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Characterization of the genomic structures and selective expression profiles of nine class I small heat shock protein genes clustered on two chromosomes in rice (*Oryza sativa* L.)

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Abstract

The cytosolic class I small heat shock proteins (sHSP-CI) represent the most abundant sHSP in plants. Here, we report the characterization and the expression profile of nine members of the sHSP-CI gene family in rice (*Oryza sativa* Tainung No.67), of which *Oshsp16.9A*, *Oshsp16.9B*, *Oshsp16.9C*, *Oshsp16.9D* and *Oshsp17.9B* are clustered on chromosome 1, and *Oshsp17.3*, *Oshsp17.7*, *Oshsp17.9A* and *Oshsp18.0* are clustered on chromosome 3. *Oshsp17.3* and *Oshsp18.0* are linked by a 356-bp putative bi-directional promoter. Individual gene products were identified from the protein subunits of a heat shock complex (HSC) and from *in vitro* transcription/ translation products by two-dimensional gel electrophoreses (2-DE). All sHSP-CI genes except *Oshsp17.9B* were induced strongly after a 2-h heat shock treatment. The genes on chromosome 3 were induced rapidly at 32 and 41 °C, whereas those on chromosome 1 were induced slowly by similar conditions. Seven of these genes, except *Oshsp16.9D* and *Oshsp17.9B*, were induced by arsenite (As), but only genes on chromosome 3 were strongly induced by azetidine-2-carboxylic acid (Aze, a proline analog) and cadmium (Cd). A similar expression profile of all sHSP-CI genes at a lower level was evoked by ethanol, H_2O_2 and $CuCl_2$ treatments. Transient expression assays of the promoter activity by linking to GUS reporter gene also supported the *in vivo* selective expression of the sHSP-CI genes by Aze treatment indicating the differential induction of rice sHSP-CI genes is most likely regulated at the transcriptional level. Only Oshsp16.9A abundantly accumulated in mature dry seed also suggested additionally prominent roles played by this HSP in development.

Abbreviations: ACD, α-crystallin domain; As, arsenite; Aze, L-azetidine-2-carboxlyic acid; Cd, cadmium; GUS, β-glucuronidase; HSC, heat shock complex; HSE, heat shock response element; ROS, reactive oxygen species; sHSP, small heat shock protein; sHSP-CI, class I small heat shock protein; 2-DE, two-dimensional gel electrophoresis

Introduction

The induction of HSPs and thermotolerance in plants are highly correlated in a time- and temperature-dependent manner. Based on this correlation, it has been hypothesized that accumulation of HSPs is essential for plants to prevent and recover from heat damages (Key *et al.*, 1981; Lin *et al.*, 1984). Plants synthesize HSPs of diverse spectrums in response to heat stress, but the roles

of individual HSP families in stress tolerance are different. sHSP and ClpB/HSP100 families are necessary for specific adaptation processes involved in acquired thermotolerance. Other HSP families such as HSP60s, HSP70s, and HSP90s also contribute to thermotolerance, but in general they appear to be important for growth at elevated temperatures (Hong and Vierling, 2000). Genetic and biochemical evidences have shown that sHSPs are essential for thermotolerance in *Neurospora crassa* (Plesofsky-Vig and Brambl, 1995), *Synechcystis* sp. strain PCC6803 (Nakamoto *et al*., 2000), and *Saccharomyces cerevisiae* (Haslbeck *et al*., 2004). Overexpression of a sHSP also resulted in increased thermotolerance of carrot suspension cells and tobacco plants (Malik *et al*., 1999; Sanmiya *et al*., 2004).

sHSPs are the most abundant and complex subset of HSPs in plants and their synthesis is induced by a rapid increase of temperature. They are encoded by members of a multi-gene family in eukaryotes and defined by possessing a conserved α-crystallin domain (ACD). More plant sHSPs have been found recently (Scharf *et al*., 2001). They are divided into at least six classes based on amino acid sequence homology, immunological cross-reactivity, and subcellular localization. Class I, II, and III sHSPs are present in both cytosol and nucleus. Members of the other three classes are localized in the plastids, endomembranes, and mitochondria. sHSP-CIs are the major sHSP in plants. In soybean, sHSP-CIs represent up to 1% of the total protein after heat-shock treatment (Hsieh *et al*., 1992). sHSPs typically exist as a large multimer HSC with a molecular weight ranging from 200 to 800 kDa. Numerous biochemical and structural studies have demonstrated that the chaperone activity of sHSPs functions as a reservoir for the intermediates of denatured proteins, thus preventing proteins from aggregation caused by heat damages (van Montfort *et al*., 2001).

The expression of the heat shock genes is mainly attributed to activation of the heat shock factors (Hsf) under heat stress. Hsfs as trimers recognize the highly conserved HSE, which has been defined as adjacent and inverse repeats of the motif 5′-nGAAn-3′, such as 5′-nGAAnnTTCnn-GAAn-3′ (Schöffl *et al*., 1998). In addition to heat stress, alcohol (Kuo *et al*., 2000), amino acid analogs (Lee *et al*., 1996), chilling (Sabehat *et al*., 1998) and heavy metals such as As and Cd (Lin *et al*., 1984; Edelman *et al*., 1988; Tseng *et al*., 1993) also induce expression of one subset of sHSP genes. Recent microarray studies in *Arabidopsis* revealed that a subset of sHSP genes was induced by various stresses such as salt, drought, chilling, oxidative stress, and wounding (Desikan *et al*., 2001; Cheong *et al*., 2002). Moreover, members of the sHSP gene families are also developmentally regulated in seeds, storage organs, and vegetative tissues in plants (Wehmeyer and Vierling, 2000; Lubaretz and Nieden, 2002; Jofré *et al*., 2003). The chaperone function of sHSP is usually emphasized under heat stress condition; however, the versatile expression patterns strongly suggest that sHSP may be important for other stresses and developmental conditions. Although it is known that the above described stresses elicit sHSP expression, the molecular mechanisms underlying the induction and the relationship between heat stress and other stresses remain unclear.

