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Isolation and characterization of the third gene encoding a 16.9 kDa class I low-molecular-mass heat shock protein, *Oshsp 16.9C*, in rice

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Abstract. Using rice cDNA *pTS1* encoding a 16.9 kDa heat shock protein (HSP) as a probe for Southern hybridization analysis, we observed five prominent bands of 9.5, 5.9, 3.4, 2.5, and 1.7 kb in the *Eco* RI digests of rice genomic DNA and found them to contain six individual genes. The 5.9 kb DNA fragment was further digested with *Hind* III to generate three fragments of 3.5, 1.7 and 0.7 kb, and we found, using *pTS1* cDNA as a probe, the 3.5 kb fragment contained a putative low-molecular-mass (LMM) HSP gene. The DNA sequencing of 3.5 kb fragment revealed encoding of a presumptive 16.9 kDa HSP (149 amino acid residues) with predicted pI value of 6.42. The nucleotide sequence of this gene was highly homologous to the coding regions of two rice class I LMM HSP genes, *Oshsp 16.9A* and *Oshsp16.9B*, sharing 93.1% and 94.3% sequence identity, respectively, as published previously in our laboratory. The deduced amino acid sequence of this gene is similar to those of the *Oshsp16.9A* and *Oshsp16.9B* genes with a difference of only 11 and 10 amino acids, respectively. It was hence designated as *Oshsp16.9C* (accession no. U81385). We used 3' *UTRs* (untranslated regions) for analysis of *Oshsp16.9C* gene expression since the 3' *UTRs* of these three genes showed very low sequence homology.

Keywords: Heat shock protein; Heat shock gene; Low-molecular-mass heat shock protein; *Oryza sativa* L.

Abbreviations: **HS,** heat shock; **HSP,** heat shock protein; **LMM,** low-molecular-mass; ***UTR*,** untranslated region.

Introduction

Heat shock proteins (HSPs) have been induced during thermal stress in all organisms ever examined, ranging from bacteria to human beings (Schlesinger et al., 1982), and they appear to be involved in thermoprotection (Lin et al., 1984; Chou et al., 1989; Krishnan et al., 1989; Vierling, 1991; Jinn et al., 1997). The HSPs are usually divided into high-molecular-mass (HMM) proteins of more than 30 kDa and low-molecular-mass (LMM) proteins of about 17 to 28 kDa (Lindquist and Craig, 1988; Vierling, 1991). In contrast to animal systems, plants synthesize more abundant LMM HSPs than HMM HSPs. The LMM HSPs superfamily is unusually complex, consisting of at least five gene families (LaFayette et al., 1996; Waters et al., 1996). The role of LMM HSP in heat stress is not completely clear yet. However, the sequence conservation of genomic and cDNA clones of plant LMM HSP genes isolated and characterized from a number of species suggests that they may play an important role in plants coping with HS.

We have been studying the physiological function of class I LMM HSPs in soybean and rice (Lin et al., 1984; Chou et al., 1989; Jinn et al., 1989; Jinn et al., 1993; Jinn et al., 1995; Yeh et al., 1995; Jinn et al., 1997; Yeh et al., 1997). We have isolated and characterized three cDNA clones: *pTS1* (encoding a 16.9 kDa HSP, Tseng et al., 1992), *pTS3* (encoding a 17.3 kDa HSP, Tseng et al., 1992), and *pYL* (encoding a 18.0 kDa HSP, Lee et al., 1995), and also five genomic clones, *Oshsp16.9A*, *Oshsp16.9B*, *Oshsp18.0*, *Oshsp17.3*, and *Oshsp17.7* of rice class I LMM HSPs (Tzeng et al., 1992; 1993; Guan et al., 1998, respectively). The *Oshsp16.9A* contains the sequence of *pTS1* cDNA. The three cDNA clones are highly homologous in their sequences except for the 3' untranslated regions (*UTRs*), which show a low degree of homology. Because of the abundance and complexity of these proteins, we have tried to isolate and characterize additional genes for rice class I LMM HSPs for the purpose of studying their differences in gene expression under heat stress. So far, we have isolated and characterized all the genes for rice class I LMM

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HSPs in our laboratory by use of *pTS1* cDNA as a probe. Here we report the nucleotide sequence and deduced amino acid sequence of a third rice gene, which encodes a protein product of about 16.9 kDa and was designated as *Oshsp16.9C*.

