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Cloning and characterization of a cDNA encoding an 18.0-kDa class-I low-molecular-weight heat-shock protein from rice

(Recombinant DNA; gene family; gene expression; fusion protein; Western blot; *Oryza sativa* L.)

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SUMMARY

A novel cDNA clone, *Oshsp18.0* cDNA, encoding a rice (*Oryza sativa* L. cv. Tainong 67) 18.0-kDa heat-shock protein (HSP), was isolated from a cDNA library of heat-shocked rice seedlings by use of the rice *HSP* cDNA, *Oshsp17.3* cDNA, as a probe. The sequence showed that *Oshsp18.0* cDNA contains a 749-bp insert encoding an ORF of 160 amino acids, with a predicted molecular mass of 18.0 kDa and a p*I* of 7.3. Sequence comparison reveals that *Oshsp18.0* cDNA is highly homologous to other low-molecular-weight (LMW) *HSP* cDNAs. Also, the results of hybrid-selected in vitro translation clearly establish that *Oshsp18.0* cDNA is the rice 18.0-kDa LMW HSP-encoding cDNA clone. The recombinant *Os*hsp18.0 fusion protein produced in *Escherichia coli* was of the size predicted, and was recognized by the class-I rice 16.9-kDa HSP antiserum. The results suggest that *Oshsp18.0* cDNA is an 18.0-kDa class-I LMW HSP- encoding cDNA clone from rice.

INTRODUCTION

Heat-shock proteins (HSP) are induced during thermal stress in all organisms examined, ranging from bacteria to human (Schlesinger et al., 1982), and appear to be involved in thermoprotection (Chou et al., 1989; Krishnan et al., 1989; Vierling, 1991). The HSP are usually divided into the high-molecular-weight (HMW) proteins of more than 30 kDa, and the low-molecular-weight (LMW) proteins of about 17 to 28 kDa (Lindquist and Craig, 1988; Vierling, 1991). In contrast to animal systems, plants synthesize more LMW HSP than HMW HSP. The LMW HSP superfamily is unusually complex, consisting of at least four gene families. There is greater identity among certain genes from different species than there is among different genes of the same species (Vierling, 1991). The role of LMW HSP in the heat stress is not completely clear yet.

We have been studying the physiological function of LMW HSP in soybean and rice (Lin et al., 1984; Chou et al., 1989; Jinn et al., 1989; 1995). We have also isolated two cDNA clones of rice LMW HSP and studied their expression in response to heat stress and other environmental factors (Tseng et al., 1992; 1993). Because of the abundance and complexity of these proteins, we have tried to isolate additional cDNA clones for rice LMW

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Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; DTT, dithiothreitol; GST, glutathione *S*-transferase; *GST*, gene (DNA, RNA) encoding GST; HMW, high molecular weight; HS, heat shock; HSP (hsp), HS protein(s); *HSP* (*hsp*), gene (DNA, RNA) encoding HSP; IEF, isoelectric focusing; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; LB, Luria-Bertani (medium); LMW, low molecular weight; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; *Os*, *Oryza sativa*; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; p*I*, isoelectric point; PMSF, phenylmethylsulfonyl fluoride; PVP, polyvinylpyrrolidone; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na_3·citrate pH 7.6; *UTR*, untranslated region(s).

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HSP to study their difference in gene expression under heat stress and their roles in thermoprotection. Here we report the isolation of a novel cDNA clone, *Oshsp18.0* cDNA, for a rice class-I LMW HSP.

EXPERIMENTAL AND DISCUSSION

(a) Isolation and characterization of a 18.0-kDa LMW HSP-encoding cDNA from rice

Rice (*Oryza sativa* L. cv. Tainong 67) seedlings were germinated in dark at 28°C in rolls of moist paper towels as described by Lin et al. (1984). Total RNA was extracted from heat-treated (at 40°C for 2 h) two-day-old rice seedlings (Yeh et al., 1991). The rice cDNA libraries were established in λgt11 from poly(A)$^+$RNA. The libraries (10^6 clones) were screened by hybridization with rice *Oshsp17.3* cDNA (Tseng et al., 1992). The insert from one positive clone was subcloned into pGEM-7Zf(+), and designated as *Oshsp18.0* cDNA. The nt sequence of 0.7-kb cDNA insert was determined and submitted to GenBank under the accession No. X75616 (Fig. 1).

