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Induction of a cDNA clone from rice encoding a class II small heat shock protein by heat stress, mechanical injury, and salicylic acid

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Abstract

This is the first report of a full-length cDNA clone for a class II small heat shock protein (sHSP) isolated from rice *(Oryza sativa* L., cv. Tainong No. 67) etiolated seedlings heat shocked at 41 °C for 2 h. The coding sequence consists of 501 bp, and the clone encodes a protein of 18.0 kDa with a predicted pI value of 5.61. The obtained full-length cDNA clone, designated *Oshsp18.0-CII*, is almost identical to a putative class II sHSP gene located on rice (cv. Nipponbare) chromosome one and another putative class II sHSP rice gene. *Oshsp18.0-CII* was induced by mechanical injury and salicylic acid treatment, which is not common in this class of sHSP genes. Only one copy of class II sHSP genes is present in the rice genome, and western blot analysis with anti-PsHSP17.7 (a class II pea sHSP) also showed only one protein of ~18 kDa in the 2D gel of heat-shocked rice proteins. *Oshsp18.0-CII* is GC-rich and contains a secondary structure in its RNA sequence.

Keywords: Gene expression; Heat shock (HS); Heat shock protein (HSP); *Oryza sativa* L.; Wounding

1. Introduction

Living organisms greatly alter their patterns of gene expression on exposure to sublethal high temperatures [1]. Elevated temperatures induce the expression of heat-shock proteins (HSPs), and suppress, at least in part, the synthesis of normal cellular proteins. Such response allows organisms to become tolerant to nonpermissive (or lethal) high temperatures. Therefore, thermoprotection by HSPs appears to be important for survival during heat stress [2,3]. The major HSPs studied to date are highly homologous among eukaryotes and, in some cases, among prokaryotes. The evolutionary conservation of genes for HSPs further suggests that the production of HSPs is a basic mechanism for coping with heat shock [2,3].

HSPs are divided into low molecular mass proteins of approximately 15–28 kDa (sHSPs) and high molecular mass proteins of more than 30 kDa (HMM HSPs) [2,3]. The HMM HSPs of plants, in contrast to those of animals, represent only a relatively small fraction of the total HSPs. Sequence analysis of sHSPs has revealed a high degree of homology among plant species, although variations in electrophoretic patterns were observed [4]. The sHSPs are classified into six classes: classes I and II are cytosolic proteins, and the others are proteins targeted to chloroplasts, mitochondria, and endomembranes [5,6]. Recently, Scharf et al. [7] identified other sHSP genes outside of the six classes.

The physiological functions of sHSPs in rice have been studied previously [8–13]. Several cDNA clones and genomic clones of rice class I sHSPs have been isolated, and their expression in response to heat stress and other environmental factors has been studied [14–18]. Because of the abundance and complexity of these proteins and because two classes of sHSPs are localized to the cytosol, we were interested in isolating the cDNA clones of class II sHSPs to further analyze the functional differences between these two classes.

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Table 1
PCR primers

Primer	Sequence and length (nt)	F/R[a]	Position[b]
ADVKEL	5′-GCCGACGT(C/G)AAGGAGCT(C/G)-3′ (18)	F	+163 to +180
KKPKTI (GSP1)	5′-GATGGTCTTGGGCTTCTT-3′ (18)	R	+466 to +483
ATG	5′-ATGGAGAGCGCCATGTTCGG-3′ (20)	F	+1 to +20
3′UTR	5′-CAGATAAGCAGCAGAACATCC-3′ (21)	R	+701 to +721
GSP2 (MGKFMRK)	5′-TTGCGCATGAACTTGCCCA-3′ (19)	R	+347 to +365
5′UTR	5′-CCCAGCGCGATCCGAGGTAG-3′ (20)	F	−63 to −44

[a] F and R denote forward and reverse primer, respectively.
[b] Position in *Oshsp18.0-CII* as shown in Fig. 2.

The class II sHSP genes in plants in general are induced by different developmental programs or by microsporogenesis [19–22]. However, *AtHsp17.6-II* isolated from Arabidopsis [23], *PsHsp17.7* isolated from pea [24] and a tomato HSP17.6 gene [25] were inducible by heat stress, and class II sHSP genes of sunflower showed tissue-specific expression in response to water stress [26]. Recently, *AtHSP17.6-II* and *AtHSP17.6A* were reported to be induced by wounding [27], which is not common for HSP genes. The level of two class II sHSP transcripts (*HSP17.4* and *HSP17.6*) has also been reported to accumulate in tomato fruit during ripening, chilling storage, and methyl jasmonate (MeJA) and methyl salicylate (MeSA) treatments [25]. In Arabidopsis, *Hsp17.6* but not *Hsp101* was induced by salicylic acid (SA) [28]. SA also affected Hsp70/Hsc70 expression in tomato cell suspension cultures [29]. In this report, we describe the isolation of a cDNA clone, *Oshsp18.0-CII*, encoding a putative rice class II sHSP, and show that it was also inducible by mechanical injury and SA treatment.

2. Materials and methods

2.1. Plant materials and treatments

Rice (*Oryza sativa* L. cv. Tainong No. 67) seedlings were germinated in the dark at 28 °C for 3 days in rolls of moist paper towels as described by Chang et al. [14]. The endosperm-removed etiolated seedlings were subjected to 28 °C (control), 41 °C, wounding (cutting seedlings into 1 cm fragments) and SA (1 mM) treatment for 1, 2, 4, 8, 16, and 24 h by submerging the seedlings in a buffer solution containing 5 mM potassium phosphate (pH 6.8), and 1% sucrose in a 28 °C shaking water bath at 30 rpm.

2.2. RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extraction from the treated rice seedlings and poly(A)$^+$ RNA purification by oligo (dT) cellulose chromatography were performed according to Chang et al. [30] and Lee et al. [16]. The first-strand cDNAs were synthesized from the poly(A)$^+$ RNAs of 3-day-old heat-shocked (2 h at 41 °C) rice seedlings using SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen). The RT-PCR product was prepared using a PCR kit (Perkin-Elmer Centus) and the first-strand cDNAs as templates [31]. The primers used (listed in Table 1) for PCR were the two 18-nt oligomers ADVKEL and KKPKTI primers. The sequences of these primers were in correspondence with those of the conserved amino acid domains (ADVKEL and KKPKTI) among the coding regions of three maize *(Zea mays* L.) class II sHSP cDNA clones, *cMHSP18-1*, *cMHSP18-3*, and *cMHSP18-9* [19]. The ATG primer (with the sequences corresponding to the first 20 nt started with the ATG start codon) paired with the KKPKTI primer or the 3′ UTR primer (with the sequences corresponding to those of the last 21 nt in the 3′ UTR of the *pOSHSP2* as described below) were also used for RT-PCR (see Table 1 for primer sequences). For all PCR programs, the reaction mixture was initially denatured at 94 °C for 5–7 min, followed by 35 cycles of reaction as listed in Table 2 and ended with 10 min of extension at 72 °C. The lengths of the PCR bands are listed in Table 2.

