Molecular characterization of *Oryza sativa* 16.9 kDa heat shock protein

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A rice class I low-molecular-mass heat shock protein (LMM HSP) Oshsp 16.9 was overexpressed in *Escherichia coli*. Oligomerized complexes of Oshsp16.9 were harvested and electron microscopic observations of purified complexes revealed globular structures of 10–20 nm in diameter (with majority of 15–18 nm) and calculated to comprise approx. 12 monomers per complex. In comparison, complexes from native rice class I LMM HSPs were observed as larger ellipsoid- or globular-like random aggregated hetero-oligomers. To characterize the biochemical functions of the hydrophobic N-terminal region of Oshsp16.9, a truncation in the N-terminal region was constructed and introduced into *E. coli*. Results showed that the N-terminal truncated Oshsp16.9 mutant was capable of forming complexes similar to the full-length Oshsp16.9; however, the deletion protein failed

to confer *in vitro* protein thermostability under elevated temperatures. Protein assays from *in vivo* treatments at higher temperatures exhibited that non-specific interactions of *E. coli* cellular proteins only occurred with full-length Oshsp16.9 complexes but not with the mutant complex. *In vitro* immunoprecipitation of cellular proteins from *E. coli* overexpressing full-length Oshsp16.9 showed that interactions between plant LMM HSP and *E. coli* cellular proteins are temperature-dependent. Taken together, the hydrophobic N-terminal region of rice class I LMM HSP is critical in the ability of the protein to interact/bind with its potential substrates.

Key words: chaperone activity, Oshsp16.9, overexpression in *Escherichia coli*, thermotolerance.

INTRODUCTION

All organisms respond to heat shock (HS) by synthesizing a number of distinct polypeptides termed heat shock proteins (HSPs). Based on sequence homology, HSPs are grouped into families of HSP110, HSP90, HSP70, HSP60, and the LMM HSPs or small HSPs (sHSPs), which range in size from approx. 15–30 kDa [1].

Ingolia and Craig [2] discovered that mammalian α -crystallins was related to LMM HSPs and subsequent investigations indicated additional structural and functional similarities. Homology of these related families of LMM HSPs is confined to a region of about 85 aa (termed the α -crystallin domain) which may contribute to the structural characteristics similar in them: (1) aggregation into complexes with a molecular weight range of 200–800 kDa, (2) similar hydropathy profiles, and (3) similar quaternary structures of 10–18 nm globular-like forms [3–5]. Several lines of research have indicated the importance of this α -crystallin domain in protection against heat-induced denaturation [6]. To date, the presence and accumulation of LMM HSPs and α -crystallins under stress conditions have been shown to have protective functions that confer thermotolerance in different cell types [7,8]. Overexpression of bovine eye lens protein α A-crystallin has been shown to increase cellular thermoresistance [9]. In the human HSP27, it functions by stabilizing actin filaments in modulating cellular thermoresistance [10]. It has also been assumed that the chaperone activity of LMM HSPs is strictly correlated with the ability of these LMM HSPs to form oligomerized complexes [11]. Recently, Kim et al. [12] reported the first crystal structure of a 16.5 kDa sHSP from *Methanococcus jannaschii* and deduced the quaternary structure of the oligomerized complex.

Among many higher plants, LMM HSPs are the most abundant proteins produced under heat stress and have been reported to accumulate up to as much as 1 % of the total protein content

of soybean [13]. The LMM HSP family in plants is complex, with up to 25 different polypeptides produced during heat stress [14]. These vast amount of LMM HSPs are divided into at least five classes, based on localization in different cellular compartments, aa sequence homology, and immunological cross-reactivity [15]. Recent advances in the knowledge of plant LMM HSPs have revealed its molecular structure by electron microscopic observations [16], *in vitro* chaperone activity that partially improves the re-activation of unfolded proteins [17] and *in vivo* enhancement of thermotolerance to *E. coli* cells overexpressing plant LMM HSP under high temperatures [8]. Despite these molecular chaperone activities that are similar to mammalian sHSPs and α -crystallins, a significant characteristic of plant LMM HSPs is that the oligomerized complexes observed to date were found to present in a relatively uniform size (approx. 200–320 kDa) [18]. This confined behaviour of plant LMM HSP complexes may be attributable to the lack of phosphorylation and post-translational modifications in the N-terminal and α -crystallin domain [19]. In addition, our previous study on a rice class I LMM HSP, Oshsp16.9, had shown that the N-terminal 78 aa fragment alone is sufficient to confer thermotolerance to *E. coli* cells under heat stress [8]. This fragment contains the N-terminal region and the consensus II region, but not the consensus I region of the α -crystallin domain. This result contrasts with reports on mammalian LMM HSPs and α -crystallin systems that emphasize the importance for chaperone function of the α -crystallin domain.