The *Oshsp18.0* cDNA contains 749 nt with 37-bp poly(A) tail, 81-bp 5′ *UTR* and 151-bp 3′ *UTR*. The ORF encodes a 160-aa protein presumably initiating from an ATG (nt 82) and terminating at TAG (nt 562). The ACAATGTC sequence around the ATG start codon matches the consensus sequence (ACAATGGC) associated with translation initiation in 79 plant genes proposed by Joshi (1987). This suggests that the A^{82}TG is the real start codon. A defined polyadenylation signal (AATAAA) was observed in the 3′ *UTR*, 72 nt upstream from the poly(A) tail (Fig. 1). In addition, there is a consensus sequence before the AATAAA signal spanning between nt 564 and 645 (Fig. 1) for 3′ end processing of mRNA as proposed by Wu et al. (1993) to add the poly(A) tail.

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CGCCGCTTGAGAATTGAGATCACCCTCTTTCCATCTAGAACACAAACCAGACAACCAAAA  60

AACAGCAAGACACAATACACAATGTCGCTGATCCGCCGCAGCAACGTGTTCGACCCCTTC 120
                     M  S  L  I  R  R  S  N  V  F  D  P  F   13

TCCCTCGACCTCTGGGACCCCTTCGACGGCTTCCCCTTCGGCTCCGGCAGCAGAAGCAGC 180
S  L  D  L  W  D  P  F  D  G  F  P  F  G  S  G  S  R  S  S   33

GGCACGATCTTCCCGTCCTTCCCCCGCGGCACCTCCTCCGAGACCGCGGCCTTCGCCGGC 240
G  T  I  F  P  S  F  P  R  G  T  S  S  E  T  A  A  F  A  G   53

GCGCGCATCGACTGGAAGGAGACGCCGGAGCACGTGTTCAAGGCGGACGTGCCGGGGCTG 300
A  R  I  D  W  K  E  T  P  E  H  V  F  K  A  D  V  P  G  L   73

AAGAAGGAGGAGGTCAAGGTGGAGGTGGAGGACGGCAACGTCCTCCAGATCAGCGGCGAG 360
K  K  E  E  V  K  V  E  V  E  D  G  N  V  L  Q  I  S  G  E   93

CGCAGCAAGGAGCAGGAGGAGAAGACGGACAAGTGGCACCGCGTGGAGCGCAGCAGCGGC 420
R  S  K  E  Q  E  E  K  T  D  K  W  H  R  V  E  R  S  S  G  113

AAGTTCCTCCGCAGGTTCCGGCTGCCGGAGAACACCAAGCCGGAGCAGATCAAGGCGTCC 480
K  F  L  R  R  F  R  L  P  E  N  T  K  P  E  Q  I  K  A  S  133

ATGGAGAATGGCGTGCTCACCGTCACCGTGCCCAAGGAGGAGCCCAAGAAGCCCGACGTC 540
M  E  N  G  V  L  T  V  T  V  P  K  E  E  P  K  K  P  D  V  153

AAGTCCATCCAGGTTACCGGCTAGTAAGAAACTTCGGGTGTGACATGCACGGTGGAGAGG 600
K  S  I  Q  V  T  G  *                                      160

CTTCGATTCGAGGCTTCGGTTTGTTGATCAATTGCAGTAAATAAAACGGTCAAATCTGGT 660

CCTCAGTGTTTATGCTGTGAAAAAGTTCAAAGCTATGTTGGAAGTGAGCAATAAAAAAAA 720

AAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                749
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Fig. 1. Nucleotide sequence of the rice LMW HSP *Oshsp18.0* cDNA clone and deduced aa sequence. The stop codon is denoted by an asterisk (*), a putative polyadenylation signal is underlined. The consensus sequence for mRNA 3′ end processing upstream the AATAAA signal is indicated by dashed lines. EMBL accession No. X75616. **Methods:** The *Oshsp18.0* cDNA clone was isolated from an unamplified λgt11 cDNA library generated from rice seedling poly(A)$^+$RNA using the rice *Oshsp17.3* cDNA insert as a probe. Screening was under high stringency (18 h in 50% formamide/5 × SSC/0.1% SDS/20 mM Na·phosphate pH 6.5/0.1% Ficoll/0.1% PVP/250 μg per ml denatured salmon sperm DNA at 43°C with a final wash in 0.1 × SSC/0.1% SDS at 50°C for 1 h).

The predicted protein properties and hydropathy profile are quite similar to those of *Oshsp16.9* cDNA and *Oshsp17.3* cDNA (reported as pTS1 and pTS3, respectively in Tseng et al., 1992). The total nt sequence of *Oshsp18.0* cDNA shares 83.1% and 73.4% similarity with *Oshsp17.3* cDNA and *Oshsp16.9* cDNA total nt sequences, respectively; while the coding region of *Oshsp18.0* cDNA shares 94.8% and 82.5% similarity with *Oshsp17.3* cDNA and *Oshsp16.9* cDNA coding regions, respectively.