2.3. Cloning and sequence analysis of the RT-PCR products

The RT-PCR products were gel purified, treated with T4 DNA kinase and cloned into the *Sma* I site of pGEM-7Zf(+)

Table 2
PCR reactions used in this study and the resulted product sizes

Primer pair	1st denaturation	35 PCR cycles	Size of PCR product (bp)
ADVKEL/KKPKTI	94 °C 5 min	94 °C 40 s, 50 °C 40 s, 72 °C 40 s	321
ATG/KKPKTI	94 °C 5 min	94 °C 40 s, 63 °C 40 s, 72 °C 40 s	390–436
ATG/3′UTR	94 °C 5 min	94 °C 40 s, 48 °C 40 s, 72 °C 40 s	625–658
5′RACE	94 °C 7 min	94 °C 30 s, 65 °C 30 s, 72 °C 1 min	568 (for SUP/GSP1 primers) 450 (for SUP/GSP2 primers)
5′UTR/3′UTR	94 °C 7 min	94 °C 1 min, 48 °C 50 s, 72 °C 1 min	784

vector (Promega), or the gel-purified products were cloned directly using the pGEM-T Easy system (Promega). The DNA sequence was determined using the Sequenase Version 2.0 DNA sequencing kit (USB). The 321 bp insert of the RT-PCR clone was used as a probe for library screening and northern blot analysis.

2.4. Northern blot analysis

Equal amounts (10 μg) of total RNA from the control (28 °C), heat-shocked, wounded and SA-treated rice seedlings were separated on formaldehyde/agarose gels and transferred to Hybond-C extra membranes (Amersham). Filters were subjected to northern hybridization using ^{32}P-labeled 321 bp RT-PCR probe according to Chang et al. [14].

2.5. cDNA library screening

The rice cDNA libraries were established in a ZAP Express vector (Stratagene) from poly(A)$^+$ RNAs of 3-day-old heat-shocked rice seedlings using the ZAP Express cDNA Gigapack II Gold cloning kit (Stratagene). The libraries (10^6 clones) were screened by hybridization with a ^{32}P-labeled 321 bp RT-PCR probe ($\sim 8 \times 10^7$ cpm) as described above. High stringency screening was performed with a final wash in 0.1× SSC/0.1% SDS at 50 °C for 1 h [16]. The insert from one positive clone was in vivo excised from the ZAP Express vector and maintained in the pBK-CMV phagemid vector (Stratagene) [32]. The sequence of the resulting clone, designated as *pOSHSP2*, was determined as mentioned above.

2.6. Rapid amplification of 5′ cDNA ends (5′ RACE)

The 5′ RACE was performed using the Smart™ RACE cDNA Amplification Kit (Clontech). The first-strand cDNAs were synthesized from the poly(A)$^+$ RNAs of 3-day-old heat-shocked rice seedlings with the PowerScript™ reverse transcriptase (Clontech), the 5′ RACE CDS primer and the SMART II A oligonucleotide according to the manufacturer's protocol. The resulting cDNAs underwent PCR with Advantage 2 Polymerase Mix, Universal Primer A Mix, including the Long Universal Primer (LUP) and the Short Universal Primer (SUP) supplied by the kit, and a gene-specific primer 1 (GSP1, the KKPKTI primer mentioned above). A second round of PCR involved amplification with a gene-specific primer 2 (GSP2, see Table 1 for sequence, the antisense sequence corresponding to MGKFMRK), and the SUP. The PCR programs are listed in Table 2. The 5′ RACE products were further subcloned in pGEM-T Easy vector (Promega) and underwent sequencing analysis as described above.

2.7. Cloning of the full-length Oshsp18.0-CII

The first-strand cDNAs were synthesized from the poly(A)$^+$ RNAs of 3-day-old heat-shocked rice seedlings using the SuperScript in RNase H$^-$ Reverse Transcriptase (Invitrogen), and then subjected to PCR. The primers used for PCR were the 5′ UTR primer, with the sequence corresponding to the first 20 nt of the 5′ RACE clone, and the 3′ UTR primer described above. The PCR program is listed in Table 2. The PCR products of about 800 bp were further subcloned in pGEM-T Easy vector (Promega) and underwent sequencing analysis as described above.

2.8. RNA structure prediction, sequence alignment, and phylogenetic analysis

The RNA secondary structure of the full-length clone was predicted using the mfold server (Version 3.1) web service (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) developed by Zuker [33] and Mathews et al. [34]. DNA and protein sequences were aligned using the Biology WorkBench 3.2 (http://workbench.sdsc.edu) CLUSTAL W (1.81) multiple sequence alignments program [35]. For phylogenetic analysis, protein sequences with the out-group LEA (U57639) were aligned using the CLUSTAL X (1.81) multiple sequence alignments program [36]. The relationship between plant classes I and II sHSPs was determined using the PAUP* [37] and neighbor-joining analysis. Protein distance matrices were computed using the Kimura-two parameter. The neighbor-joining method was implemented by the bootstrap procedure with 1000 re-samplings of the data.

2.9. DNA isolation and Southern blot analysis

Total rice DNA was isolated according to Chang et al. [14]. DNA was digested with different restriction enzymes (New England Biolabs), separated on 1% agarose gels and transferred to Hybond-C extra membranes (Amersham). Hybridization conditions and the ^{32}P-labeled 784 bp *Oshsp18.0-CII* probe were as described above. The final wash for the filter was in 0.1× SSC/0.1% SDS twice at 70 °C for 30 min.

2.10. Protein extraction, PAGEs, and western blot analysis

Five-day-old rice seedlings without endosperm were heat-shocked at 41 °C for 2 h, then transferred to 28 °C for 3-h recovery. Seedlings were harvested and homogenized and the postribosomal supernatant was prepared as described by Jinn et al. [9]. The postribosomal supernatant was fractionated by 70–100% saturation with ammonium sulfate, and the resulting sHSP-enriched fraction was separated by 2D-PAGE [9]. The protein profile of the sHSP-enriched fraction was observed by silver nitrate staining or was transferred to PVDF membranes (Millipore) for western blot analysis. Equal amounts (40 μg) of total protein from the control (28 °C), heat-shocked, wounded, and SA-treated rice seedlings were separated by SDS-PAGE (12.5% gel) and transferred to PVDF membranes (Millipore) according to Jinn et al. [38]. Protein blots of SDS-PAGE and 2D-PAGE were subjected to western blot analysis with rabbit anti-Oshsp16.9 (a class I sHSP isolated from rice donated by Dr. Chu-Yung Lin) [10] or rabbit anti-PsHSP17.7 (a class II sHSP isolated from pea donated by Dr. Elizabeth Vierling) as the primary antisera,

followed by HRP-conjugated goat anti-rabbit (Jackson) as the secondary antisera according to the manufacturer's protocol. The antisera were detected by assaying alkaline phosphate activity conjugated to second antisera by color development according to Jinn et al. [38].

3. Results

3.1. Isolation of a rice class II sHSP gene, Oshsp18.0-CII

By comparing the alignment of deduced amino acid sequences of several class II sHSPs from diverse species reported by Bartling et al. [23], we found two highly conserved domains (ADVKEL and KKPKTI, located near the center and C terminal of the amino acid sequences, respectively) in four cDNA clones, namely, *cMHSP18-1*, *cMHSP18-3*, and *cMHSP18-9* of maize [19], and *Tahsp17.3* of wheat [39]. Two 18-nt degenerated oligomers, ADVKEL and KKPKTI, were synthesized and used as primers for RT-PCR to obtain a partial cDNA clone encoding a putative class II sHSP of rice. The RT-PCR product consisted of approximately 320 bp (Fig. 1A). Sequence analysis showed that the obtained RT-PCR product contained 321 bp, encoding 107 amino acid residues (Fig. 2). Northern blot analysis revealed that this RT-PCR product recognized a message of about 800 nt in the RNA samples prepared from heat-shocked rice seedlings (Fig. 1B).