To gain further information on the structure–function relationship of plant LMM HSPs, we have investigated the biochemical properties of the N-terminal region of Oshsp16.9 with respect to the oligomerization of complexes by the expression of a full-length and an N-terminal deletion mutant of Oshsp16.9 in *E. coli* cells. In addition, the relationship of complex formation to the conferred protein thermotolerance of *E. coli* cellular proteins under heat stress was also studied. Furthermore, we

Abbreviations used: A_{600} , absorbancy at 600 nm; aa, amino acid; IPTG, isopropyl β -D-thiogalactopyranoside; LB, Luria–Bertani; O.D., optical density; HS, heat shock; HSPs, heat shock proteins; sHSPs, small HSPs; LMM, low molecular mass; PRS, post-ribosomal supernatant; PPT, precipitated portion; SNT, supernatant portion.

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provide evidence that interaction modes occurred between Oshsp16.9 and *E. coli* cellular proteins at elevated temperatures that are associated with increased protein thermostability. Finally, structures of recombinant Oshsp16.9 complexes and native rice class I LMM HSP complexes are shown and compared by electron microscopic observations. To our knowledge, this is the first report to characterize native rice class I LMM HSPs in the form of oligomerized complexes.

MATERIALS AND METHODS

Preparation of expression constructs for the full-length and the N-terminal deletion mutant of Oshsp16.9

To prepare recombinant full-length Oshsp16.9, the 5' primer is designed as 5'-CGGGGATCCATGTCGCTGGTGAGG-3' and the 3' primer is 5'-GCCGAAGCTTTAACCGGAGATCTC-3' based on the coding region of Oshsp16.9 cDNA substrate which encodes a full-length 150 aa polypeptide. *Bam*HI and *Hind*III restriction sites were introduced into the primers, respectively (underlined). PCR reactions were performed for 30 cycles. After gel purification of the PCR product, the amplified DNA was ligated into the polycloning sites of *pUC8* vector (both restricted by *Bam*HI and *Hind*III) following standard procedures [20], and was designated as 'pUC-FL' recombinant vector. An N-terminal 42 aa deletion mutant construct of Oshsp16.9 encoding the C-terminal 108 aa of Oshsp16.9 was also amplified, cloned into the *pUC8* plasmid following the above procedures, and designated as 'pUC-C108' recombinant vector. For the N-terminal deletion mutant, the 5' primer is 5'-CGGGGATCCGCCCGCATCGA-CTGG-3' and the 3' primer is 5'-GCCGAAGCTTTAACCGG-AGATCTC-3'.

Correct insertion of each cDNA insert into the expression vector was confirmed by restriction mapping and dideoxy-sequencing [21].

E. coli JM109 cells were transformed using the standard *E. coli* transformation procedures [20]. A control transformant using only the *pUC8* vector, without Oshsp16.9 cDNA insert, was also transformed into *E. coli* JM109, and was designated as 'pUC' cells.

Expression and collection of full-length and N-terminal deletion mutant of Oshsp16.9

Transformants were grown at 37 °C in LB/ampicillin broth to $A_{600} = 0.6$, and then induced by addition of IPTG to a final concentration of 1 mM. Cells from 10 ml equal O.D. cultures were collected by centrifuging at 3000 *g* for 10 min and resuspended in 200 μ l Laemmli sample buffer [22]. Cell suspensions were disrupted by ultrasonication for 3 min using the Heat Systems Sonicator® at full speed and boiled at 95 °C for 5 min. As for the extraction of native soluble proteins, cells from IPTG-induced cell culture were resuspended in PBS (150 mM NaCl/16 mM Na₂HPO₄/4 mM NaH₂PO₄, pH 7.8, 4 °C) and disrupted by ultrasonication for 10 min at low speed under 4 °C water bath.

The bacterial lysates were then centrifuged at 13000 *g* for 10 min and supernatants were collected for further analysis.