Materials and Methods

Plant Materials and Treatments

Rice (*Oryza sativa* L. cv. Tainong 67) seedlings were germinated in darkness at 28°C for 3 days in rolls of moist paper towels as described by Lin et al. (1984). Three-day-old rice seedlings without endosperms were incubated in a medium containing 5 mM potassium phosphate (pH 6.0) and 1% sucrose before treatment at control (28°C) or heat-shocked (41°C) temperatures for 2 h. Seedlings were harvested, frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. The powders were subjected to DNA or RNA isolation as described below.

DNA Isolation and Southern Blot Analysis

Total rice DNA was isolated according to Malmberg et al. (1985). DNA was digested with *Eco* RI and *Hind* III restriction enzymes, separated on 1% agarose gels and transferred to Hybond-C extra membranes (Amersham, Buckinghamshire, UK). Filters were prehybridized in 50% formamide/5X SSC/0.1% SDS/20 mM Na phosphate pH 6.5/0.1% Ficoll/0.1% PVP/250 mg ml^{-1} denatured salmon sperm DNA at 42°C for 4-6 h followed by hybridization at 42°C overnight in prehybridization solution with ^{32}P-labeled probes (~5×10^7 cpm). The coding region of *pTS1*, 3' *UTRs* of *pTS3*, *pYL*, and *Oshsp16.9C* were labeled with (α-^{32}P) dCTP (1000 Ci $mmol^{-1}$, Amersham, Buckinghamshire, UK) for probes using the Prime-a-Gene Labeling System (Promega, Madison, WI, USA). Filters were then washed three times in 2X SSC/0.1% SDS at room temperature for 10 min with final washes in 0.1X SSC/0.1% SDS twice at 53°C for 30 min.

Construction and Screening of Size-Selected Genomic Library

Total rice genomic DNA was digested with *Eco* RI restriction enzyme. The DNA sizes between 5 and 7 kb were eluted and further ligated to the *Eco* RI site of λEMBL-3 vector (Promega, Madison, WI, USA) for library construction according to the standard methods (Sambrook et al., 1989). The size-selected genomic libraries (~10^5 plaques) were screened with rice *pTS1* probe under the same conditions described in DNA Isolation and Southern Blot Analysis. Positive plaques were identified by autoradiography.

Restriction Mapping, Subcloning and Sequencing of the Positive Clones

One positive clone (5.9 kb) was isolated and subcloned into a pGEM-3Zf(+) vector. The restriction map was determined using different combinations of restriction digestion of suitable enzymes located in the multiple cloning sites of the vector. For this 5.9 kb clone, nested deletion sets were generated using the Erase-a-Base System (Promega, Madison, WI, USA) according to the manufacturer's protocol and DNA sequences were determined using the Sequenase Version 2.0 DNA Sequencing Kit (USB, Cleveland, OH, USA).