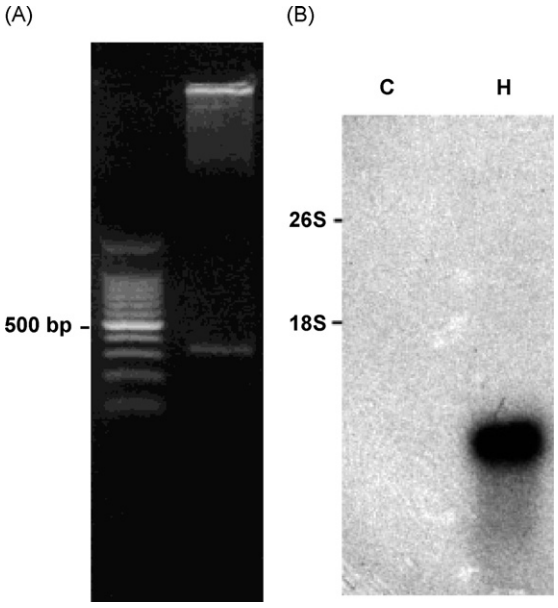

Fig. 1. Agarose gel analysis of RT-PCR products and the induction of the RNA transcript by heat shock using the 321 bp RT-PCR clone as a probe. (A) A total of 10 μl of the RT-PCR products (100 μl/reaction) was separated on a 1% agarose gel. The gel was stained with ethidium bromide and illuminated on a UV light box to show the DNA bands. The RT-PCR (right lane) produced a band between 300 and 400 bp. The 100 bp DNA ladders (Promega) are shown in the left lane. (B) Equal amounts (10 μg) of total RNA isolated from control (C; 28 °C 2 h) and heat shock (H; 41 °C 2 h) rice seedlings were analyzed on a formaldehyde/agarose gel. The northern hybridization was performed at 42 °C with ^{32}P-labeled RT-PCR clone used as a probe. The locations of 26S and 18S rRNAs are indicated on the left.

The 321 bp RT-PCR clone was used as a probe to screen the cDNA library made from mRNA of 3-day-old heat-shocked rice seedlings. One putative clone out of 10^6 recombinant plaques screened was isolated, and the insert of the resulted cDNA clone, designated *pOSHSP2*, consisted of 477 bp with the 3′ UTR of 220 bp before the poly(A)$^+$ tail, and included 257 bp of coding region. The *pOSHSP2* encodes about 85 amino acids, a sequence identical to that of nucleotides 82 to 321 in the RT-PCR clone (Fig. 2). However, this was not the full length of the cDNA clone. The 5′ region (including the 5′ UTR and coding sequence) of *pOSHSP2* was further extended by 5′ RACE. The obtained 5′ RACE clone contained 456 bp, the sequence corresponding to 63 bp of the 5′ UTR and 365 bp of the 5′ coding sequence shown in Fig. 2. The oligomers corresponding to the first 20 nt of the 5′ UTR and the last 21 nt of the 3′ UTR (see Table 1 for primer sequences) were used for RT-PCR to obtain a full-length cDNA clone of 784 bp, including 63 bp of 5′ UTR, 501 bp of coding region, and 220 bp of 3′ UTR.

The coding sequence of the clone consisted of 501 bp, suggesting the coding of a protein of 18.0 kDa with a predicted pI of 5.61. The obtained full-length cDNA clone was hence designated *Oshsp18.0-CII* (GenBank accession number DQ180746), to be distinguished from the isolated class I sHSP gene *Oshsp18.0*. The entire *Oshsp18.0-CII* clone contained 60% GC, but the first 300 nt in the coding region consisted of 73% GC. Computer analysis of the RNA secondary structure suggested a strong and complex stem loop structure in the first 300 nt of *Oshsp18.0-CII* coding sequence (Fig. 3). The predicted structure was low in free energy (−156) and consequently was very stable (Fig. 3).

3.2. Comparisons of the nucleotides and deduced amino acid sequences of Oshsp18.0-CII with other plant cytosolic sHSP genes

Comparisons of the nucleotides and deduced amino acid sequences of the *Oshsp18.0-CII* with other plant sHSP genes are summarized in Table 3. *Oshsp18.0-CII* shares 82.5–84.2% DNA identity, 80.2–83.1% amino acid identity, and 85.9–88.9% amino acid similarity with three maize clones (*cMHSP18-1*, *cMHSP18-3*, and *cMHSP18-9*) and the wheat clone *Tahsp17.3*. The DNA and deduced amino acid sequences of seven published class I sHSP genes from rice (*Oshsp16.9A*, *Oshsp16.9B*, *Oshsp16.9C*, *Oshsp17.3*, *Oshsp17.7*, *Oshsp17.9*, and *Oshsp18.0*) shared less identity with the sequences of the *Oshsp18.0-CII*, only 56.1–60.3% DNA identity and 36.3–41.7% amino acid identity. Overall, by comparing deduced amino acid sequences, the similarity of *Oshsp18.0-CII* to other published class II sHSP genes in plants was more than 71%, while that to the published class I sHSP genes in plants was less than 53% (Table 3). At the DNA level, about 75% of the 3′ coding sequence of *Oshsp18.0-CII* was homologous with that of the published class II sHSP genes in plants (data not shown), whereas at the protein level, the C terminal of the deduced amino acid sequences of the published class II sHSP genes in plants showed the highest homology as compared with the

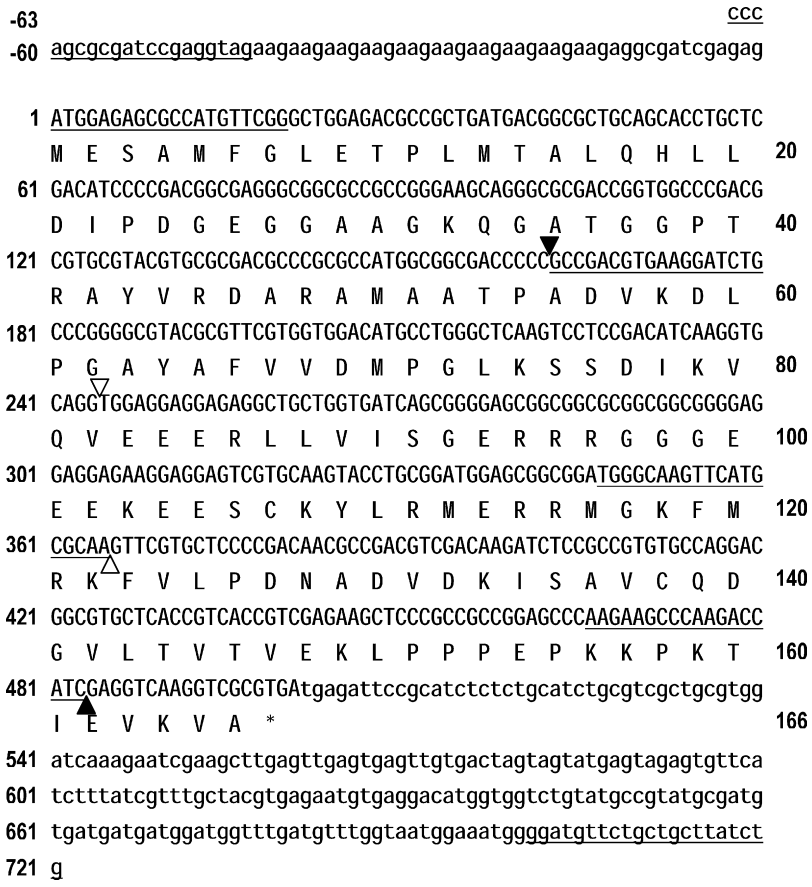

Fig. 2. The DNA and corresponding deduced amino acid sequences of the isolated *Oshsp18.0-CII* gene encoding a putative class II sHSP of rice. The number of nucleotides and amino acids are indicated on the left and right, respectively. The beginning of the *pOSHSP2* is indicated as ▽; the beginning and the end of the RT-PCR clone are indicated as ▼ and ▲, respectively. The end of the longest 5′ RACE clone is indicated as △. Star (*) indicates stop codon. The primers used in this study as indicated in Section 2 are underlined. The GenBank accession number for *Oshsp18.0-CII* is DQ180746.