Extraction of rice post-ribosomal supernatant (PRS)

Rice seedlings (*Oryza sativa* L. cv. Tainong No. 67) were rinsed thoroughly in water and then germinated in a roll of moist paper towel at 28 °C in a dark growth chamber. Four-day-old seedlings without endosperm were harvested and homogenized with a polytron in 0.2 M Tris/HCl buffer, pH 8.8, containing 0.5 M

sucrose, 0.1 M KCl, 30 mM MgCl₂, 1 mM DTT, and 1 mM PMSF. The homogenate was filtered through a layer of Miracloth, and the filtrate was further centrifuged at 300000 *g* for 90 min, as described previously [23] for the preparation of the post-ribosomal supernatant.

Polyacrylamide gel electrophoresis and Western blotting

SDS/PAGE was performed using 15% acrylamide gels as described [19]. Non-denaturing PAGE was performed on a 5–20% gradient acrylamide gel according to the method of Anderson et al. [24]. Western blots were performed with rabbit anti-Oshsp16.9 antibodies as previously described [25].

Purification of oligomerized complexes

Purification of the *E. coli* expressed full-length Oshsp16.9 complexes and the native rice class I LMM HSP complexes (obtained from an ammonium sulphate-enriched fraction as described previously [23]) were performed by using 5–20% non-denaturing PAGE. Subsequently, the gel bands containing the complexes were cut out from the non-denaturing acrylamide gel and were eluted using a Model 422 electro-eluter (Bio-Rad) for 6 h at 4 °C in the 25 mM Tris/HCl buffer containing 192 mM glycine (pH 7.0).

Electron microscopic observations

Purified Oshsp16.9 complex samples (50 μ g/ml) were applied to a formvar carbon film, and negatively stained with 1.5% phosphotungstic acid (pH 7.0). Samples were viewed by Hitachi H-600 electron microscope operating at 75 kV. Micrographs were taken at 100000 $\times$ magnification.

Assay for thermal denaturation of cellular proteins from transformed *E. coli*

Cells of transformant cultures, with or without IPTG inductions, were collected and suspended in PBS (4 °C). Native soluble proteins were extracted as previously described. Concentrations of soluble protein extracts were determined by the Bradford dye reagent method [26]. Two mg/500 μ l of native soluble protein samples were incubated at 55 °C for 30 min. After heating, supernatants and precipitated pellets were separated by centrifuging at 300000 *g* for 90 min, and the protein concentration of each supernatant and precipitated portion was determined.

Immunoprecipitation

Native proteins from pUC-FL cells (1.5 mg/200 μ l) after 6 h IPTG induction were incubated at 45 °C for 15 min or left at room temperature as non-heated controls. After centrifuging at 13000 *g* for 15 min, supernatants were collected. To test for any non-specific binding of proteins to the Protein A-agarose, the supernatants were mixed with Protein A-agarose by shaking for 3 h at 4 °C.

Following pre-mixing, the Protein A-agarose was removed by centrifugation at 13000 *g* for 1 min. Anti-Oshsp16.9 antibodies were added to the supernatant solution and incubated for 1 h, followed by the addition of Protein A-agarose and incubation for an additional 3 h at 4 °C. Treated samples were then centrifuged at 13000 *g* for 1 min to yield a Protein A-agarose precipitated portion (PPT) and a supernatant portion (SNT). The Protein A-agarose precipitates were further washed with buffer (50 mM Tris/HCl, pH 7.5/500 mM NaCl/0.1% Nonidet® P40/0.05% sodium deoxycholate) three times for removing any

non-specific protein binding, resuspended in 50 μ l Laemmli sample buffer and boiled at 95 °C for 5 min to collect for interacted proteins. Equal quantities of protein samples (50 μ g) from PPT and SNT were loaded and analysed by 15% SDS/PAGE (the methods above were modified from the immunoprecipitation kit of Boehringer–Mannheim).