Comparison of the deduced aa sequences of the *Oshsp18.0* cDNA and seven other class-I LMW HSP shows 70.0%, 87.0%, 71.3%, 66.9%, 69.1%, 66.9% and 66.9% identity to that of *Oshsp16.9* cDNA, *Oshsp17.3* cDNA (Tseng et al., 1992), *Peahsp18.1*, *Peahsp17.9a* (DeRocher et al., 1991), *Whthspc5-8* (McElwain and Spiler, 1989), *Soyhsp17.5E* (Nagao et al., 1985) and *Athhsp17.6* (Helm and Vierling, 1989), respectively. We also found that the sequence identity is higher in the C-terminal regions. This is true for all HSP (Lindquist and Craig, 1988; Vierling, 1991). The sequence identity suggests that *Os*hsp18.0 belongs to class-I LMW HSP.

(b) Identification of the *Os*hsp18.0 protein by in vitro translation

In order to determine if *Oshsp18.0* cDNA encodes a LMW HSP, in vitro translation of the hybrid-selected *Oshsp18.0* poly(A)$^+$RNA was performed. Purified poly(A)$^+$RNA isolated from the 28°C-grown and 41°C-treated rice etiolated seedlings was used. The analysis of the two-dimensional PAGE showed that most of the mRNA synthesized at 28°C was repressed by 41°C treatment. However, 41°C treatment induced some newly-synthesized LMW HSP ranging from 16 to 30 kDa. Some 70 to 90-kDa HMW HSP were also found (Fig. 2B). These results agree with the observations of Lindquist (1986), Mansfield and Key (1987) and Vierling (1991). The products of *Oshsp18.0* cDNA hybrid-selected in vitro translations (as shown in Fig. 2C,D) included seven major LMW HSP found in Fig. 2B. This result