N terminal (Fig. 4A). The C terminal of the published class I sHSP genes in rice and *Oshsp18.0-CII*, however, also showed some sequence similarity as indicated in Fig. 4A. When the deduced amino acid sequences of the published classes I and II sHSP genes in plants were compared with those of *Oshsp18.0-CII* (Fig. 4A), the C-terminal sequences all showed some level of similarity. According to the dendrogram drawn from the similarity of deduced amino acid sequences (Fig. 4B), *Oshsp18.0-CII* was grouped with the class II sHSP genes and separated from the other class I sHSP genes.

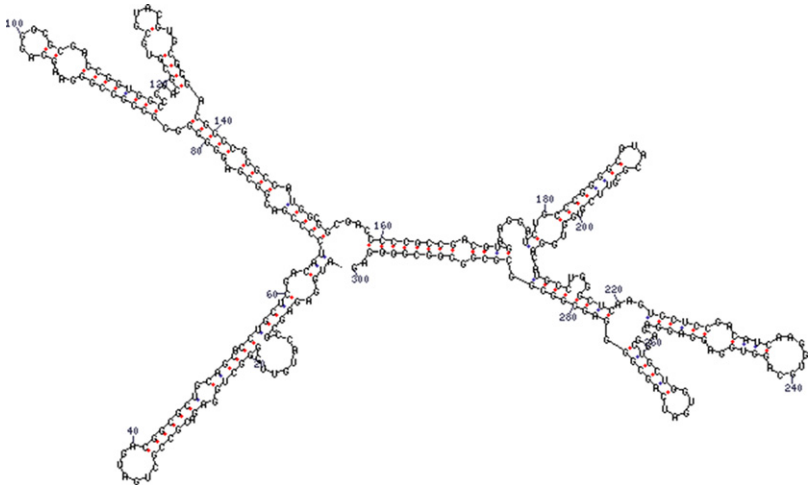

Fig. 3. The secondary structure of the first 300 nt of the coding sequence *of Oshsp18.0-CII* mRNA predicted by mfold server. Only one secondary structure predicted, with the lowest free energy, is shown. The free energy ($\triangle G$) is −150.52 (initially −155.4).

Table 3
Relationships of nucleotide sequences and deduced amino acid sequences between the coding region of *Oshsp18.0-CII* and other plant sHSP genes

Species	Gene (accession number)	Identity or similarity (%)		
		DNA	AA-I[a]	AA-S[b]
Class II				
Zea mays	*cMHSP18.1* (X54075)	84.2	82.1	88.2
Triticum aestivum	*Tahsp17.3* (X58279)	83.7	80.2	85.9
Zea mays	*cMHSP18.9* (S59777)	83.4	82.4	88.9
Zea mays	*cMHSP18.3* (X54076)	82.5	83.1	87.5
Pharbitis nil	*sHSP-2* (M99430)	67.2	61.7	72.2
Pharbitis nil	*sHSP-1* (M99429)	66.2	60.0	71.6
Helianehus annuus	*HaHSP17.9* (Z29554)	65.6	67.0	75.3
Glycine max	*GmHSP17.9-D* (X07159)	65.0	66.6	75.4
Arabidopsis thaliana	*AtHSP17.6-II* (X63443)	61.9	63.2	74.1
Pisum sativum	*PsHSP17.7* (M33901)	61.6	65.7	75.6
Class I				
Oryza sativa	*Oshsp16.9B* (M80939)	60.3	38.5	50.0
Oryza sativa	*Oshsp16.9A* (X60820)	59.8	38.5	50.0
Oryza sativa	*Oshsp16.9C* (U81385)	59.1	39.1	49.6
Oryza sativa	*Oshsp17.7* (U83671)	58.5	41.7	52.6
Oryza sativa	*Oshsp18.0* (U83670)	57.2	36.3	46.1
Triticum aestivum	*C5-8* (X13431)	57.0	36.0	48.3
Oryza sativa	*Oshsp17.3* (M80186)	56.3	39.7	52.1
Oryza sativa	*Oshsp17.9* (AY034057)	56.1	39.3	50.9
Arabidopsis thaliana	*AtHSP17.6* (X16076)	48.2	37.0	48.7
Glycine max	*GmHSP17.5-E* (M11395)	48.1	39.7	51.7
Pisum sativum	*PsHSP17.9* (M33900)	47.8	37.0	50.0

[a] Amino acid identity.
[b] Amino acid similarity.

3.3. Induction of Oshsp18.0-CII by heat shock, wounding and SA treatment

Oshsp18.0-CII was inducible by heat shock at 41 °C during 24 h. The transcript level was highest at 1 h after heat shock, remained high at 2 h, and then decreased rapidly with time. After prolonged heat stress of 16–24 h, however, *Oshsp18.0-CII* was still induced but to a much lower level (Fig. 5A). The Oshsp18.0-CII protein started to accumulate at 8 h and gradually increased in level during 24 h (Fig. 5B). *Oshsp18.0-CII* also was inducible by mechanical injury and SA, but to a much lower level as compared with heat shock (Figs. 5A and 6). However, mechanical injury and SA did not induce Oshsp18.0-CII protein accumulation (Fig. 5B).

3.4. Determination of copy numbers of class II sHSP gene in rice

According to Southern blot analysis, the 784 bp *Oshsp18.0-CII* recognized only one to two major bands of total rice DNA digested with different restriction enzymes (Fig. 7). This result indicated that one copy or low copy numbers of class II sHSP gene was present in the rice genome of cv. Tainong No. 67, a japonica cultivar. Western blot analysis of the sHSP-enriched fraction (Fig. 8A) with anti-PsHSP17.7 also showed only one protein spot of ~18 kDa with acidic pI (Fig. 8C and D), even though more than four protein spots of 15–18 kDa with acidic to basic pI were recognized by anti-Oshsp16.9 (Fig. 8B and D).

4. Discussion

An RT-PCR product of about 320 bp (Fig. 1A) was obtained from the rice heat-shocked mRNA using the primer pair designed from the conserved regions of four cDNA clones of two monocots. This RT-PCR clone hybridized with a message of about 800 nt in the RNA samples prepared from heat-shocked rice seedlings (Fig. 1B), but did not cross-hybridize with *Oshsp16.9A* (Chang, unpublished data), a rice gene encoding a class I sHSP [18]. These results suggested that the obtained fragment represented a partial cDNA clone of a heat-inducible transcript different from the class I sHSPs of rice. Sequence of the obtained full-length cDNA clone (Fig. 2), *Oshsp18.0-CII*, shared 100% identity with a putative class II sHSP gene (accession number AP002484) located on rice (cv. Nipponbare) chromosome one [40]. The *Oshsp18.0-CII* coding region also differed only in a 3-nt (AAG) insertion in nucleotides −16 to −14 of the 5′ UTR and a 1-nt difference (C versus A) in nucleotide 387 of that of another putative class II sHSP gene (accession number AK071240, cv. Nipponbare) [41], with no difference in deduced amino acid sequences.