RESULTS

Expression of the full-length and the N-terminal deletion mutant of recombinant Oshsp16.9 in transformed *E. coli* cells

Nucleic acids encoding full-length and N-terminal deleted coding regions of rice class I LMM HSP, Oshsp16.9, were cloned into the polycloning sites of the expression vector *pUC8*, downstream of the *LacZ'* promoter. These recombinant vectors, pUC-FL and pUC-C108 respectively, were transformed into *E. coli* JM109 cells, in which the expression of foreign proteins was induced by IPTG at 1 mM. Protein expression of both transformants reached a climax 6 h after IPTG induction, and the production ratio of the full-length and the N-terminal deletion mutant of Oshsp16.9 was 1 to 1.2 (Figures 1A and 1B). These proteins could accumulate up to 15% of the total protein content (data not shown). Growth curves of the transformants and the control pUC cells were identical under normal conditions and reached stationary phase 6 h after IPTG induction (data not shown). The molecular mass of the expressed full-length and N-terminal deletion mutant of recombinant Oshsp16.9 were estimated at 18.3 and 14.4 kDa, respectively (Figure 1A and 1B). These values reflect the presence of 9 extra aa (TMITNSRGS) from the *LacZ'* gene upstream of the first methionine residue of Oshsp16.9 (data not shown).

The pattern of *E. coli* HSP synthesis following IPTG induction was also analysed. Immunoblot analysis using individual antibodies against the HSPs, DnaK and DnaJ, did not detect any induced synthesis of these major stress proteins of *E. coli* (data not shown). This result is in agreement with previous over-expression studies in which the accumulation of Oshsp16.9 and other LMM HSPs does not appear to induce a stress-like response in *E. coli* cells [8,9].

Analysis of oligomerized complexes in transformants

A common feature of the LMM HSP families and α -crystallin is the aggregation into higher molecular-weight complexes [27]. The molecular weight of the oligomerized recombinant full-length Oshsp16.9 complex is 220 ± 10 kDa (Figure 1C, lane 2, filled triangle). The N-terminal deletion mutant expressed in *E. coli* also aggregated into complexes, but with an altered molecular weight of 260 ± 10 kDa (Figure 1C, lane 3, open triangle).

Electron microscopic observations of oligomerized complexes of recombinant Oshsp16.9 and native rice class I LMM HSPs

Purified complexes of recombinant full-length Oshsp16.9 and native rice class I LMM HSPs were reproducible when checked by re-electrophoresis on 5–20% gradient non-denaturing PAGE and immunostained by anti-Oshsp16.9 antibodies. Results are shown in the left of each micrograph in Figure 2. The purity of the purified full-length Oshsp16.9 and native rice class I LMM HSP complexes were checked by SDS/PAGE and analysed by densitometer; they contained up to 95% of the individual monomers of Oshsp16.9 and class I LMM HSPs, respectively (data not shown).

Mammalian LMM HSPs and α -crystallin have a similar globular-like structure with a diameter of 10–18 nm [28–30]. In this study, the recombinant Oshsp16.9 complexes, observed by

Figure 1 Expression of the recombinant and the N-terminal deletion mutant of Oshsp16.9 in *E. coli* transformants

E. coli JM109 cells transformed with pUC-FL and pUC-C108 vectors were induced by 1 mM IPTG for the indicated time. Soluble proteins from equal O.D. cells were extracted and analysed by 15% SDS/PAGE. Coomassie Blue R-250 stained gels are shown, indicating time-course of expression of the recombinant full-length (A) and the N-terminal deletion mutant (B) of Oshsp16.9, respectively. Lane 1, no IPTG induction of the individual transformants; lane 2, 3 h IPTG induction; lane 3, 6 h IPTG induction. In (C), native protein samples (50 μ g each) of pUC-FL and pUC-C108 cells induced by IPTG for 6 h were subjected to 5–20% gradient non-denaturing PAGE, transferred to a PVDF membrane and immunostained by anti-Oshsp16.9 antibodies. Lane 1, total proteins from control pUC cells; lane 2, total proteins from pUC-FL cells producing recombinant full-length Oshsp16.9; lane 3, total proteins from pUC-C108 cells producing N-terminal deletion mutant of Oshsp16.9. Filled triangle indicates the recombinant Oshsp16.9 oligomerized complex and the open triangle indicates the N-terminal deletion mutant oligomerized complex.

Hitachi H-600 electron microscope, are globular with a diameter of approx. 10–20 nm, and with the majority within 15–18 nm (Figure 2A). In comparison, purified rice class I LMM HSP complexes, obtained from ammonium sulphate-enriched fraction, observed in the electron microscope appear as larger ellipsoid- or globular-like random aggregated hetero-oligomers (Figure 2B).