Rice plant is sensitive to heat stress at all stages of development (Maestri *et al*., 2002). Because of the distinct abundance and complexity of sHSP-CI in rice, much research has concentrated on the identification of sHSP-CI genes in our laboratory (Tseng *et al*., 1993; Tzeng *et al*., 1993; Lee *et al*., 1995; Chang *et al*., 2001; Guan *et al*., 2003). In this report, we identified and characterized nine members of the rice sHSP-CI gene family on chromosome 1 and 3 and examined during seed maturation and the effects of various stresses including HS, Aze, As, Cd, and ethanol on expression profiles of these genes in etiolated seedlings. Our results indicate that different mechanisms may be involved in the selective induction of sHSP-CIs by heat stress and chemical agents.

Materials and methods

Plant materials

Rice (*Oryza sativa* L. cv. Tainung No. 67) seedlings were germinated in rolls of moist paper towels at 28 °C in a dark growth chamber as described by Lin *et al*. (1984). Tainung No.67 belongs to the *japonica* subspecies and is widely grown in paddy fields in Taiwan. Three-day-old rice seedlings without endosperms were incubated in shaking buffer (1% (w/v)) sucrose and 5 mM

potassium phosphate buffer pH 6.0) in shaking baths at various temperature regimes. For seed development, rice plants were grown in a 28 °C growth chamber with a 16-h day length. For chemical stress treatments, seedlings were incubated at 28 °C in shaking buffer with added chemicals as indicated. Samples were harvested and flash-frozen in liquid nitrogen and stored at −80 °C for subsequent RNA or protein extraction.

RNA isolation and RT-PCR

Samples were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using TRIZOL reagent (Invitrogen, Rockville, Maryland, USA) according to the manufacturer's protocol. The quantity of total RNA was determined by optical density measurement using a Hitachi U-3200 UV spectrophotometer. Total RNA (1 μg) was treated with one unit of DNase I (Promega, Madison, WI, USA) for 30 min at room temperature prior to RT-PCR to remove residual DNA contamination. The RT-PCR analyses were conducted using Superscript one-step RT-PCR kit (Invitrogen, Rockville, Maryland, USA) according to the manufacturer's protocol. Table 1 shows gene-specific primers for the nine genes examined in this study. Sixteen nanograms of total RNA were reverse transcribed into cDNA using random primer, $d(N)_6$, and then amplified with gene specific primers (10 pmol for each primer) in the same tube. For each RT-PCR reaction, a pair of plant 18S internal standard primers (Ambion, Austin, TX, USA) was conducted as an internal PCR control. PCR reactions for all genes were subjected to 25 cycles of 95 °C (30 s), 54 °C (30 s), and 72 °C (30 s) with GeneAmp PCR System 2400 (Perkin–Elmer Applied Biosystems, Foster City, CA, USA). For all treatments, three replicates of RT-PCR were conducted with three batches of total RNA samples isolated independently. DNA from 10 μl of each PCR reaction was fractionated by electrophoresis through a 2.0% (w/v) agarose gel with 0.01% (w/v) ethidium bromide in 0.5 × Tris-borate EDTA buffer. The gels were digitally photographed with a FloGel-1 fluorescent gel digital imaging system (TOPBIO, Taipei, Taiwan). Scion Image for Windows (Scion, http://

Table 1. Oligonucleotide primers used in RT-PCR.

Gene	Primer	Sequence	Products (bp)
Oshsp16.9A	169A-Fw	5′-GCTCCTGAAGATGTGATCGG-3′	206
	169A-Rv	5′-CCTCAACGAGCAAGAACTAA-3′	
Oshsp16.9B	169B-Fw	5′-AATGGTGAAAACGGGAGTTT-3′	152
	169B-Rv	5′-GCTTCGTGCAAACGCGCTCA-3′	
Oshsp16.9C	169C-Fw	5′-GAAGGAGAGAAGCTATATAC-3′	187
	169C-Rv	5′-TAGCTCATTCATTCAGACTC-3′	
Oshsp16.9D	169D-Fw	5′-CATATCACCGTCCGTGTTCGGCTG-3′	261
	169D-Rv	5′-CATATCCGCGGTAGACAGGTACATG-3′	
Oshsp17.3	173-Fw	5′-AGCATTGGGCTAATCTAAAA-3′	189
	173-Rv	5′-AGAACATAATATAGTTCACT-3′	
Oshsp18.0	180-Fw	5′-TAAGAAACTTCGGGTGTGAC-3′	148
	180-Rv	5′-ATTGCTCACTTCCAACATAG-3′	
Oshsp17.7	177-Fw	5′-AGCCCCGTTTGTTTATTCTG-3′	211
	177-Rv	5′-CATTGGTACATTAATCAAGC-3′	
Oshsp17.9A	179A-Fw	5′-GCATCGCCGGCGTGCCGCGTGCGC-3′	158
	179A-Rv	5′-CTGACACGACGCGACACACGACTG-3′	
Oshsp17.9B	179B-Fw	5′-GATGCGATGAACACACACACACAC-3′	230
	179B-Rv	5′-CGTAAGGGAACTAAGATGGAACATG-3′	

Fw, forward primer; Rv, reverse primer.

www.scioncorp.com) software was used to quantify the intensity of the ethidium bromide stained DNA bands from the negative images of the gels.