When comparing the nucleotides and deduced amino acid sequences with those of other plant sHSP genes (Table 3), *Oshsp18.0-CII* shared more than 80% identity with four monocot clones, but less than 61% identity with the rice class I sHSP genes. From the dendrogram shown in Fig. 4B, it is clear that *Oshsp18.0-CII* is a class II sHSP gene. The 3′ coding sequence (Chang, unpublished data) and C terminal but not N

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Oshsp18.0-CII     MESA---------MFGLETPLMTALQHLLDIPDGEGGAAGKQ—GATGGP 39
cMHSP18.9         MDAR---------MFGLETPRVAALHHLLDVPDGDK---------AGGGA 32
cMHSP18.1         MDAV---------MFGLETPLMAALQHLLDVPDGDAGAGGDNKTGSGGSA 41
cMHSP18.3         MDGR---------MFGLETPLMVALQHLLDVPDGDAGAGGDK—AGGGGP 39
Tahsp17.3         MAGM---------VFGLDAPMMAALQHLLDIPDGEAEPPPEK-----QGP 36
AtHSP17.6-II      MDLG-----------RF-PIISILEDMLEVPEDHNNEKTRN----N—P 31
HaHSP17.9         MDID---------SLMGFDPLLRNLHYILEATDDNTTGNKSN----NSGP 37
PsHSP17.7         MDLD-------------SPLFNTLHHIMDLTDD-TTEKN------LNAP 29
GmHSP17.9-D       MDFR---------VMGLESPLFHTLQHMMDMSED-GAGDNKT----HNAP 36
sHSP-1            MDLR---------LMGFDHPLFH---HIMDYAGD-DKSSN------SSAP 31
sHSP-2            MDLRNFGLS----NFGLEPQLLSTIQDMLDFADDHDRAGRAP----PEQP 42
PsHSP17.9         --IIPRVFGTGRRTNAFDPFSLDLW-DPFQNFQLAR-SATG--------- 37
GmHSP17.5-E       MSLIPGFFG-GRRSNVFDPFSLDMW-DPFKDFHVPT-SS---------- 36
AtHSP17.6         MSLIPSIFG-GRRTNVFDPFSLDVF-DPFEGFLTPSGLANA--------- 39
Oshsp16.9A        MSLV-------RRSNVFDPFSLDLW-DPFDSVFRSVVPATS--------- 33
Oshsp16.9B        MSLV-------RRSNVFDPFSLDLW-DPFDSVFRSVVPATS--------- 33
Oshsp16.9C        MSLV-------RRSNVFDPFA-DFW-DPFDGVLRSLVPATS--------- 32
C5-8              MSIV-------RRSNVFDPFA-DLWADPFD-TFRSIVPAISG-------- 33
Oshsp17.9         MSLI-------RRSNVFDPFSLDLW-DPFDGFPFGSGGSSS—GSIFPSF 40
Oshsp18.0         MSLI-------RRSNVFDPFSLDLW-DPFDGFPFGSGSRSS—GTIFPSF 40
Oshsp17.3         MSMI-------RRSNVFDPFSLDLW-DPFDGFPFGSG---S--GSLFP-- 35
Oshsp17.7         MSLI-------RRGNAFDPFSLDLW-DPVDGFPFGSGGSSSSSGSLFP-- 40
                                                    ..

Oshsp18.0-CII     TRAYVRDARAMAATPADVKDLPGAYAFVVDMPGLKSSDIKVQVEEERLLV 89
cMHSP18.9         TRTYVRDARAMAATPADVKELAGAYAFVVDMPGLSTGDIRVQVEDERVLV 82
cMHSP18.1         TRTYVRDARAMAATPADVKELPGAYAFVVDMPGLGTGDIRVQVEDERVLV 91
cMHSP18.3         TRTYVADARAMAVTPADVKELPGAYAFVVDMPGLGTGDIKVQVEDERVLV 89
Tahsp17.3         TRAYVRDARAMAATPADVKELPGAYAFVVDMPGLGSGDIKVQVEDERVLV 86
AtHSP17.6-II      SRVYMRDAKAMAATPADVIEHPNAYAFVVDMPGIKGDEIKVQVENDNVLV 81
HaHSP17.9         SRAYVRDARAMAATPADVKECPNSYVFIVDMPGLKSGDIKVQVERDNVLV 87
PsHSP17.7         TRTYVRDAKAMAATPADVKEHPNSYVFMVDMPGVKSGDIKVQVEDENVLL 79
GmHSP17.9-D       TWSYVRDAKAMAATPADVKEYPNSYVFEIDMPGLKSGDIKVQVEDDNLLL 86
sHSP-1            SRTFMLDAKAMAATPADVKEYPNSYVFIIDMPGLKSGDIKVQVDGDNVLS 81
sHSP-2            IRAYVRDAKAMAATPADVKEYPNSYVFIADMPGVKAAEIKVQVEDDNVLV 92
PsHSP17.9         ---TTNETAAFANAHIDWKETPEAHVFKADLPGVKKEEVKVEIEEDRVLK 84
GmHSP17.5-E       ---VSAENSAFVSTRVDWKETPEAHVFKADIPGLKKEEVKVQIEDDRVLQ 83
AtHSP17.6         ---PAMDVAAFTNAKVDWRETPEAHVFKADLPGLRKEEVKVEVEDGNILQ 86
Oshsp16.9A        ----DNDTAAFANARIDWKETPESHVFKADLPGVKKEEVKVEVEEGNVLV 79
Oshsp16.9B        ----DNDTAAFANARIDWKETPESHVFKADLPGVKKEEVKVEVEEGNVLV 79
Oshsp16.9C        ----DRDTAAFANARVDWKETPESHVFKADLPGVKKEEVKVEVEEGNVLV 78
C5-8              ---GSSETAAFANARVDWKETPEAHVFKVDLPGVKKEEVKVEVEDGNVLV 80
Oshsp17.9         PRGASSETAAFAGARIDWKETPEAHVFKADVPGLKKEEVKVEVDDGNILQ 90
Oshsp18.0         PRGTSSETAAFAGARIDWKETPE-HVFKADVPGLKKEEVKVEVEDGNVLQ 90
Oshsp17.3         -R-ANSDAAAFAGARIDWKETPEAHVFKADVPGLKKEEVKVEVEDGNVSR 83
Oshsp17.7         -R-ANSDAAAFAGARIDWKETPEVHVFKADVPGLKKEEVKVEVDDGNILQ 88
                        :  *:. :  *  : .  :.*  *:**:   :::*:::  .:
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Fig. 4. Deduced amino acid sequence alignment of the *Oshsp18.0-CII* with published class I and class II sHSP genes in plants and the phylogenetic relationships. (A) The number of amino acids is indicated on the right. The class I sHSP genes compared are *GmHSP17.5-E*, *PsHSP17.9*, *Oshsp16.9A*, *Oshsp16.9B*, *Oshsp16.9C*, *C5-8*, *Oshsp17.3*, *Oshsp18.0*, *Oshsp17.7*, *Oshsp17.9*, and *AtHSP17.6*, and class II genes are *HaHSP17.9*, *sHSP-1*, *GmHSP17.9-D*, *PsHSP17.7*, *AtHSP17.6-II*, *sHSP-2*, *cMHSP18.1*, *cMHSP18.3*, *cMHSP18.9*, *Tahsp17.3*, and *Oshsp18.0-CII* as in Table 3. Protein sequences were aligned by use of Biology WorkBench 3.2 CLUSTAL W (1.81) multiple sequence alignments program. Consensus keys shown are: '*' for single, fully conserved residue; ':' for conservation of strong groups; '.'for conservation of weak groups; '-' for no consensus. (B) Phylogenetic relationships of the plant class II sHSP genes based on sequence alignments of the mature encoded proteins. Sequences were aligned by use of CLUSTAL X (1.81) multiple sequence alignments program. The analysis was based on alignment of 22 unique sHSP amino acid sequences, with the rice LEA protein sequence (U57639) used as an out-group for comparison. The evolutionary tree was constructed by the Neighbor-Joining method and drawn by the Tree View program. The bootstrap values are shown on each branch (% of 1000 re-sampled data set); only values greater than 60% are shown.

terminal of the deduced amino acid sequence (Fig. 4A) of the *Oshsp18.0-CII* showed high identity with those of the published plant class II HSP genes. The C terminal but not N terminal also showed some degree of homology with those of published rice and other plant class I sHSP genes (Fig. 4A). Sequence conservation of the C-terminal amino acid sequences appeared to exist in most of the sHSP genes in plants [6].