Figure 2 Electron microscopic observations of oligomerized complexes

Purified recombinant Oshsp16.9 (**A**) and native rice class I LMM HSP (**B**) complexes were negatively stained with 1.5% phosphotungstic acid and observed by Hitachi H-600 electron microscope. Electron micrographs were taken at 100 000 $\times$ magnification. Bar, 100 nm. Shown in the left of each micrograph is the immunostained gel from the re-electrophoresis of purified complexes after 5–20% gradient non-denaturing PAGE, respectively.

***In vitro* assessment of the recombinant proteins from transformants for thermostabilization effects under elevated temperature**

Previously we showed that LMM HSP complexes isolated from soybean seedlings were able to protect up to 75% of the soluble proteins from heat denaturation *in vitro* at 55 °C for 30 min [23]. This type of analysis was performed on total proteins extracted from *E. coli* cells expressing either full-length or N-terminal deleted mutant of Oshsp16.9, and the results are shown in Figure 3. For control pUC cells (open bars), between 50 and 60% of the extracted total cellular proteins remain soluble following the heat treatment. Similar analysis from pUC-FL cells (dotted bars), which express full-length Oshsp16.9, shows that over 80% of the proteins remain soluble. However, analysis of total extracted cellular proteins from pUC-C108 cells (striated bars), which express the N-terminal deleted mutant of Oshsp16.9, showed that these extracts are essentially identical to the control pUC cells, i.e. approx. 50–60% of the total proteins remain soluble. Total protein recovery following heat treatment in each assay was over 95% (data not shown). Values represent means $\pm$ S.D. of at least three separate experiments.

Interactions of Oshsp16.9 with *E. coli* cellular proteins

There are several reports concerning the interaction of mammalian LMM HSPs and α -crystallin with non-native proteins [10,31,32]. In plants, LMM HSPs were proposed to interact similarly with non-native proteins [16], and they were subsequently reported to stably bind to heat-denatured model substrates in a folding-competent state [17]. However, demonstrations of interaction mode between cellular soluble proteins and plant LMM HSPs that might provide insights to the ac-

Figure 3 *In vitro* thermostability of *E. coli* cellular proteins at 55 °C for 30 min

Soluble proteins of pUC, pUC-FL, and pUC-C108 cells were extracted after the indicated induction time with 1 mM IPTG. Protein samples (2 mg/500 μ l) were subjected to 55 °C heat treatment for 30 min. Denatured protein precipitate and soluble supernatant were separated by centrifuging at 300 000 g for 90 min, and individual protein concentrations were measured. The percentage of proteins protected from heat denaturation was measured by the ratio of the amount of supernatant soluble proteins remained after heat treatment to soluble proteins initially subjected. Recovery of total proteins in each assay was greater than 95%. Open bars, dotted bars, and striated bars indicate the percentage of soluble supernatant proteins protected from heat denaturation of pUC, pUC-FL and pUC-C108 cells, respectively. Values represent means $\pm$ S.D. of at least three separate experiments.

quisition of thermotolerance, rather than using model substrates, is lacking and needs further investigation.

For this reason, non-denaturing PAGE was used to detect whether interactions of native LMM HSPs with cellular proteins occurred during the increase of temperature. Purified complexes of recombinant full-length (Figure 4A, lanes 1–7) and N-terminal deletion mutant (data not shown) of Oshsp16.9 were collected for *in vitro* assays of temperature stability under temperatures up to 55 °C for 30 min. This result is consistent with previous results in soybean and mammalian LMM HSP systems [18,32]. However, when the purified recombinant Oshsp16.9 complex solution was added into rice PRS, the major complex band was shifted. This effect was observed between 32.5–55 °C (Figure 4A, lanes 8–14). Identical results were obtained by treating transformants in temperatures ranging from 37 to 50 °C for 5 min; we detected the *in vivo* molecular weight of the full-length Oshsp16.9 complexes to expand as the temperature increased (Figure 4B, lanes 3–5), similar to the results of soybean 280-kDa oligomerized complexes [23]. This expanded property of Oshsp16.9 complexes

Figure 4 Non-specific interaction of recombinant full-length Oshsp16.9 with substrate proteins *in vitro* and *in vivo* under elevated temperatures