Preparation of the 3' UTRs of pTS3 and pYL cDNAs and Oshsp16.9C

The 3' *UTRs* of *pTS3*, *pYL*, and *Oshsp16.9C* were prepared by polymerase chain reaction (PCR) using the PCR kit (Perkin Elmer Centus, Norwalk, CT, USA). The primers for PCR were 5' AGCATTGGGCTAATCT 3' (the 5' end primer) and 5' ACAACAGGTTTTACCG 3' (the 3' end primer) for *pTS3*, 5' AGAAACTTCGGGTGTG 3' (the 5' end primer) and 5' TCACTTCCAACATAGC 3' (the 3' end primer) for *pYL*, and 5' GAAGGAGAGAAGCTATATAC 3' (the 5' end primer) and 5' TAGCTCATTCATTCAGACTC 3' (the 3' end primer) for *Oshsp16.9C*. The PCR reactions for *pTS3* and *pYL* were 40 sec at 94°C, 40 sec at 50°C, 40 sec at 72°C for 32 cycles; and that for *Oshsp16.9C* was 30 sec at 94°C, 30 sec at 42°C, 30 sec at 72°C for 35 cycles followed by 10 min at 72°C for 1 cycle. The PCR products were gel-purified, ligated into a pGEM-7Zf(+) vector, confirmed by sequencing, and used for labeling as probes. The lengths of PCR products were 158 bp, 141 bp and 187 bp for 3' *UTRs* of *pTS3*, *pYL*, and *Oshsp16.9C*, respectively.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from control and heat-shocked 3-day-old rice seedlings according to Chang et al. (1993). RNA samples were separated on 1.2% formaldehyde agarose gels and transferred to Hybond-C extra membranes (Amersham, Buckinghamshire, UK) as described by Sambrook et al. (1989). Filters were prehybridized in 50% formamide/5X SSC/0.1% SDS/20 mM Na phosphate pH 6.5/0.1% Ficoll/0.1% PVP/250 mg ml^{-1} denatured salmon sperm DNA at 42°C for 4-6 h followed by hybridization at 42°C for overnight in prehybridization solution with ^{32}P-labeled probes (~5×10^6 cpm). The 3' *UTR* of *Oshsp16.9C* was labeled with (α-^{32}P) dCTP (1000 Ci $mmol^{-1}$, Amersham, Buckinghamshire, UK) for a probe using the Prime-a-Gene Labeling System (Promega, Madison, WI, USA). Filters were then washed three times in 2X SSC/0.1% SDS at room temperature for 10 min with final washes in 0.1X SSC/0.1% SDS twice at 53°C for 30 min.

Primer Extension Analysis

Poly $(A)^+$ RNA was purified from total RNA isolated from heat-shocked 3-day-old rice seedlings by oligo (dT) cellulose chromatography (Zurfluh and Guilfoyle, 1982). Ten pmole of the oligonucleotide 5'-tgttggtttgtcgtgatc-3', which is complementary to 65-82 bases downstream from the TATA sequence of the *Oshsp16.9C* gene, was labeled at the 5' terminus with (γ-^{32}P) ATP (3000 Ci $mmol^{-1}$,

Amersham, Buckinghamshire, UK) and T4 polynucleotide kinase using the Primer Extension System (Promega, Madison, WI, USA). One µg of the poly $(A)^+$ RNA samples were annealed with 1 pmole of the labeled primer for primer extension reaction according to the manufacturer's protocol. Primer extension products were electrophoresed in parallel with the sequencing reaction, which had been primed with the same oligonucleotide.

Results and Discussion

Isolation of a 5.9 kb Genomic Clone Encoding a Putative Rice Class I LMM HSP

Five prominent bands of 9.5, 5.9, 3.4, 2.5, and 1.7 kb in the *Eco* RI digests of rice genomic DNA were observed by Southern hybridization analysis using rice *pTS1* cDNA, encoding a 16.9 kDa HSP, as a probe (Tzeng et al., 1993). The 3.4 and 2.5 kb fragments have been shown to contain two rice HSP genes: *Oshsp16.9B* and *Oshsp16.9A*, respectively (Tzeng et al., 1993). In order to isolate other genes of rice class I LMM HSPs for expression analysis, the same probe was used to screen a size-selected rice genomic library established in λEMBL-3 vector. A 5.9 kb *Eco* RI fragment from the positive clone was isolated and subcloned into a pGEM-3Zf(+) vector. The 5.9 kb DNA fragment could be further digested with *Hind* III to generate three fragments of 3.5, 1.7 and 0.7 kb, and we found the 3.5 kb fragment contained a putative class I LMM HSP gene as observed by Southern hybridization with *pTS1* cDNA (Figure 1). The restriction map of the 5.9 kb fragment was determined, as shown in Figure 1C, to contain two *Xbal* I sites, one *Sac* I site, one *Xho* I site, one *Bgl* II 1site, and two *Hind* III sites.