Oshsp18.0-CII was induced by heat shock, mechanical injury and SA (Figs. 5A and 6). Recently, *AtHSP17.6-II* and *AtHSP17.6A* were found to be induced by wounding [27]. The level of two class II sHSP transcripts (*HSP17.4* and *HSP17.6*) increased in tomato fruit during ripening, chilling storage, and MeJA and MeSA treatments [25]. SA has been reported to induce *Hsp17.6* but not *Hsp101* in Arabidopsis [28], and influence Hsp70/Hsc70 expression in tomato cell suspension cultures [29]. The induction level of our putative class II sHSP gene *Oshsp18.0-CII* by mechanical injury and SA treatment was much less than that by heat shock. However, only heat shock but not mechanical injury or SA treatment induced Oshsp18.0-CII protein accumulation (Fig. 5B). Our results

```
Oshsp18.0-CII     ISGERR-RGGGEEEKE-ESCKYLRMERRMGKFMRKFVLPDNADVDKISAV 137
cMHSP18.9         ISGERR-R----EERE-D-AKYLRMERRMGKFMRKFVLPDNADVDKVAAV 125
cMHSP18.1         VSGERR-R----EERE-DDAKYLRMERRMGKFMRKFVLPDNADVDKVAAV 135
cMHSP18.3         ISGERR-R----EERE-D-AKYLRMERRMGKFMRKFVLPDNADMDKISAV 132
Tahsp17.3         ISGERR-R----EEKE-D-AKYLRMERRMGKLMRKFVLPENADMEKISP- 128
AtHSP17.6-II      VSGERQ-R----ENKENEGVKYVRMERRMGKFMRKFQLPENADLDKISAV 126
HaHSP17.9         ISGKRN-R----E-EEKEGVKYVRMERRMGKFMKKFALPEDANTDKISAI 131
PsHSP17.7         ISGERK-R----E-EEKEGVKYLKMERRIGKLMRKFVLPENANIEAISAI 123
GmHSP17.9-D       ICGERK-R----D-EEKEGAKYLRMERRVGKLMRKFVLPENANTDAISAV 130
sHSP-1            ISGERK-R----EAEEKEGAKYVRMERRVGKLMRKFVLPENANKEKITAV 126
sHSP-2            VSGERTER----EKDEKDGVKYLRMERRVGKFMRKFVLPENANVEAINAV 138
PsHSP17.9         ISGERK------TEKEDKNDTWHRVERSQGSFLRRFRLPENAKVDQVKAA 128
GmHSP17.5-E       ISGERN------VEKEDKNDTWHRVERSSGKFTRRFRLPENAKVNEVKAS 127
AtHSP17.6         ISGERS------NENEEKNDKWHRVERSSGKFTRRFRLPENAKMEEIKAS 130
Oshsp16.9A        ISGQRS------KEKEDKNDKWHRVERSSGQFMRRFRLPENAKVDQVKAG 123
Oshsp16.9B        ISGQRS------KEKEDKNDKWHRVERSSGQFMRRFRLPENAKVDQVKAG 123
Oshsp16.9C        ISGQRS------KEKEDKNDKWHRVERSSGQFMRRFRLPENAKVDQVKAS 122
C5-8              VSGERS------REKEDKNDKWHRVERSSGKFVRRFRLPEDAKVEEVKAG 124
Oshsp17.9         ISGERN------KEQEEKTDQWHRVERSSGKFLRRFRLPDNAKPEQIKAS 134
Oshsp18.0         ISGERS------KEQEEKTDKWHRVERSSGKFLRRFRLPENTKPEQIKAS 134
Oshsp17.3         SAGERI------KEQEEKTDKWHRVERSSGKFLRRFRLPENTKPEQIKAS 127
Oshsp17.7         ISGERS------REQEEKSDKWHRVERSSGKFLRRFRLPENTKPEQIKAS 132
                   .*:*          *.   : ::**  *.: ::* **:::. : : .

Oshsp18.0-CII     CQDGVLTVTVEKLPPPEPKKPKTIEVKVA- 166
cMHSP18.9         CRDGVLTVTVEKLPPPEPKKPKTIEIKVA- 154
cMHSP18.1         CRDGVLTVTVEKLPPPEPKKPKTIEVKVA- 164
cMHSP18.3         CRDGVLTVTVEKLPPPEPKKPKTIEVKVA- 161
Tahsp17.3         CRDGVLTVTVDKLPPPEPKKPKTIQVQVA- 157
AtHSP17.6-II      CHDGVLKVTVQKLPPPEPKKPKTIQVQVA- 155
HaHSP17.9         CQDGVLTVTVEKLPPPEPKKPKTIQVQVA- 160
PsHSP17.7         SQDGVLTVTVNKLPPPEPKKPKTIQVKVA- 152
GmHSP17.9-D       CQDGVLSVTVQKLPPPEPKKPRTIQVKVA- 159
sHSP-1            CQDGVLTVTVENVPPPEPKKPRTIEVKIG- 155
sHSP-2            YQDGVLQVTVEKLPPPEPKKPKTVEVKVA- 167
PsHSP17.9         MENGVLTVTVPKE---EVKKPEAKPIQITG 155
GmHSP17.5-E       MENGVLTVTVPKE---EVKKPDVKAIEISG 154
AtHSP17.6         MENGVLSVTVPKV---PEKKPEVKSIDISG 157
Oshsp16.9A        LENGVLTVTVPKA---EVKKPEVKAIEISG 150
Oshsp16.9B        MENGVLTVTVPKA---EVKKPEVKAIEISG 150
Oshsp16.9C        MENGVLTVTVPKA---EVNKPEVKAIEISG 149
C5-8              LENGVLTVTVPKA---EVKKPEVKAIEISG 151
Oshsp17.9         MENGVLTVTVPKE---EAKKPDVKSIQISG 161