In (A), purified recombinant full-length Oshsp16.9 complexes were tested for *in vitro* heat stability for 30 min at the temperature indicated (lanes 1–7) or with the addition of rice PRS (lanes 8–14). Immunoblot of gradient non-denaturing PAGE is shown. *In vivo* assay for protein interaction of full-length (B) and the N-terminal deletion mutant (C) of Oshsp16.9 complexes are shown. After pUC-FL (B) and pUC-C108 (C) cells were induced by 1 mM IPTG for 6 h, 10 ml of both cell cultures were subjected to the indicated temperature for 5 min and immediately frozen in liquid nitrogen. Soluble proteins were then extracted with PBS and subjected to 5–20% gradient non-denaturing PAGE and immunostained with anti-Oshsp16.9 antibodies. Lane 1, native proteins of pUC cells; lanes 2–5, native proteins of individual transformants treated at the indicated temperatures for 5 min. (D) Shows that each pUC-FL cell total protein samples loaded in (A) is checked for any loss of proteins by 15% SDS/PAGE and staining by Coomassie Blue R-250. Lanes 2'–5' corresponded to the protein samples loaded in (A) lanes 2–5.

was analysed by SDS/PAGE and there was no detectable loss of proteins in the soluble supernatants (Figure 4D). In contrast, the N-terminal deletion mutant complex of Oshsp16.9 from *E. coli* did not change in size as the temperature was increased (Figure 4C) and substantial amounts of proteins were denatured (data not shown).

Temperature-dependent interaction of Oshsp16.9 with *E. coli* cellular proteins

In light of the above result, *in vitro* immunoprecipitation methods were used to assess the interactions that occur between full-length Oshsp16.9 and *E. coli* cellular proteins. In our results

Figure 5 Temperature-dependent interaction of *E. coli* cellular proteins with Oshsp16.9

After 6 h IPTG induction of pUC-FL cells, soluble proteins were extracted with PBS and 1.5 mg/200 μ l of protein samples were subjected to 45 °C treatment for 15 min (+) or left at room temperature as control (—). Supernatants were collected by centrifugation, precleaned with Protein A-agarose, and immunoprecipitated with anti-Oshsp16.9 antibodies. The immunoprecipitated portion (PPT) and supernatant portion (SNT) was dissolved in Laemmli sample buffer and equal quantities (50 μ g) of protein samples were then subjected to 15% SDS/PAGE analysis. Coomassie Blue R-250 stained gel is shown. Lane 1, pUC-FL total cell proteins; lane 2, protein profile of anti-Oshsp16.9 antibodies; lane 3, protein profile of Protein A-agarose; lane 4, supernatant portion of heat-treated samples after immunoprecipitation; lane 5, immunoprecipitated portion of heat-treated samples; lane 6, supernatant portion of control samples after immunoprecipitation; lane 7, immunoprecipitated portion of control samples. Arrowheads indicate protein bands selectively immunoprecipitated with Oshsp16.9 after heat treatment. Filled circle indicates recombinant Oshsp16.9. Open circle indicates the heavy chain of IgG.

shown in Figure 5 stained by Coomassie Blue R-250, the amount of Oshsp16.9 immunoprecipitated (filled circle) were identical in both heat-treated (lane 5) and the control at room temperature (lane 7). The efficiency (approx. 40%) of Oshsp16.9 immunoprecipitation was seemingly low; however, it was sufficient enough to differentiate between them. Apart from some backgrounds and the antibody (open circle), there were approx. 10 more protein bands that interacted with Oshsp16.9 on incubation at 45 °C (Figure 5, lane 5) which were not present in the sample from the room temperature control (Figure 5, lane 7). The proteins that interacted with Oshsp16.9 at 45 °C ranged in size from 22 to 70 kDa (indicated by the arrowheads). Our preliminary tests with pUC cells that do not express Oshsp16.9 indicated that no specific interaction of anti-Oshsp16.9 antibodies with *E. coli* cellular proteins at 45 °C or room temperature was observed (data not shown). In addition, Protein A-agarose bound only to antibodies (IgG) (data not shown).

DISCUSSION

Plant LMM HSPs are unique in their complexity in number and the localization of different classes to individual cellular compartments. In contrast, only one LMM HSP has been found in human and yeast, and at most four sets in *Drosophila* [33,34], and most of these are localized primarily in the cytosol [35]. Due to the low copy number of LMM HSPs in these organisms, their function and characteristics were proficiently uncovered by loss- or gain-of-function studies on the specific gene. However, such studies are not feasible in higher plants which produce up to five classes of LMM HSPs that differentially localize to individual cellular compartments in a single species. For this reason, we have focused on elucidating the structure and function of individual plant LMM HSPs using an *E. coli* system, which does not normally synthesize similar LMM HSPs.