Identification of a New Gene Encoding a Putative Rice Class I HSP

The sequences of three cDNA clones—*pTS1* (encoding 16.9 kDa HSP), *pTS3* (encoding 17.3 kDa HSP), and *pYL* (encoding 18.0 kDa HSP)—of rice class I LMM HSP are highly homologous in their coding regions. However, the 3' *UTRs* show a low degree of homology. Thus, we used the 3' *UTRs* of *pTS3* and *pYL* cDNAs as probes to identified the possible corresponding genes, since the *Oshsp16.9A* has been shown to contain the *pTS1* cDNA (Tzeng et al., 1993). The results of Southern blot analysis suggested that the 5.9 kb fragment was not recognized by either the 3' *UTRs* of *pTS3* or *pYL* (data not shown); hence, this 5.9 kb fragment contained a new gene of putative rice class I LMM HSP which has not been characterized before.

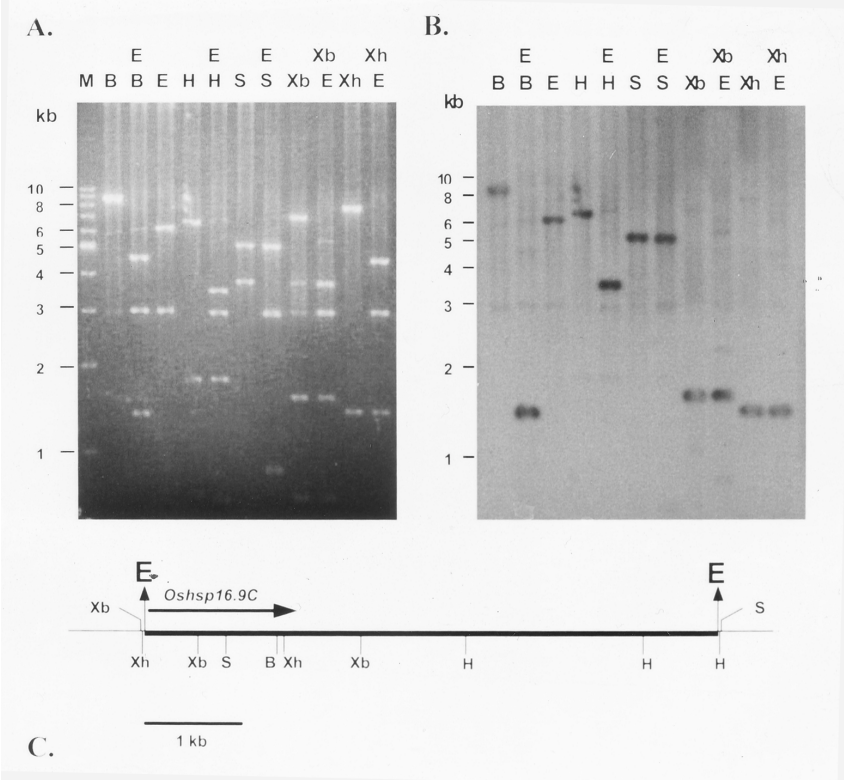

Figure 1. Southern blot analysis of rice 5.9 kb DNA fragment probed with the coding region of *pTS1* cDNA and the restriction map of the rice 5.9 kb *Eco* RI fragment. The clone of rice 5.9 kb *Eco* RI fragment in pGEM-3Zf(+) vector was digested with *Eco* RI (E), *Bgl* II (B), *Hind* III (H), *Sac* I (S), *Xba* I (Xb), *Xho* I (Xh) or subjected to double digestion with *Bgl* II and *Eco* RI (BE), *Hind* III and *Eco* RI (HE), *Xba* I and *Eco* RI (XbE), *Xho* I and *Eco* RI (XhE), or *Sac* I and *Eco* RI (SE). Panel A: ethidium bromide staining; Panel B: Southern blot analysis; Panel C: restriction map of the rice 5.9 kb *Eco* RI fragment. The molecular mass markers (M) are shown and indicated.