Primer extension analysis

Using the AMV Reverse Transcriptase Primer Extension System (Promega, Madison, WI, USA), 10 pmol of the gene-specific oligonucleotide, which is complementary to 2–18 bases upstream from the start codon, was labeled at the 5′ terminus with γ-^{32}P-ATP (>5000 Ci/mmol, Amersham, Buckinghamshire, UK) and T4 polynucleotide kinase (Promega, Madison, WI, USA). The sequences for the gene-specific oligonucleotides are pTS3-PE (5′-GTCGGAATAGCTGCG AAT-3′) and pYL-PE (5′-GTGTATTGTGT CTTGCTG-3′) for *Oshsp17.3* and *Oshsp18.0*, respectively. Thirty micrograms of the total RNA was annealed with 1 pmol of the labeled primer for primer extension according to the manufacture's protocol. The reaction products were electrophoresed through 6% (w/v) polyacrylamide gel and visualized with autoradiography in parallel with the sequencing reaction products primed with the same oligonucleotide. DNA was sequenced using the Sequenase version 2.0 DNA sequencing kit (USB, Cleveland, Ohio, USA) following the manufacturer's protocol.

Coupled in vitro *transcription/translation and expression of recombinant proteins*

The TNT Coupled Reticulocyte System (Promega, Madison, WI, USA) was used for the characterization of the cloned sHSP gene products according to the manufacture's protocol. To use this system, the gene including the coding region and 3′-untranslated region (3′-UTR) was amplified by PCR and cloned into pGEM-7Zf (+) vector downstream of the SP6 RNA polymerase promoter. For each construct, 1 μg plasmid DNA was linearized by restriction enzyme digestion, purified and then added to the in vitro transcription/translation reaction mixture which was allowed to proceed for 1.5 h at 30 °C. For autoradiograph detection of the product, L-[^{35}S]-methionine (>1000 Ci/mmol, Amersham, Buckinghamshire, UK) was included in the reaction mixture. The translation products were separated by 2-DE gels as described by Lin *et al.* (1984). For *Oshsp17.9A*, *Oshsp17.9B*, and *Oshsp16.9D*, the coding region of the gene was cloned into the *Eco*RI and *Hin*dIII digested pUC8 vector and so that the recombinant proteins contained five amino acid residues (M-I-T-N-S) before the first methionine residue. The plasmid was transformed into *E. coli* DH5α strain and induced by addition of IPTG (final concentration, 1 mM) to produce the recombinant protein.

Fractionation of the HSCs, 2-DE and western blotting analysis

HSCs were purified and analyzed as described by Yeh *et al.* (1995). Protein samples were fractionated with ammonium sulfate (between 70% and 100% saturation). After dialysis in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) at 4 °C for overnight, the extracts were analyzed on a non-denaturing gradient gel (5–20 % acrylamide). A protein complex of ~310 kDa on the non-denaturing gradient gel was recovered and then further analyzed on a 2-DE gel as described by Yeh *et al.* (1995). For immunoblotting, proteins were transferred from acrylamide gels to Immobilon PVDF membranes (Millipore, Bedford, CA, USA). The anti-Oshsp16.9A antiserum was used as first antibody and antibodies raised in goat against rabbit IgG, conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA, USA), were used as second antibody following the user's manual. For immunological detection in Figures 5B and 7B (Aze treatment and dry embryo), the Western Lightning™ Chemiluminescence Reagent *Plus* (PerkinElmer Life Sciences, Boston, MA, USA) was used according to the manufacture's protocol. The KODAK BioMax Light Film (Kodak, Rochester, NY, USA) was used for chemiluminescent light detection.

Chromosome mapping

For chromosome mapping, the 3′-untranslated regions (3′-UTRs) of rice sHSP-CI genes were used as hybridization probes. Chromosomal mapping was conducted as described previously (Guan *et al.*, 2003).

Particle bombardment and transient expression assays

The coleoptile of a etiolated rice seedling was cut from embryonic root and positioned on the

middle of a 10-cm Petri dish containing MS salts supplemented with 0.6% (w/v) agarose and 3% (w/v) sucrose. The mixture (in a 1:1 molar ratio) of a test DNA construct and a maize ubiquitin-luciferase internal control construct were coated onto a 10-μl aliquot of 3 mg gold particles (Shen *et al.*, 1996). A helium biolistic particle-delivery system (model PDS-1000, Bio-Rad, Hercules, CA, USA) was used for particle bombardment. All bombardments were performed at 1350 psi under a vacuum of 26 mm Hg, with a distance of 6 cm between the targets and the barrel of the particle gun. Following the bombardments, the Petri dishes were incubated at 28 °C in the dark for at least 6 h and then subjected to experimental treatments indicated. After incubation under heat shock or 5 mM Aze, separately, for 2 and 4 h, the bombarded coleoptiles were homogenized in 600 μl grinding buffer (Shen *et al.*, 1996). After centrifugation at 12,000 $\times$ *g* at 4 °C for 15 min, 50 μl of the supernatant was assayed for luciferase activity by Bright-Glo^{TM} luciferase assay system (Promega, Madison, WI, USA) according to the technical manual. The luminescence was detected by an OPTOCOMP I luminometer (MGM Instruments, CT, USA). For the GUS activity assay, 50 μl of the supernatant was diluted into 200 μl of GUS assay buffer (Shen *et al.*, 1996) and incubated at 37 °C for 20 h. One hundred microliters of the reaction mixture was then diluted into 900 μl of 0.2 M Na_2CO_3 (pH 11.2). After aliquot every 300 μl into three separate wells of a 96-well plate, the resulting fluorescence was measured in a Fluoroskan Ascent FL fluorometer (Labsystems, Helsinki, Finland). Normalized GUS activity was calculated by dividing GUS activity by luciferase activity of each respective sample.