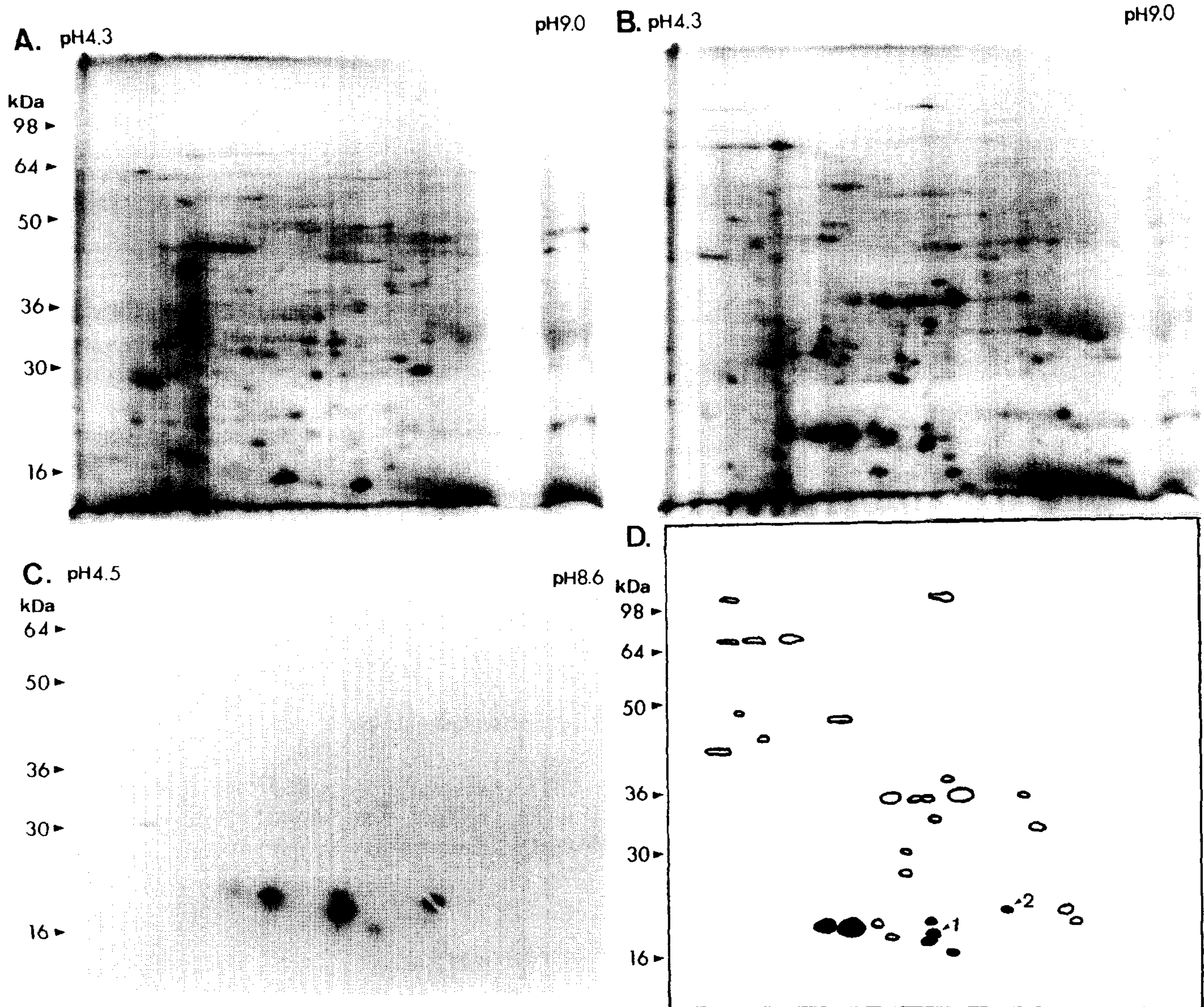

Fig. 2. Products of in vitro translation of hybrid-selected mRNA from poly(A)$^+$RNA isolated from heat-shocked rice seedlings. In panel **C**, RNA was selected with *Oshsp18.0* cDNA clone and translated in vitro in a rabbit reticulocyte lysate (Promega, Madison, WI, USA). Panels **A** and **B** show products translated in vitro from poly(A)$^+$RNA isolated from rice seedlings grown at 28°C (**A**) or from rice seedlings treated at 41°C for 1 h (**B**). Panel **D** shows the diagram of heat-induced proteins (as open circles), the corresponding spots of hybrid-selection products in panel **C** were filled with black (as black spots). The predicted products of *Oshsp16.9* cDNA and *Oshsp18.0* cDNA are labelled as number 1 and 2, respectively. **Methods:** Total RNA from rice was extracted from heat-treated (41°C for 1 h) seedlings or 28°C-grown seedlings by the procedure of Yeh et al. (1991). The hybrid-selected in vitro translation assay was performed as described by Maniatis et al. (1982) in a rabbit reticulocyte lysate. The products of in vitro translation were subjected to two-dimensional IEF (pH 4.3–9.0)/0.1% SDS-12.5% PAGE followed by autoradiography using a 425 PhosphoImager (Molecular Dynamics).

confirms that *Oshsp18.0* cDNA is a cDNA clone encoding a rice LMW HSP.

In Fig. 2C,D there are at least seven LMW HSP ranging from 16 to 20 kDa synthesized by *Oshsp18.0* cDNA hybrid-selected poly(A)$^+$RNA. The size of these proteins is in agreement with the predicted molecular mass (18.0 kDa). According to the previous study (Tseng et al., 1993), spot 1 (16.9 kDa, p*I* 6.4) was predicted to be encoded by *Oshsp16.9* cDNA. Correspondingly, it appears that spot 2 (18.0 kDa, p*I* 7.3) was predicted to be encoded by *Oshsp18.0* cDNA.

To confirm that *Oshsp18.0* cDNA encodes a class-I LMW HSP, immunoblotting was performed using a rice class-I LMW HSP antiserum. The *Oshsp18.0* cDNA coding region was introduced to pGEX-2T expression vector (Pharmacia). The sequence was confirmed to be in-frame. The GST-*Os*hsp18.0 fusion protein was obtained from *E. coli* and then purified as described by Johnson et al. (1989). The fusion protein was cleaved with thrombin to separate *Os*hsp18.0 protein from GST then subjected to SDS-PAGE analysis.