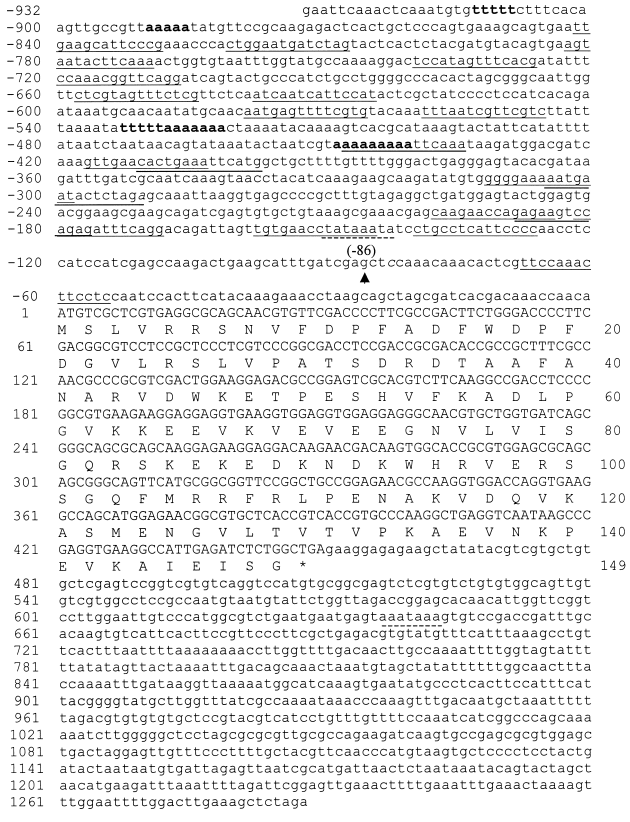

Figure 2. DNA sequence and corresponding amino acid sequence of the rice gene *Oshsp16.9C*. The sequence of coding region is capitalized. Arrow indicates site of transcriptional initiation (-86). HSE-like sequences are underlined. A/T-runs are in boldface. Polyadenylation signal and putative TATA box are designated by dashed lines. The GenBank accession number is U81385 for *Oshsp16.9C*.

Sequence Analysis and Characterization of the Putative Rice HSP Gene

The 5.9 kb fragment cloned into a pGEM-3Zf(+) vector was subjected to sequencing. The DNA sequence between the *Eco* RI site and *Xba* I site (as shown in Figure 2) of this fragment revealed an open reading frame predicted to encode a polypeptide of 16.9 kDa (149 amino acid residues) with a pI value of 6.4 and was hence designated as *Oshsp16.9C*. The GenBank accession number is U81385 for *Oshsp16.9C*. The coding region of *Oshsp16.9C* was compared with those of *Oshsp16.9A* and *Oshsp16.9B* as shown in Figure 3. The base composition in the coding region of the *Oshsp16.9C* gene is 20.44% of A, 35.33% of G, 14.00% of T, and 30.22% of C. No intron is present in the *Oshsp16.9C* gene, just as in the *Oshsp16.9A*, *Oshsp 16.9B* genes and other class I LMM HSP genes of rice. According to the results of nucleotide sequence comparison (Figure 3A), *Oshsp16.9C* shares 93.1% and 94.3% identity with *Oshsp16.9A* and *Oshsp16.9B*, respectively, in their coding regions. The composition of 149 amino acid residues deduced from the coding sequence of *Oshsp 16.9C* is 25 strong basic (+) amino acids, 26 strong acidic (-) amino acids, 52 hydrophobic amino acids, and 26 polar amino acids. The deduced amino acid sequence of *Oshsp16.9C* is identical to those of the *Oshsp16.9A* and *Oshsp16.9B* genes with a difference of only 11 and 10 amino acids, respectively (Figure 3B). Within the class I LMM HSPs in general, there is a high degree of sequence conservation in the carboxyl-terminal portion of the proteins while the amino-terminal shows significantly less (Vierling, 1991). The deduced amino acid sequence alignment of *Oshsp16.9A*, *Oshsp16.9B*, and *Oshsp16.9C* genes, as shown in Figure 3B, showed that only two or three out of 100 amino acids were different in the carboxyl-terminal, whereas, eight out of 50 amino acids were different in the amino-terminal.