Preparations of DNA constructs

The 631-bp promoter region of *Oshsp16.9A* and the 567-bp promoter regions of *Oshsp17.3* and *Oshsp18.0* were separately cloned into the *Eco*RI and *Bam*HI digested pGN100 vectors, a pUC19 vector bearing the β-glucuronidase (GUS) gene fused with *nos* termination sequence. All the promoter regions contain the regulatory sequence and 5′ transcribed region. All of the constructs were verified by restriction enzyme digestions and sequencing.

Results

Rice sHSP-CI gene family contains nine members

In our previous studies, we identified seven sHSP-CI genes in rice using rice *Oshsp16.9A* as a screening probe (Tzeng *et al.*, 1993; Lee *et al.*, 1995; Chang *et al.*, 2001; Guan *et al.*, 2003). These genes were located on the rice chromosomes by RFLP using gene-specific 3′-UTRs. As shown in Figure 1A, *Oshsp16.9A*, *Oshsp16.9B*, and *Oshsp16.9C* were clustered on the short arm of chromosome 1; *Oshsp17.3*, *Oshsp17.7*, *Oshsp17.9A*, and *Oshsp18.0* were clustered on chromosome 3. Since the rice genome (cv. Nipponbare) has been completely sequenced, in order to find out if there were additional members of rice class sHSP-CI gene family, we used the conserved coding sequence encoding the ACD to search against GenBank database, and two putative genes located at chromosome 1 with high homology to sHSP-CI genes were found. Based on rice genome sequence, we cloned these genes using PCR from cultivars Tainung No.67 referring to them as *Oshsp16.9D*, and *Oshsp17.9B*. They were clustered closely with three genes of *Oshsp16.A*, *Oshsp16.9B*, and *Oshsp16.9C* previously found on chromosome 1. Amino acid alignment indicated that the sHSP-CI proteins encoded by the genes on chromosome 3 contain an insertion between the partially conserved N-terminal region and the highly conserved ACD domain (data not shown). Phylogenetic analysis with other monocot sHSP-CI genes from barley, corn, creeping bentgrass, pearl millet, and wheat, three clades were revealed in the rectangular cladogram (Figure 1B).

Genomic organization and promoter sequences of the rice sHSP-CIs gene family

Schematic diagrams of the genomic organization of the nine rice sHSP-CI genes are shown in Figure 1C. Interestingly, *Oshsp17.3* and *Oshsp18.0* on chromosome 3 are arranged closely in a head-to-head orientation. There are 567 bp between the two translation start sites of *Oshsp17.3* and *Oshsp18.0*. The nucleotide sequence of the intergenic region of *Oshsp17.3* and *Oshsp18.0* was shown in Figure 1D. To determine the transcription initiation sites of these two genes, we conducted primer extension analyses. The 5′ end of *Oshsp17.3* transcripts was