Among the plant LMM HSPs we cloned recently [36,37], a rice class I LMM HSP, Oshsp16.9, that is specifically expressed under heat stress was extensively studied. In contrast to other mammalian LMM HSPs and α -crystallin systems, our study demonstrates that the N-terminal 78 aa of Oshsp16.9 alone functions to confer thermotolerance to *E. coli* cells under heat stress regardless of the lack of most of the region of the α -crystallin domain [8]. Consistent with our results, photolabelling of a pea LMM HSP with a hydrophobic probe, bis-ANS, has revealed a putative protein interaction site in the consensus II region [17]. Furthermore, a mutational study on α B-crystallin indicates several important residues in the N-terminal region that are critical for the chaperone activity [38]. Clearly, biochemical roles can be diversely assigned to domains within and among classes of LMM HSPs, indicating a complex collaboration between individual domains that contribute to the observed formation of oligomerized complexes and their functional activities. However, the biochemical properties of individual domains in plant LMM HSPs are still not clear.

In this report, the biochemical contribution of the N-terminal region of Oshsp16.9 to the oligomerization of high molecular mass complexes, and the relationship of these complexes to chaperone activities were investigated. We utilized the pUC8 plasmid, rather than the GST expression system, to express Oshsp16.9 and its deletion mutant to study the native conformation of the multimerized complexes. From our native protein analysis, both the full-length and N-terminal deletion mutant of Oshsp16.9 were capable of forming oligomerized complexes, revealing that the conserved C-terminal region of Oshsp16.9 which contains the α -crystallin domain is involved in multimerization despite deletions in the N-terminal end (Figure 1C, lane 3). This result is expected, since almost all α -crystallin-related families have been reported to aggregate into higher-molecular-weight complexes [10,30]. In electron microscopic observations, the purified recombinant Oshsp16.9 complexes (Figure 2A) appeared as globular structures with a diameter between 10 and 20 nm and were similar to those reported previously for other LMM HSPs and α -crystallin [16,28,29]. We estimate that these globular complexes, with a molecular weight of approx. 220 kDa are composed of approx. 12 Oshsp16.9 monomers, which is equivalent to the monomer composition of the pea HSP18.1 and HSP17.7 complexes reported previously [16]. We also observed native rice class I LMM HSP complexes, with a molecular weight of approx. 310 kDa (for review, see [18]), which have larger ellipsoid- or globular-like random aggregated hetero-oligomers (Figure 2B). This difference in molecular weight and appearance may be due to different class I LMM HSPs clustered in the rice complexes to form hetero-oligomers [25].

Another aim of this study was to provide additional evidence

Concluding from the above *in vivo* and *in vitro* results, we suggest that the interactions between the LMM HSP and cellular substrates are both non-specific and temperature-dependent.

Upon recovery to physiological temperatures, the transient binding of LMM HSPs to cellular proteins is reversed, as observed by the recovery of the complexes to their original molecular weight (data not shown). This recovery of the complex size may be related to ATP-dependent chaperone activities in *E. coli* cells as proposed in a previous report [17]. Interestingly, upon recovery from heat shock, NaF or anaerobiosis inhibits the relocalization of organelle-associated LMM HSPs in soybean seedlings (C. Y. Lin, unpublished results).

In this report, we have shown that the hydrophobic N-terminal region of Oshsp16.9 is critical for the chaperone activities and interactions with cellular proteins. Our results highlight one difference between plant LMM HSPs and several sHSPs of other organisms; in the latter, the conserved C-terminal region has been shown to be important in thermoprotection. This difference may be due to the dynamic phosphorylation and other post-translational modifications in the N-terminal region and α -crystallin domains in other mammalian systems that are not observed in plant LMM HSPs [19]. We have also presented the molecular structure of native rice class I LMM HSPs and Oshsp16.9 complexes, and our observations show that structures of purified Oshsp16.9 are similar to other known LMM HSPs and α -crystallin. Finally, we have provided evidence of non-specific protein interactions by this LMM HSP with cellular proteins that are temperature-dependent. However, currently we are studying which amino acid(s) in the N-terminal region of Oshsp16.9 are critical for protein interactions. Furthermore, we hope to determine the quaternary structure of this rice class I LMM HSP by X-ray crystallographic studies, which have not yet been reported for any plant LMM HSPs.

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