In Fig. 3A it is clear that a 18.2-kDa protein was pro-

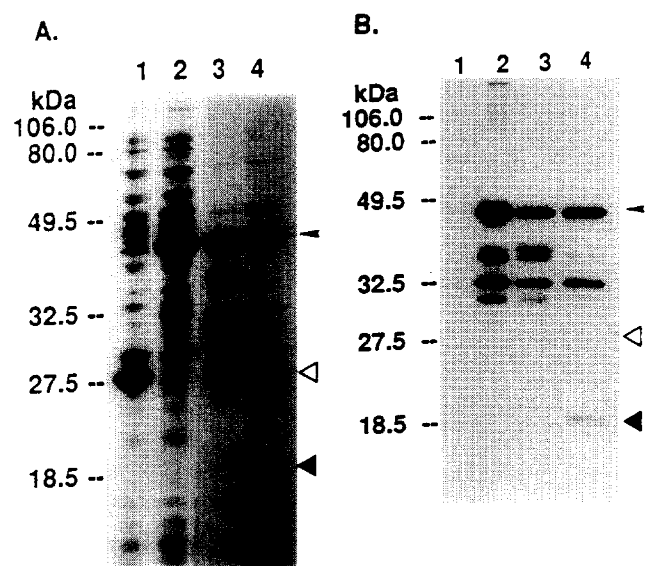

Fig. 3. Expression and purification of GST-*Os*hsp18.0 fusion protein produced from *E. coli* cultures, and immunological reactivity of the recombinant *Os*hsp18.0 protein with anti-*Os*hsp16.9 (rice class-I LMW HSP) antiserum. Panel **A** shows the Coomassie-blue-stained SDS-PAGE of proteins from *E. coli* cultures. Panel **B** shows the subsequent Western blots with anti-*Os*hsp16.9. Lane 1, control sample of total proteins from *E. coli* XL1-Blue transformed with pGEX-2T expression vector (no insert contained); lane 2, total proteins from *E. coli* expressing the fusion protein of GST-*Os*hsp18.0; lane 3, fusion protein purified by affinity chromatography with a glutathione-Sepharose 4B column; lane 4, purified fusion protein cleaved with thrombin. Small filled arrow head indicates GST-*Os*hsp18.0 fusion protein (about 46 kDa); big open arrow head indicates GST protein (about 27.5 kDa); big filled arrow head indicates *Os*hsp18.0 HSP (about 18.2 kDa). **Methods:** *E. coli* cultures and induction conditions were previously described by Smith and Johnson (1988). The cultures (induced by IPTG for 2 h) were harvested and cell pellets were obtained by centrifugation. Pellets were resuspended in PBS buffer (150 mM NaCl/16 mM Na_2HPO_4/4 mM NaH_2PO_4, pH 7.5) and centrifuged twice to remove LB media and secreted proteins of *E. coli*. The pellets were then resuspended in PBS buffer containing 1 mM PMSF/10 mM DTT/1% Triton X-100/1% Tween-20, sonicated, and subjected to centrifugation to obtain the supernatant containing the fusion protein. The fusion protein was purified by one step through a Glutathione-Sepharose 4B affinity column (Pharmacia). The recombinant *Os*hsp18.0 protein was obtained by cleavage of the fusion protein with thrombin (24 units/mg fusion protein). Protein samples (50 μg) were analyzed by 0.1% SDS-15% PAGE. For immunoblotting, proteins were transferred from polyacrylamide gels to Immobilon PVDF transfer membranes (Millipore) with glycine electrode buffer.

duced after thrombin treatment (lane 4). The size of the *Os*hsp18.0 protein was larger than the predicted 18.0 kDa because three extra aa were introduced to the N-terminal of *Os*hsp18.0 when using the *GST* gene fusion system. A duplicate SDS-PAGE was prepared for subsequent Western blots with rice *Os*hsp16.9 antiserum. The *Os*hsp18.0, but not GST (27.5 kDa), cross-reacted with anti-*Os*hsp16.9 (Fig. 3B). Thus, we have enough evidence to confirm that *Oshsp18.0* cDNA is a cDNA clone for rice LMW HSP. The 32.5-kDa protein (in Fig. 3B, lanes 2–4) is probably the product of incompletely translated fusion protein for some unknown reasons. In our experience, 20 to 30% *E. coli* died with the IPTG treatment. This may cause the fusion proteins with different lengths. These fusion proteins may have special conformations such that thrombin can not cleave the peptides completely and they are recognized by the rice *Os*hsp16.9 antiserum.

(c) Conclusions

(*1*) We have isolated a novel rice LMW HSP-encoding cDNA clone, *Oshsp18.0* cDNA.

(*2*) The sequence of *Oshsp18.0* cDNA suggests that a 18.0-kDa protein (p*I* 7.3) is encoded by this cDNA clone.

(*3*) Sequence comparison suggests that *Os*hsp18.0 is highly homologous to other class-I LMW HSP.

(*4*) The results of *Oshsp18.0* cDNA hybrid-selected in vitro translation and the cross reaction of *Os*hsp18.0 fusion protein with antiserum of rice class-I LMW HSP confirm that the *Oshsp18.0* cDNA clone belongs to the gene family of class-I LMW HSP.

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