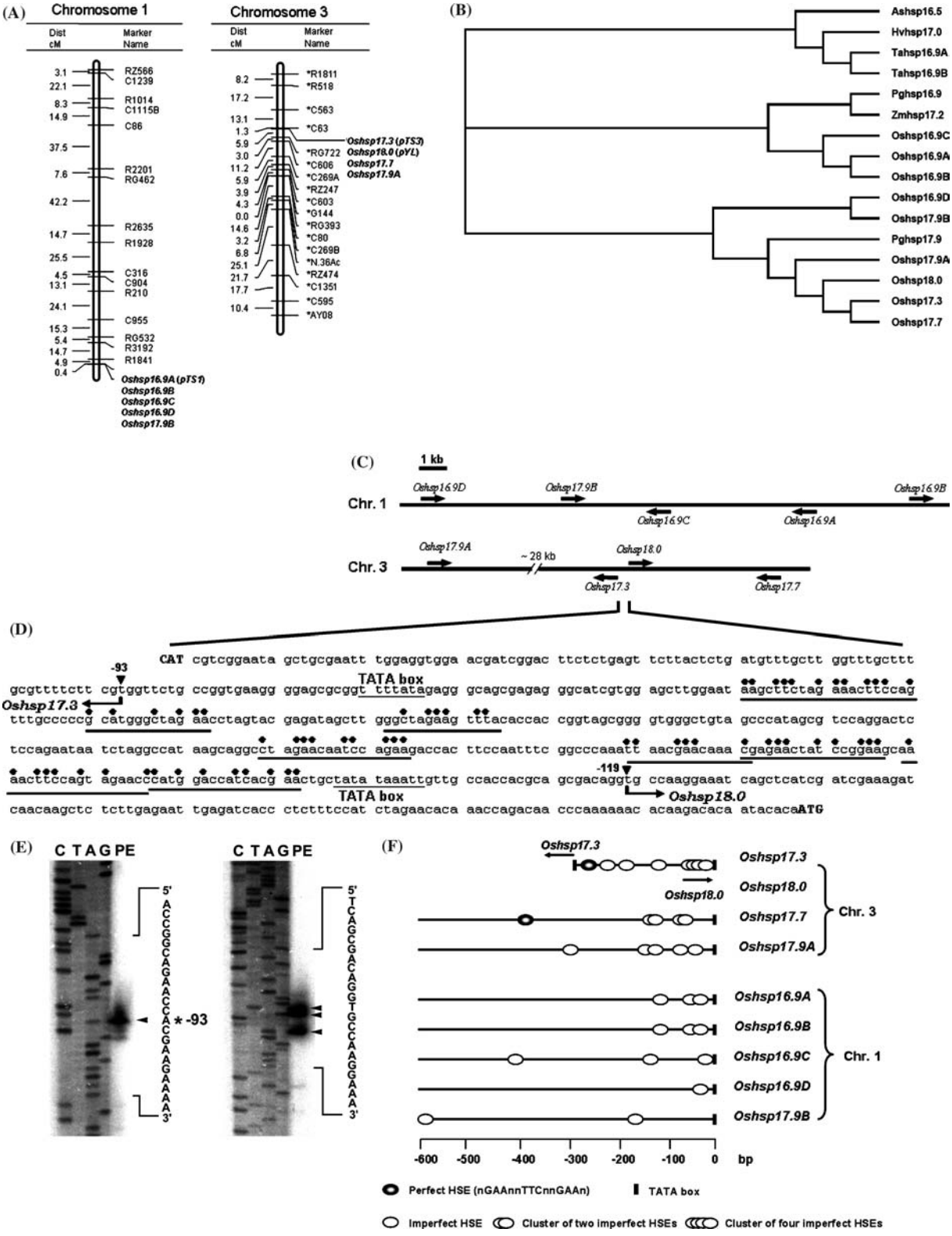
(A)
Chromosome 1
Dist cM
Marker Name
3.1 RZ566
22.1 C1239
8.3 R1014
14.9 C1115B
C86
37.5
7.6 R2201
RG462
42.2
R2635
14.7 R1928
25.5
4.5 C316
13.1 C904
R210
24.1
C955
15.3
5.4 RG532
14.7 R3192
4.9 R1841
0.4
Oshsp16.9A (pTS1)
Oshsp16.9B
Oshsp16.9C
Oshsp16.9D
Oshsp17.9B
Chromosome 3
Dist cM
Marker Name
8.2 *R1811
*R518
17.2
*C563
13.1
1.3 *C63
5.9 Oshsp17.3 (pTS3)
3.0 *RG722 Oshsp18.0 (pYL)
11.2 *C606 Oshsp17.7
5.9 *C269A Oshsp17.9A
3.9 *RZ247
4.3 *C603
0.0 *G144
14.6 *RG393
3.2 *C80
6.8 *C269B
25.1 *N.36Ac
21.7 *RZ474
17.7 *C1351
*C595
10.4 *AY08
(B)
Ashsp16.5
Hvhsp17.0
Tahsp16.9A
Tahsp16.9B
Pghsp16.9
Zmhsp17.2
Oshsp16.9C
Oshsp16.9A
Oshsp16.9B
Oshsp16.9D
Oshsp17.9B
Pghsp17.9
Oshsp17.9A
Oshsp18.0
Oshsp17.3
Oshsp17.7
(C)
1 kb
Chr. 1
Oshsp16.9D
Oshsp17.9B
Oshsp16.9B
Oshsp16.9C
Oshsp16.9A
Chr. 3
Oshsp17.9A
~ 28 kb
Oshsp18.0
Oshsp17.3
Oshsp17.7
(D)
CAT cgtcggaata gctgcgaatt tggaggtgga acgatcggac ttctctgagt tcttactctg atgtttgctt ggtttgcttt
-93
TATA box
gcgttttctt cgtggttctg ccggtgaagg ggagcgcggt tttatagagg gcagcgagag ggcatcgtgg agcttggaat aagcttctag aaacttccag
Oshsp17.3
tttgcccccg catgggctag aacctagtac gagatagctt gggctagaag tttacaccac cggtagcggg gtgggctgta gcccatagcg tccaggactc
tccagaataa tctaggccat aagcaggcct agaacaatcc agaagaccac ttccaatttc ggcccaaatt aacgaacaaa cgagaactat ccggaagcaa
-119
aacttccagt agaacccatg gaccatcacg aactgctata taaattgttg ccaccacgca gcgacaggtg ccaaggaaat cagctcatcg atcgaaagat
TATA box
Oshsp18.0
caacaagctc tcttgagaat tgagatcacc ctctttccat ctagaacaca aaccagacaa cccaaaaaac acaagacaca atacacaATG
(E)
C T A G P E
C T A G P E
5' ACCGGCAGAACCACGAAGAAAA 3'
* -93
5' TCAGCGACAGGTGCCAAGGAAA 3'
(F)
Oshsp17.3
Oshsp18.0
Oshsp17.3
Oshsp18.0
Oshsp17.7
Oshsp17.9A
Chr. 3
Oshsp16.9A
Oshsp16.9B
Oshsp16.9C
Oshsp16.9D
Oshsp17.9B
Chr. 1
-600 -500 -400 -300 -200 -100 0 bp
Perfect HSE (nGAAnnTTCnnGAAn)
TATA box
Imperfect HSE
Cluster of two imperfect HSEs
Cluster of four imperfect HSEs

