

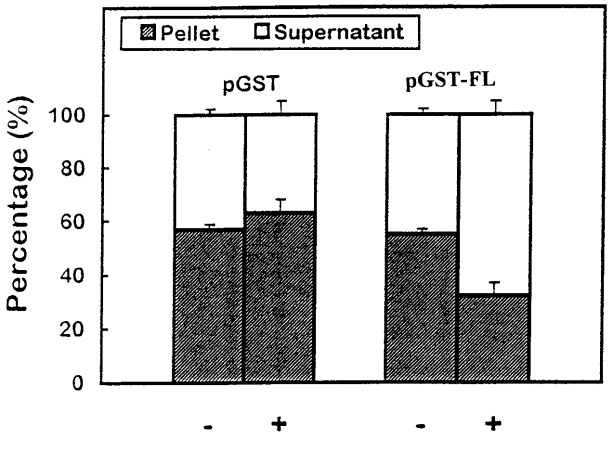

FIG. 4. Thermostabilization of proteins prepared from cell lysates of pGST cells and pGST-FL cells (with or without IPTG treatment) after heating at 55°C for 30 min. pGST cells and pGST-FL cells, with (+) or without (−) 2 h IPTG induction, were sonicated in PBS and subjected to centrifugation as described. Protein samples from the supernatant were heated at 55°C for 30 min and centrifuged at 16,000 × *g* for 10 min. The amount of protein in pellet (hatched bars) and supernatant (open bars) was quantified and expressed as a percentage. The recovery of protein obtained from pellet and supernatant was greater than 90%.

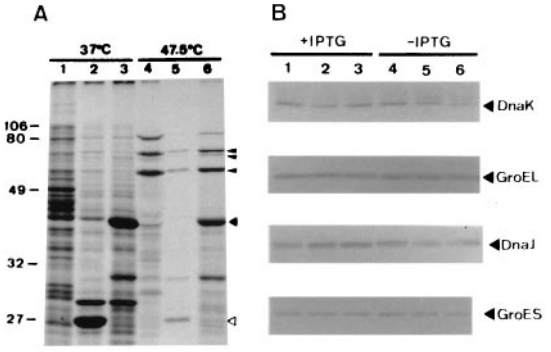

FIG. 5. Induction of HSPs in wild-type cells, pGST cells and pGST-FL cells. (*A*) Fifteen microliters of ^{35}S-labeled protein samples (extracted from an equal number of cells) in Laemmli sample buffer as described were subjected to SDS/PAGE (12.5% acrylamide) followed by autoradiography. In *A*, protein samples shown in lanes 1 and 4, 2 and 5, and 3 and 6 were from wild-type *E. coli* XL1-Blue cells, pGST cells, and pGST-FL cells, respectively. Lanes 1–3 and 4–6, protein samples subjected to 37°C and 47.5°C, respectively. The open triangle indicates the GST (27.5 kDa) protein, the filled triangle indicates the GST–Oshsp16.9 (≈45 kDa) fusion protein, and the arrowheads indicate HMM HSPs of *E. coli*. (*B*) Samples (30 μg protein) prepared from cultures of wild-type, pGST, and pGST-FL cells as described were taken after 2 h incubation with or without IPTG at 37°C, subjected to SDS/PAGE (12.5% acrylamide) and subsequently immunoblotting using antibodies against DnaK, GroEL, DnaJ, and GroES proteins of *E. coli*. Samples shown in lanes 1 and 4, 2 and 5, and 3 and 6 were from cultures of wild-type, pGST, and pGST-FL cells, respectively. Lanes 1–3 and 4–6, samples from *E. coli* cells incubated with (+) or without (−) IPTG, respectively.

treatment, we observed no difference in the synthesis of *E. coli* HSPs at normal growth temperature as shown in the Western bolt analyses using the antibodies for *E. coli* DnaK, GroEL, DnaJ, and GroES proteins (Fig. 5*B*).