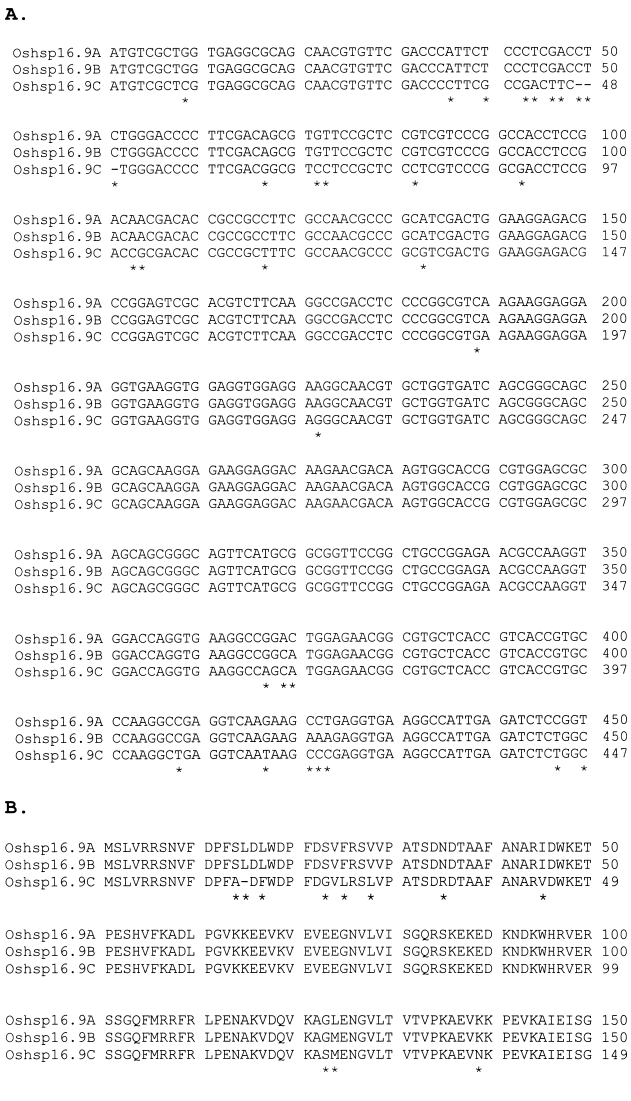

Figure 3. Comparison of DNA and deduced amino acid sequences of three rice HSP genes *Oshsp16.9A*, *Oshsp16.9B*, and *Oshsp16.9C*. DNA (A) and deduced amino acid (B) sequences of *Oshsp16.9C* compared with those of two other rice genes for 16.9 kDa HSP, *Oshsp16.9A* (M80938) and *Oshsp16.9B* (M80939) reported by Tzeng et al. (1992). Dashes indicate insertions or deletions to allow for maximal alignment, and stars (★) indicate sequence difference.

The 5' upstream sequence obtained was about 932 bp (as shown in Figure 2), which contained a putative TATA box (TATAAATA), 142-base upstream from the initiation codon ATG, and multiple copies of HS consensus elements (HSEs) upstream from the TATA box. The initiation site of transcription of *Oshsp16.9C*, identified by the primer extension method, was located 86 bases upstream (-86) from the deduced start of translation and 56 bases downstream from the TATA box (Figure 4). The weak band was seen above the major band (Figure 4), which might have resulted from the cross-hybridization of RNA transcribed from closely related genes encoding HSPs in the same LMM class (Czarnecka et al., 1985; Tzeng et al., 1993). There were tandem overlapping HSEs, (from -19 to -48) proximal to the TATA box, a typical feature of class I LMM HSP genes (Nagao and Key, 1989; Schöffl et al., 1998). Among these HSEs, 5 to 9 out of 10 nucleotides matched the HS consensus, CTnGAAnnTTCnAG, as defined by Pelham (1985). The arrangement of HSEs on the 5' upstream sequence of *Oshsp16.9C* was compared with those of *Oshsp16.9A* and *Oshsp16.9B* (Figure 5). There are 8 HSEs in the upstream region of both the *Oshsp16.9A* and *Oshsp16.9B* genes, but there are 20 in that of *Oshsp16.9C*. Some HSEs even locate downstream the TATA box and transcription initiation site of the *Oshsp16.9C*, but whether these HSEs are required for heat

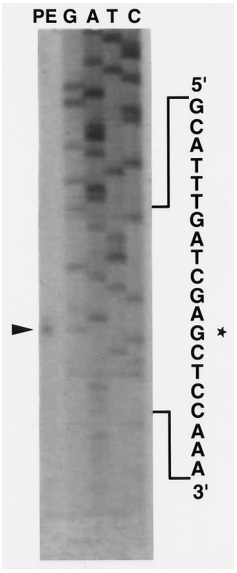

Figure 4. Identification of the site of transcription initiation for *Oshsp16.9C* by the primer extension method. Lane PE shows the primer extension products of HS. Lanes G, A, T, C are from a DNA sequencing reaction in which the same primer was used. The transcription initiation site is indicated by an arrowhead with a star (★).