Figure 1. Chromosome location and genomic organization of members of the sHSP-CI gene family in rice. (A) The locations of rice sHSP-CI genes on chromosomes 1 and 3. Map distances are given in centimorgan (cM) to the left of each chromosome. Seven genes were mapped by RFLP using gene-specific 3′-UTRs as probes. The other two genes, *Oshsp16.9D* and *Oshsp17.9B*, are assigned to chromosome 1 based on the released sequence results of Rice Genome Sequencing Project. Names of cDNA clones of their corresponding genes are indicated in parentheses. (B) Phylogenetic analysis of monocot sHSP-CI genes. Cladogram was generated by CLUSTALW program on the basis of amino acid sequences. (C) Schematic diagrams of arrangement of the rice sHSP-CI genes on chromosome 1 (Chr.1) and chromosome 3 (Chr. 3). The transcription direction of each gene is indicated by an arrow. The intergenic region between *Oshsp17.9A* and *Oshsp17.3* is approximate 28 kb. (D) Nucleotide sequence of the 567-bp intergenic region between *Oshsp17.3* and *Oshsp18.0*. The transcription start sites are indicated by arrowheads. There are 356 bp between the two transcription start sites. The arrow indicates the transcription direction of each gene. Clusters of HSE modules are indicated by bold lines and the consensus bases are indicated by black dots. The upper cases indicate the translation start codons. (E) Identification of transcription initiation sites for *Oshsp17.3* and *Oshsp18.0*. Lane PE shows the primer extension products of HS. Lanes C, T, A, G are from a DNA sequencing reaction in which the same primer was used. The transcription start sites are indicated by an arrowhead with a star (*). The number indicates the length of nucleotide upstream from the translation start site. (F) Predicted HSEs in the promoters of rice sHSP-CI genes. Approximate 0.6-kb 5′ flanking regions were analyzed. The numbers at the bottom indicate the length of nucleotides upstream to the putative TATA box. The arrows indicate the transcription orientations of *Oshsp17.3* and *Oshsp18.0*. The perfect HSE was defined by an interrupted pattern of three palindromic modules of five nucleotides (nGAAnnTTCnnGAAn or nTTCnnGAAnnTTCn). The imperfect HSE comprises three modules, of which at least one module with five nucleotides (nGAAn or nTTCn) and the others with invariant G or C, or both AA or TT residues (such as nGnAnnnnCnnGAAn or nTTnnnGAAnnTTnn).

mapped to an 'A' residue 93 nucleotides upstream of the translation start codon (Figure 1E, left panel). The *Oshsp18.0* primer, pYL-PE, yielded three distinct extension products as indicated in Figure 1E (right panel). The transcription initiation sites of these two genes were also indicated in Figure 1D. Therefore, *Oshsp17.3* and *Oshsp18.0* are linked head-to-head and share a single 356-bp putative bi-directional promoter. Approximately 600-bp promoter regions of all the rice sHSP-CI genes were searched for HSEs according to the previous reports (Schöffl *et al*., 1998; Nover *et al*., 2001). Several clusters of HSE modules, which mediate efficient Hsf binding, were located in the promoters of seven sHSP-CI genes except *Oshsp16.9D* and *Oshsp17.9B*, in which only one to two low-consensus HSEs were found (Figure 1F). In contrast, the highly efficient HSEs (5′-nGAAnnTTCnnGAAn-3′ or 5′-nTTCnnGAAn nTTCn-3′) were found in the promoters of *Oshsp17.3*, *Oshsp17.7*, and *Oshsp18.0*.

Seven major sHSP-CI are present in the rice HSCs

Rice sHSP-CIs form HSCs with a molecular weight of approximate 310-kDa (Yeh *et al*., 1995). Purified rice HSCs were resolved by 2-DE, and five prominent protein spots were recognized after western blot analysis with anti-Oshsp16.9A antibody. To identify the gene products consisting of the HSCs, we conducted a coupled *in vitro* transcription/translation assay for six of the nine sHSP-CI genes. The protein products were separated by 2-DE and the protein spot consistent with the predicted p*I* value and molecular mass for each gene product was identified (Figure 2A). A summary illustration of the components of the rice HSC separated in a 2-DE gel is shown in Figure 2B. Oshsp16.9D, Oshsp17.9A, and Oshsp17.9B were identified according to the 2-DE profiles of recombinant proteins expressed in *E. coli* (data not shown).

The heat stress responsiveness of the rice sHSP-CI genes

Although we knew sHSP-CI genes are expressed under heat stress, the kinetics and expression levels in rice were unclear. Thus, we monitored the expression patterns of the nine sHSP genes by RT-PCR. Primers were designed to yield PCR products with lengths between 150 and 240 bp (Table 1). The conditions for RT-PCR were optimized to produce unsaturated PCR amplification product accumulation that reflected a linear relationship with the original transcript levels in all samples (data not shown). First, we wanted to know whether all rice sHSP-CI genes responded similarly to the temperature fluctuations. Expression profiles under increasing temperature were determined in etiolated seedlings that were exposed to 32, 35, 38, or 41 °C for 2 h. Control plants were kept at 28 °C. The transcripts of the eight genes with the exception of *Oshsp17.9B* were easily detected at 38 °C (Figure 3). The transcript