DISCUSSION

All plants synthesize the abundant multiple LMM HSPs that are encoded by genes in the LMM HSPs superfamily. No organisms other than plants synthesize so many different LMM HSPs. Plant LMM HSPs can be divided into six gene families: class I, II, III, IV, V, and VI based on their homology and their localization to specific organelles (7, 8). A single plant species synthesizes several LMM HSPs of the same class, and the LMM HSPs of the same class are highly homologous. The LMM HSPs of different classes have much lower overall aa identity but maintain domains of amino acid conservation. Even in different species, the LMM HSPs of the same class share greater identity than the LMM HSPs of different classes from the same species. The antisera of LMM HSPs typically possess very high specificity to the LMM HSPs of the same class across different species of plants. For example, antisera of the rice class I LMM HSPs cross-react with the class I LMM HSPs of soybean, mung bean, pea, maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), cucumber (*Cucumis sativas* L.), tobacco (*Nicotiana plumbaginifolia* 43C), and *Arabidopsis* (*Arabidopsis thaliana* L.) but not with the other classes of LMM HSPs in these species (21). In *E. coli*, no protein was recognized by the rice class I LMM HSP antiserum (24), which is in agreement with the finding that no plant homologue of LMM HSPs is present in this prokaryotic organism (10).

The pea LMM HSPs have been shown to function as molecular chaperones *in vitro* to prevent thermal aggregation of model protein substrates (23). In our studies, the rice LMM HSP can protect proteins from heat denaturation, and the LMM HSPs are exchangeable among different species for this protective function (21, 22, 24). We do not know yet if this

function requires specific domains of LMM HSPs and/or specific sequences in the targeted protein or only the denatured form of proteins. In *E. coli*, a similar function to prevent protein denaturation has also been shown for DnaK, DnaJ, and GroE proteins (33). In our previous study, the GST–Oshsp16.9 fusion protein, similar to the purified rice 16.9-kDa HSP excised from the fusion protein, conferred thermoprotection *in vitro*, although the excised rice 16.9-kDa HSP is more effective for thermoprotection *in vitro* (24).

The plant class I LMM HSPs have been shown to form multimeric complexes *in vivo* (22–24), similar to the LMM HSPs found in animal systems, although the mammalian complexes were much larger in size (6, 34). The isolated GST–Oshsp16.9 fusion protein can also form a multimeric complex (data not shown), as does the excised 16.9-kDa HSP *in vitro* (24). The 16.9-kDa rice HSP forms a complex of about 310 kDa that can be estimated to consist of about 15–17 copies of the 16.9-kDa protein. However, the GST–Oshsp16.9 fusion protein, which is about 45 kDa, forms a complex of about 130–235 kDa in *E. coli*, which would be ≈3–5 units of GST–Oshsp16.9 fusion protein (data not shown). It is not yet known whether the formation of multimeric complexes is required to confer thermoprotection.

So far, the function of plant LMM HSPs has not been elucidated *in vivo*. For studying the function of HSPs *in vivo*, usually null mutants are used and by transforming these mutants with a specific HSP gene to confirm the function of the transferred gene (35, 36). To study the function of the plant HSP *in vivo*, we introduced a plant HSP gene into an organism that does not naturally produce an equivalent HSP during heat stress. In our previous study, the *Oshsp16.9* cDNA was introduced into the pGEX-2T vector with six extra amino acids in the N terminus (24). In the current study we used PCR to introduce the *Oshsp16.9* insert into the pGEX-2T vector, which resulted in a shorter peptide (only two extra amino acid residues in the N terminus of Oshsp16.9). This difference did not affect the function of the GST–Oshsp16.9 fusion protein. Shorter sequences of *Oshsp16.9* were also generated by PCR and introduced to the pGEX-2T vector to produce fusion proteins with only N78 or C108 aa residues of Oshsp16.9 attached to GST.

In pGST-FL cells and pGST-N78 cells, production of full-length or N78 amino acid residues of Oshsp16.9 protein allows *E. coli* to survive at a temperature that is lethal to pGST cells; however, production of C108 amino acid residues of Oshsp16.9 protein (pGST-C108 cells) had no such effect (Fig. 3). The protein profiles for IPTG-treated wild-type and transformed cells, as shown in Fig. 2*A*, were not different except for the presence of the GST and fusion proteins. Thus, the increased thermotolerance in pGST-FL and pGST-N78 cells resulted from the presence of specific fusion proteins in *E. coli*.