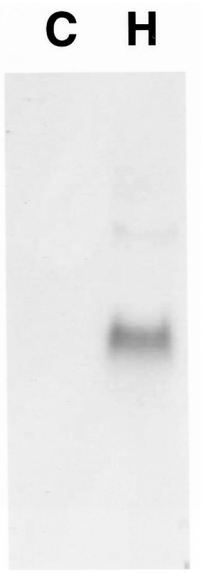

Figure 6. Northern blot analysis of rice RNA probed with the 3' *UTR* of *Oshsp16.9C*. Total RNA (15 µg per lane) isolated from 3-day-old rice seedlings after 2 h of 28°C (C) or 41°C (H) treatments were subjected to Northern blot analysis.

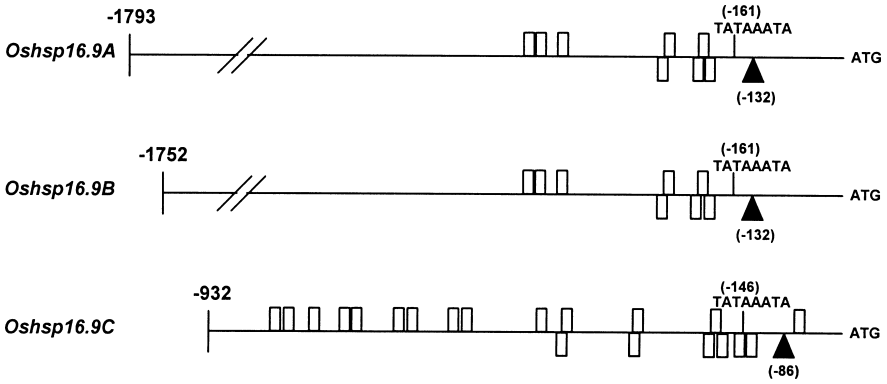

Figure 5. The arrangement of HSEs on the 5' upstream sequences of *Oshsp16.9A*, *Oshsp16.9B*, and *Oshsp16.9C*. Schematic alignment of about 930 nucleotides (lines) upstream from the coding regions of three rice HSP genes, *Oshsp16.9A*, *Oshsp16.9B*, and *Oshsp16.9C*, relative to their TATA boxes (TATAAATA, as indicated). The sites of transcriptional initiation are indicated by filled triangle. Putative HSEs (CTnGAAnnTTCnAG) are indicated as boxes. Number in parenthesis indicates position relative to the first ATG. All sequence elements and their spacing are drawn to scale.

inducible transcription of the native gene as suggested by Baumann et al. (1987) remains to be tested. Sequences other than the TATA-proximal HSEs have also been shown to be required for full expression of the promoter in plant HS genes (Czarnecka et al., 1989).

Further upstream from the putative promoter regions, the *Oshsp16.9C* gene contains a DNA sequence very rich in A+T (54.01% compared to only 34.44% within the coding region) starting with runs of "simple sequences" such as $(A)_9$, $(A)_7$, $(A)_5$ and $(T)_5$ at nucleotide position -441, -522, -886, and -903 (Figure 4). Runs of "simple sequences" $(A)_n$, $(T)_n$ or $(AT)_n$ have been observed in most HS promoters in soybean (Raschke et al., 1988). Czarnecka et al. (1990) demonstrated a binding of nuclear proteins to scattered AT-rich sequences of soybean *Gmhsp 17.5-E* gene promoter. Such sequences possibly contribute to the transcriptional regulation of HS genes. The significance of these upstream elements in gene regulation is being studied in our laboratory using the PCR technique to amplify different DNA fragments.