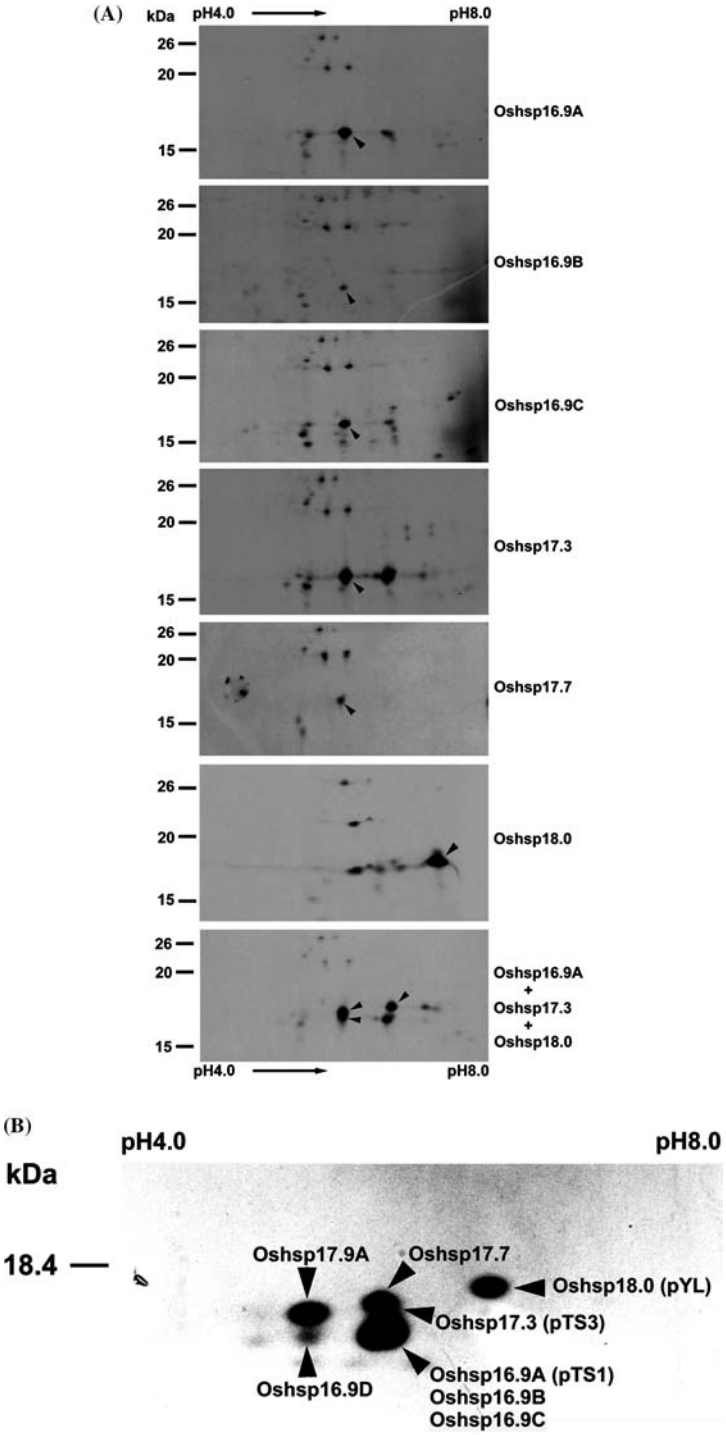

Figure 2. Identification of the sHSP-CIs consisting of heat shock complexes in rice. (A) 2-DE of ^{35}S-labeled *in vitro* transcription/translation products. Arrowhead indicates the product of each gene labeled on the right side. Three gene products, Oshsp16.9A, Oshsp17.3, and Oshsp18.0, which are different in molecular weight and p*I* were combined in an *in vitro* transcription/translation assay (bottom panel). (B) A summary illustration of the components of the purified HSCs separated in a 2-DE gel. The HSC proteins were identified by western blotting analysis. The protein spots corresponding to Oshsp16.9D, and Oshp17.9A were identified according to the western blots of the recombinant proteins expressed in *E. coli*.

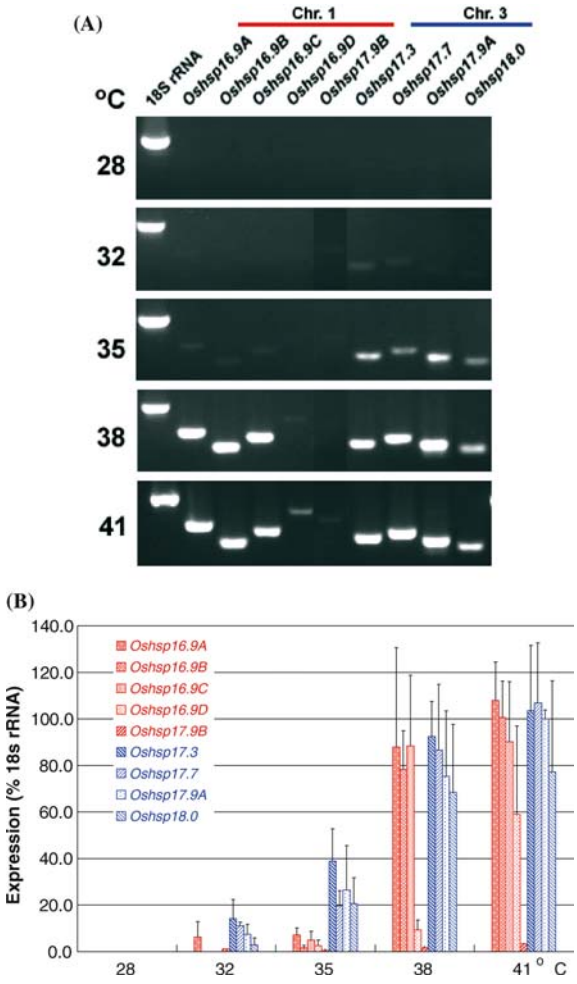

Figure 3. Expression patterns of rice sHSP-CI genes in response to temperature increase. Three-day-old rice seedlings were subjected to 28 °C (control), 32, 35, 38, and 41 °C for 2 h. (A) The RT-PCR products of sHSP-CI genes were shown by ethidium bromide staining, and the RT-PCR product of the 18S rRNA was used as an internal PCR control. (B) Quantification of the RT-PCR product for each gene. The resulting mean values are presented as percentage of the 18S rRNA signal value. Error bar represents ±SE from three separate experiments.