Oshsp18.0         MENGVLTVTVPKE---EPKKPDVKSIQVTG 161
Oshsp17.3         MENGVLTVTVPKE---EPKKPDVKSIQITG 154
Oshsp17.7         MENGVLTVTVPKE---EPKKPDVKSIQISG 159
(A)                .:*** *** :      :** .  :.:
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Fig. 4. (*Continued*)

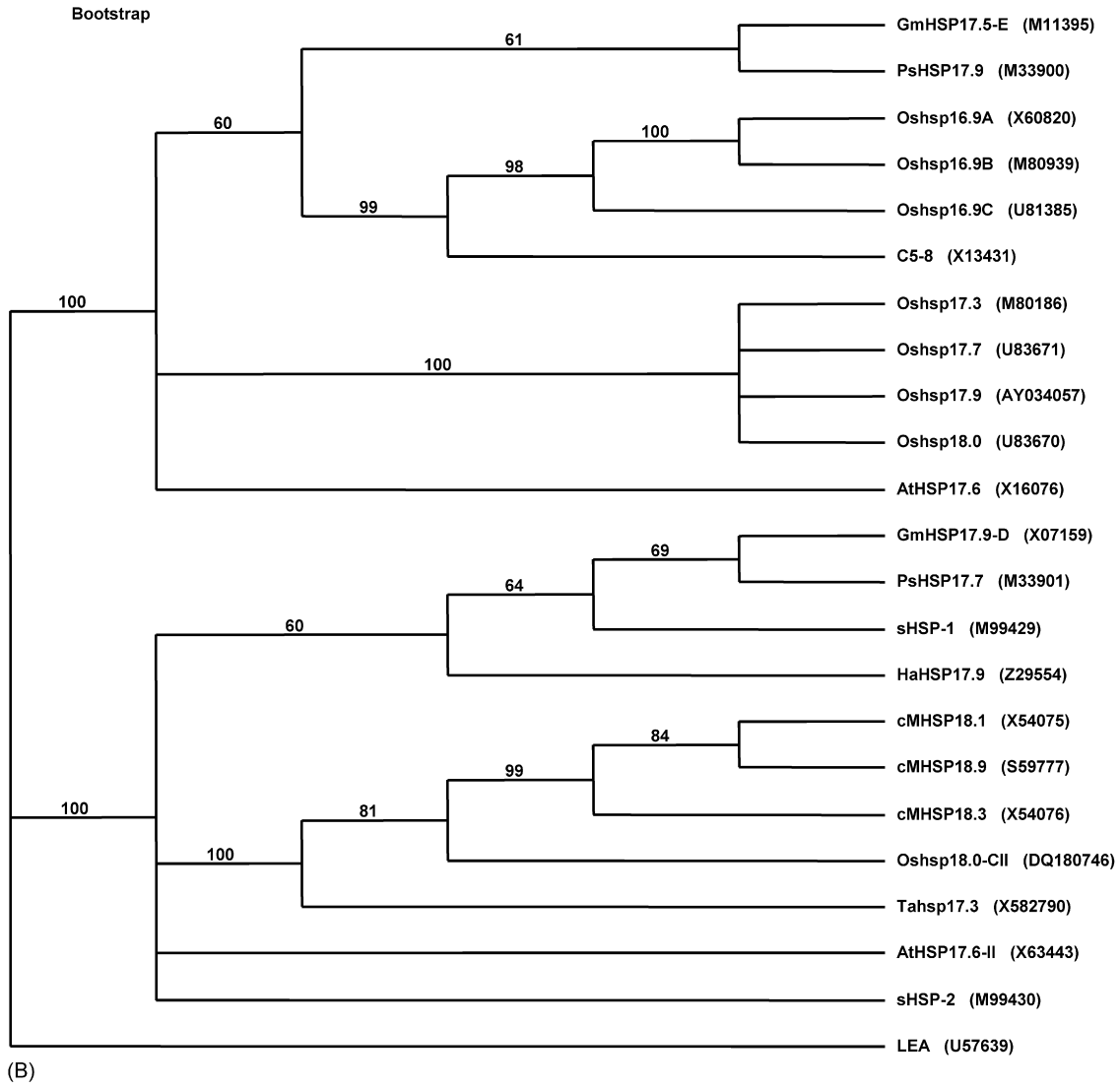

Fig. 4. (*Continued*).

suggest some posttranscriptional regulation of class II sHSP genes under stresses other than heat shock. It is conceivable that the protein level induced by SA or wounding was too low to be detected by western blot analysis.

Although induction of plant HSPs is generally considered to be an abiotic stress response [42], it has also been shown to be involved in the biotic stress response. The HSP70 gene was known to be induced by infection with a cyst nematode in

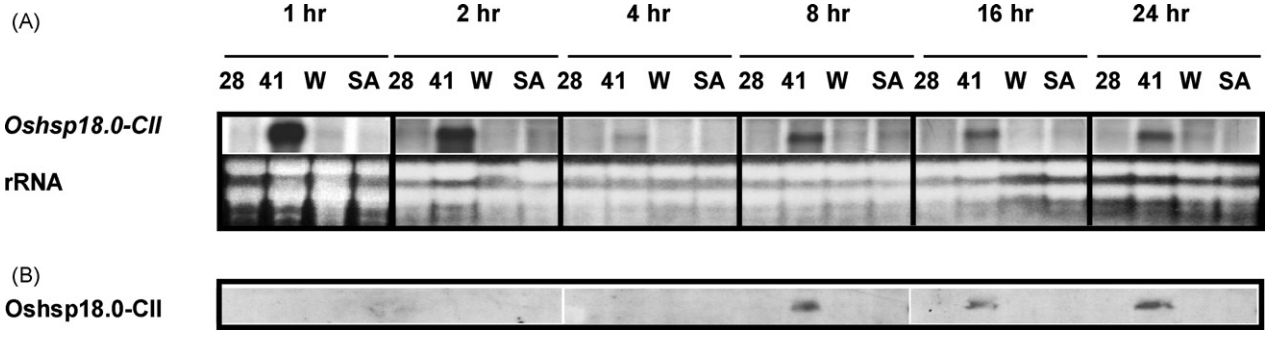

Fig. 5. Induction of the *Oshsp18.0-CII* mRNA and accumulation of the Oshsp1 8.0-CII protein in 3-day-old etiolated rice seedlings treated with 28 °C (C), 41 °C (H), wounding (W; cut seedlings), and salicylic acid (SA; 1 mM) for 1, 2, 4, 8, 16, and 24 h. (A) Equal amounts (10 μg) of total RNA were analyzed on a formaldehyde/agarose gel. Northern hybridization was performed at 42 °C with ^{32}P-labeled *Oshsp18.0-CII* used as a probe. rRNAs are shown for RNA loading reference. (B) Equal amounts (40 μg) of total protein were separated by SDS-PAGE (12.5% gel) and subjected to western analysis using rabbit anti-PsHSP17.7 (a class II sHSP isolated in pea) as the primary antisera.

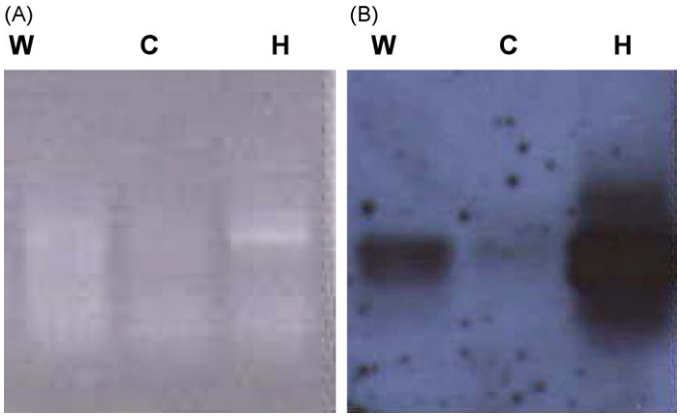

Fig. 6. RT-PCR of rice RNA isolated from control, heat-shocked, or wounded rice seedlings with PCR primers specific to class II sHSP genes. (A) A total of 10 μl of the RT-PCR products (25 μl/reaction) from RNA isolated from rice seedlings treated with 28 °C (C), 41 °C (H), and wounding (W) for 1 h were separated on a 1% agarose gel. The gel was stained with ethidium bromide and illuminated on a UV light box to show the DNA bands. RT-PCR produced a band between 300 and 400 bp. (B) The corresponding Southern hybridization was performed at 42 °C with ^{32}P-labeled RT-PCR clone used as a probe.

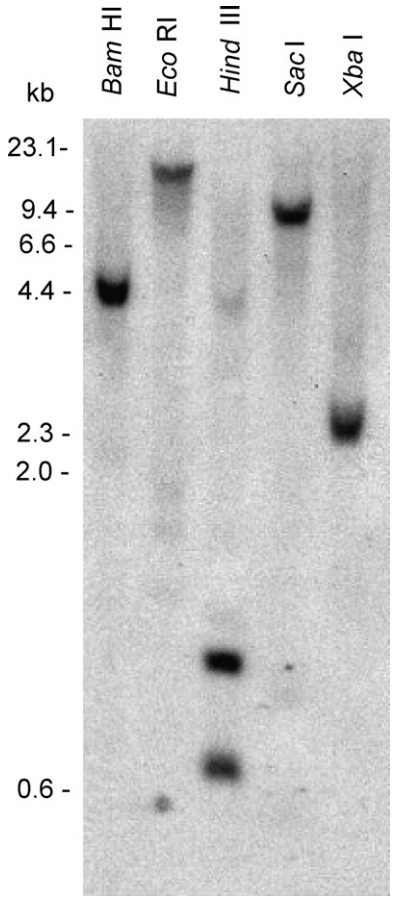

Fig. 7. Southern blot analysis of rice genomic DNA probed with *Oshsp18.0-CII*. Equal amounts (15 μg) of rice genomic DNA were digested with *Bam* HI, *Eco* RI, *Hind* III, *Sac* I, and *Xba* I restriction enzymes at 37 °C for overnight and analyzed on a 0.7% agarose gel. Southern hybridization was performed at 42 °C with ^{32}P-labeled *Oshsp18.0-CII* used as a probe. The locations of DNA size markers are indicated on the left.

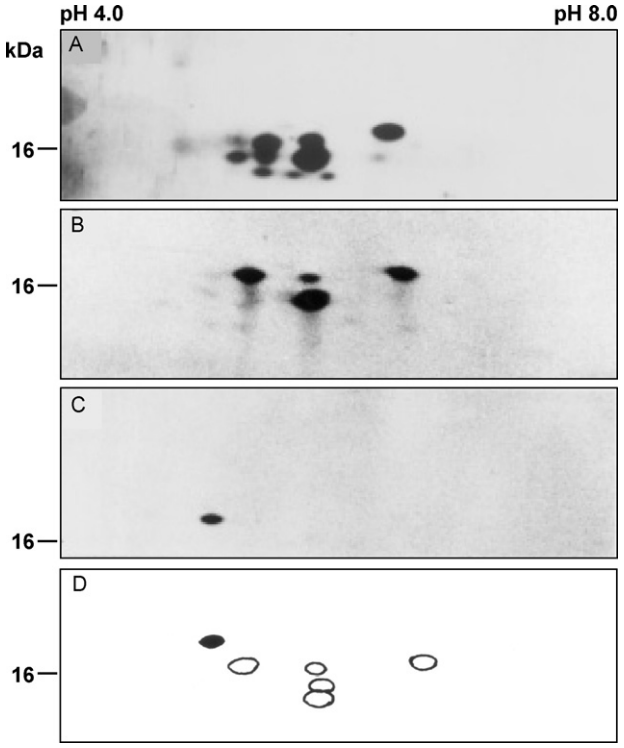

Fig. 8. Identification of class II sHSPs in rice. The sHSP-enriched fraction by 70–100% ammonia sulfate fractionation was separated by 2D-PAGE. Panel A, the total protein profile in this fraction shown by silver nitrate staining. Panel B, antisera against class I sHSPs in rice were used for western blotting; Panel C, antisera against class II sHSPs in pea were used for western blotting. The immunoreactions were detected by assaying alkaline phosphate activity conjugated to second antisera by color development. Panel D, diagrammatic representation of the total 15–18 kDa sHSPs where the proteins cross-reacted to antisera against class I (open circle) and class II (black circle) sHSPs detected in rice. Sizes of the molecular mass markers are indicated at the left.