In the protein labeling experiment, there was no difference in the level of amino acid incorporation between the pGST cells and pGST-FL cells at 37°C, but at 42.5°C and 47.5°C the incorporation of [^{35}S]methionine was about 3-fold and 6-fold higher, respectively, in the pGST-FL cells compared with the pGST cells (data not shown). The level of incorporation of [^{35}S]methionine at 47.5°C, relative to the level at 37°C, was about 10% and 50% in pGST cells and pGST-FL cells, respectively (data not shown). In the labeling experiment, the protein profiles for IPTG-treated wild-type, pGST, and pGST-FL cells at 37°C were similar except for synthesis of the GST, GST–Oshsp16.9, and unfinished products of GST–Oshsp16.9, although the incorporation of [^{35}S]methionine in the wild-type cells was slightly higher (Fig. 5*A*, lanes 1–3). The IPTG-treated wild-type cells at 47.5°C produced the HMM HSPs in abundance (Fig. 5*A*, lane 4), as did the IPTG-treated pGST-FL cells (Fig. 5*A*, lane 6). The protein profile of IPTG-treated pGST cells at 47.5°C (Fig. 5*A*, lane 5), however, showed much less radioactive protein because pGST cells could not tolerate the high temperature. Thus, the increase in survival of pGST-FL cells at a high lethal temperature compared with pGST cells (Fig. 3*A*) could be due to direct or indirect function of both Oshsp16.9 and the subsequently induced *E. coli* HSPs. The pGST-FL cells produced more *E. coli* HSPs at 47.5°C than at 42.5°C (data not shown). It is therefore possible that the role of plant class I LMM HSPs in *E. coli* during heat stress is to enhance the production of *E. coli* HSPs directly or indirectly, because in the presence of IPTG the level of pre-existing HSPs did not change (Fig. 5*B*). This enhanced HSP production in the presence of Oshsp16.9 is correlated with a higher survival rate of *E. coli* cells at the lethal temperature. *E. coli* HSPs have been suggested to play a major role in protecting cells from thermal killing. However, it appears that additional proteins other than *E. coli* HSPs may be required for thermotolerance (37). The Oshsp16.9 may fulfill such a role. The function of plant class I LMM HSPs in *E. coli* may be involved in providing thermotolerance (somewhat similar to preadaption to heat) by enhancing synthesis of *E. coli* proteins (including HSPs) or acting as chaperones in protecting *E. coli* proteins from heat denaturation as we have shown previously in soybean (22). In fact, thermoprotection of proteins from 55°C precipitation was observed in cell lysates of *E. coli* producing the GST–Oshsp16.9 fusion protein (Fig. 4). Similar results were obtained in pGST-N78 cells but not in pGST-C108 cells (data not shown). The thermotolerance and thermoprotection observed in both the pGST-FL and pGST-N78 cells was not due to the stress response by overproduction of foreign proteins because neither thermotolerance nor thermoprotection was observed in pGST and pGST-C108 cells, and there was no change in the levels of *E. coli* HSPs between the wild-type and transformed cells after IPTG induction at 37°C (Fig. 5*B*). In addition, the GST activity could not account for the difference in thermotolerance, we observed the same activity in the lysates of transformed pGST-FL, pGST-N78, and pGST-C108 cells (data not shown). Therefore, even though overproduction of GST and GST fusion proteins may cause thermosensitivity in *E. coli*, the enhanced thermotolerance and thermoprotection in pGST-FL cells appears to result from the function of Oshsp16.9 rather than the reduction of a GST effect.

The fusion protein produced in the pGST-N78 cells, with only the N-terminal 78-aa residues (1–78) of Oshsp16.9, which includes the consensus II region of plant LMM HSPs (30), maintained the function of Oshsp16.9 protein. In contrast, the fusion protein produced in the pGST-C108 cells, with only the C-terminal 108-aa residues (43–150) of Oshsp16.9, which includes both consensus I and consensus II regions of plant LMM HSPs (30), was not functional. This finding suggests that a specific domain or structure in the consensus I region is not necessary, whereas the consensus II region is essential but not sufficient for Oshsp16.9 function. This result coincides with the recent finding of Lee *et al.* (38), which suggests that the consensus II region is critical for substrate binding. Because class I LMM HSPs are highly conserved proteins in diverse species, we are now studying whether certain specific structural domains of the LMM HSPs are involved in the thermoprotection function using the GST fusion system in *E. coli*.

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