Although the sequence of *Oshsp16.9C* is highly homologous to those of the *Oshsp16.9A* and *Oshsp16.9B* in their coding regions, the 3' *UTRs* of these three genes show very low sequence homology. The sequence similarity between the 3' *UTRs* of *Oshsp16.9A* and *Oshsp 16.9B*, *Oshsp16.9A* and *Oshsp16.9C*, and *Oshsp16.9B* and *Oshsp16.9C* are 53.33%, 45.46% and 53.28%, respectively. Since the homology of 3' *UTRs* in *Oshsp16.9A*, *Oshsp 16.9B* and *Oshsp16.9C* are low, we could use these 3' *UTR* fragments as probes for gene expression studies. The role of 3' *UTR* in affecting gene expression remains to be determined. However, the effect of the 3' *UTR* in two *hsp70-adh* cDNA gene chimeras was examimed (Yost et al., 1990). Both constructs were driven by the *hsp70* promoter and contained the *adh* coding region. They differed in having either the *adh* 3' *UTR* or the *hsp70* 3' *UTR*. While transcripts with the *adh* 3' *UTR* were very stable during recovery from HS, transcripts with the *hsp70* 3' *UTR* were unstable, and decayed in a manner similar to the endogeneous *hsp70* transcripts. Thus, the 3'*UTR* of the *hsp70* message plays a critical role in regulating its degradation (Yost et al., 1990). The putative polyadenylation signal AATAAA was located between +635 and +640 in *Oshsp16.9C* (Figure 2). This signal was located between +791 and +796 in *Oshsp16.9A* and between +737 and +742 in *Oshsp16.9B* (Tzeng et al., 1993).

Expression of the Oshsp16.9C Gene

The 3' *UTR* of *Oshsp16.9C* was obtained by PCR as described in Materials and Methods. It was used as a probe to analyze the expression of the *Oshsp16.9C* gene. The results of Northern blot hybridization suggest that this gene is expressed in heat-shocked (41°C, 2 h), but not in control (28°C, 2 h), seedlings (Figure 6). It is clear the *Oshsp16.9C* gene is activated by HS with a transcript of about 900 nucleotides in length. Activation of this gene under different HS or stress conditions in comparison with *Oshsp16.9A* and *Oshsp16.9B* will be further studied.

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水稻中第三個 16.9 kDa 第一族低分子量熱休克蛋白質基因，*Oshsp16.9C*，之分離與鑑定

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利用水稻 16.9 kDa 熱休克蛋白質（HSP）基因之 cDNA，pTS1，作為南方墨點分析法之探針，可在以 *Eco* RI 限制切割之水稻基因組 DNA 樣品中，偵測到五個清晰的條帶，其大小分別為 9.5、5.9、3.4、2.5 及 1.7 kb，經過本研究室之分析，其中包含六個不同的基因。將 5.9 kb 之 DNA 片段以 *Hind* III 限制酵素作進一步切割，可得 3.5、1.7 及 0.7 kb 等三個條帶，再以 pTS1 作為探針得知 3.5 kb 片段中可能包含一低分子量熱休克蛋白質基因。經由定序分析此 3.5 kb 片段之核酸序列，推測其可能之轉譯產物為包含 149 個氨基酸之 16.9 kDa 熱休克蛋白質，其等電點（pI）值估計為 6.42。此基因和本研究室已發表的二個水稻第一族低分子量熱休克蛋白質基因，*Oshsp16.9A* 及 *Oshsp16.9B*，在轉譯區內之核酸序列具有相當高的同質性，分別有 93.1% 及 94.3% 的相同度。此基因之氨基酸推演序列和 *Oshsp16.9A* 及 *Oshsp16.9B* 基因幾乎完全相同，分別只有 11 個及 10 個氨基酸之不同，因此將此基因定名為 *Oshsp16.9C*（accession number: U81385）。已知水稻第一族低分子量熱休克蛋白質基因之 3’ 非轉譯區（3’ *UTR*）的核酸序列相似性較低，因此我們將 *Oshsp16.9C* 之 3’ 非轉譯區作為探針，以分析其基因之表現情形。

關鍵詞：熱休克蛋白質；熱休克基因；低分子量熱休克蛋白質；水稻。