soybean [43]. In Arabidopsis, AtHSP90.1 and cytosolic HSP90 have been reported to be required for disease resistance [44,45]. Moreover, HSP70 and HSP90 have also been shown to be essential for hypersensitive response and nonhost resistance in *Nicotiana benthamiana* [46]. Whether Oshsp18.0-CII is involved in crop protection against biotic stresses remains to be determined.

Computer analysis of RNA secondary structure revealed the existence of strong and complex stem loop structures in the first 300 nt (73% GC) of the coding region of *Oshsp18.0-CII* (Fig. 3) and suggested its possible folding into a stem-loop structure during RT or even PCR. This may be the reason for our failure to obtain a full-length clone by heat-shock cDNA library screening. When we later designed a specific ATG primer, which contained the predicted start codon of the putative class II sHSP gene of rice cultivar Nipponbare (AK071240) to pair with the KKPKPI or 3′ UTR antisense primer for PCR analysis of the heat-shocked cDNA, the resulted RT-PCR products had sizes ranging from 390 to 436 bp or 625 to 658 bp (Table 2). The 5′ end sequences of the RT-PCR products, generated by these two primer pairs, showed various lengths (47–96 nt) of deletion regions spanning from the DNA sequence corresponding to nucleotides 65–190 of the coding region in the *Oshsp18.0-CII* sequence (Chang, unpublished data). The remaining sequences in the entire 501 bp

coding region and the 3′ UTR were the same. The isolated 18 kDa HSP genes in maize also revealed small deletions scattered in similar regions of different cDNA clones (*cMHSP18-1*, *cMHSP18-3*, and *cMHSP18-9*) [19].

Frappier et al. [47] demonstrated that in vitro transcription of the mRNA from the cDNA clone *cMHSP18-9* is responsible for the synthesis of three members of the sHSP family in maize and suggested that *cMHSP18-3* and *cMHSP18-1* may be responsible for the synthesis of five additional members of this family. They further suggested that in maize a single gene might produce a number of different proteins [47]. Although we obtained several deletion clones some of which even encoded shorter peptides with the same open reading frame as Oshsp18.0-CII, our *Oshsp18.0-CII* has only one protein product (Fig. 8). We, therefore, speculate that the variation in deletions in our RT-PCR products for the putative class II sHSP gene was due to the strong secondary structure of the class II sHSP mRNA at that region, which is not easy to be dissociated at 42 °C by regular RT methods. When we later used SuperScript III RNase H⁻ Reverse Transcriptase and 65 °C for RT, no such deletion was found in the obtained clones (Chang, unpublished data). Moreover, when the genomic DNA was used as PCR templates, the resulting clones also contained no deletion. Our results showed that for GC-rich genes or RNA sequences containing a significant secondary structure, the cDNA clones obtained by RT-PCR may contain some deletions.

Prior to our report, there was only one putative rice class II sHSP gene (AK071240) [41] deposited in GenBank. Results from the Rice Genome Project also revealed one putative HSP gene (AP002484) in a PAC clone (P0489A01) located on chromosome one [40]. The coding sequences of these two genes and our *Oshsp18.0-CII* are almost identical. This is consistent with the evidence obtained in this study, which suggested that there is only one copy of class II sHSP gene in rice (Fig. 7).

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