of *Oshsp17.9B* was detected at 41 °C, though at a very low level. Transcripts of the sHSP-CI genes located on chromosome 3 (*Oshsp17.3*, *Oshsp17.7*, *Oshsp17.9A*, and *Oshsp18.0*) were detected at a temperature as low as 32 °C and accumulated more at 35 °C when compared with those on chromosome 1. At 35 °C, the transcript levels of sHSP-CI genes on chromosome 3 are 3- to 4-fold higher than those of the sHSP-CI genes on chromosome 1 (Figure 3B).

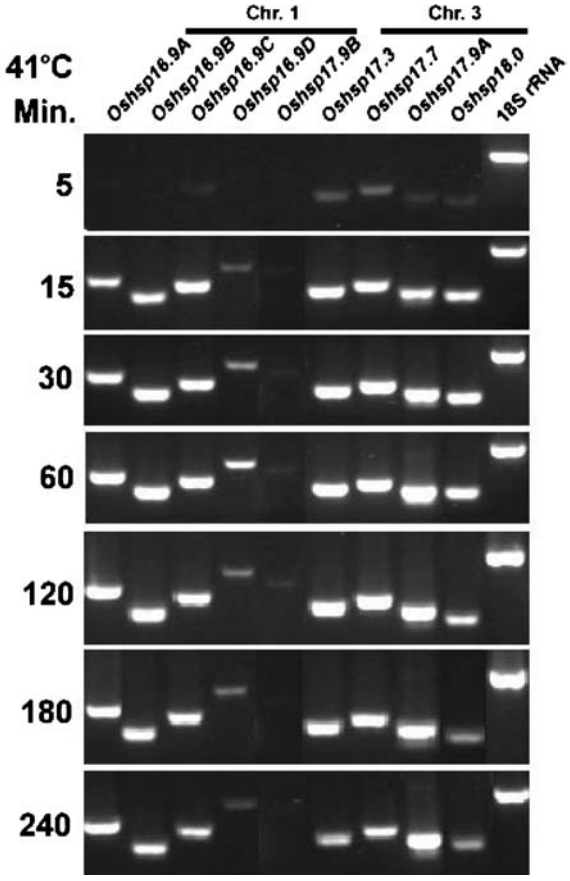

Figure 4. Time course response of the rice sHSP-CI genes under heat shock. Three-day-old rice seedlings were subject to 41 °C for 5, 15, 30, 60, 120, 180, and 240 min. The RT-PCR products were shown by ethidium bromide staining and the RT-PCR product of the 18S rRNA was used as an internal PCR control.

The levels of mRNA from sHSP-CI were analyzed by RT-PCR at different time points after initiation of the 41 °C treatment. The levels reached a maximum 30–60 min after the treatment and slowly declined afterwards (Figure 4). *Oshsp17.9A* had the highest level of expression among the members studied after 1 h of heat shock treatment. The transcript of *Oshsp16.9A* declined slower than those of other sHSP-CI genes. After prolonged HS treatment (4 h), *Oshsp16.9A* transcripts only decay for 10% of the maximal level; in contrast, transcripts of the other genes decay for approximately 30–40%. Similar to the expression profile at 32 °C for 2 h (Figure 3), the time-course experiments showed that sHSP-CI genes on chromosome 3 were induced promptly within 5 min of HS treatment (Figure 4). Overall, the expression

profiles revealed that there were differences in the transcription regulation and transcript stability among the rice sHSP-CI genes.

Rice sHSP-CI genes are induced by various chemical inducers in a selective manner

Chemicals such as As, Aze, Cd, and ethanol are cytotoxic agents that cause cellular damages and have been shown to induce a HS-like response in plants. They were known to induce the expression of sHSP-CIs (Lee *et al*., 1996; Banzet *et al*., 1998); however, the characteristics of the induction were not known. We determined the transcript levels of individual sHSP-CI genes to examine whether individual members of the family respond differently to these agents. First, the time-course responsiveness of individual sHSP-CI genes by As treatment was determined. With the exception of *Oshsp16.9D* and *Oshsp17.9B*, seven out of all sHSP-CI genes were induced (Figure 5A). When the seedlings were treated with Aze, Cd, or ethanol, sHSP-CI genes on chromosome 3 were selectively induced to various degrees, whereas those on chromosome 1 showed little or no response (Figure 5A). To examine whether the RT-PCR data are reflected by changes at the protein level, we examined the total proteins from seedlings after Aze treatment by 2-DE and western blotting analysis. As shown in Figure 5B, only proteins encoded by genes on chromosome 3 were detected.

ROS may have a role in the selective induction of rice sHSP-CI genes in response to chemical inducers

Banzet *et al*. demonstrated that the synthesis of sHSP was induced by H_2O_2 in tomato cells (Banzet *et al*., 1998). Lee *et al*. found that expression of the chloroplast-localized sHSP was induced by oxidative stress and H_2O_2 in rice (Lee *et al*., 2000). In

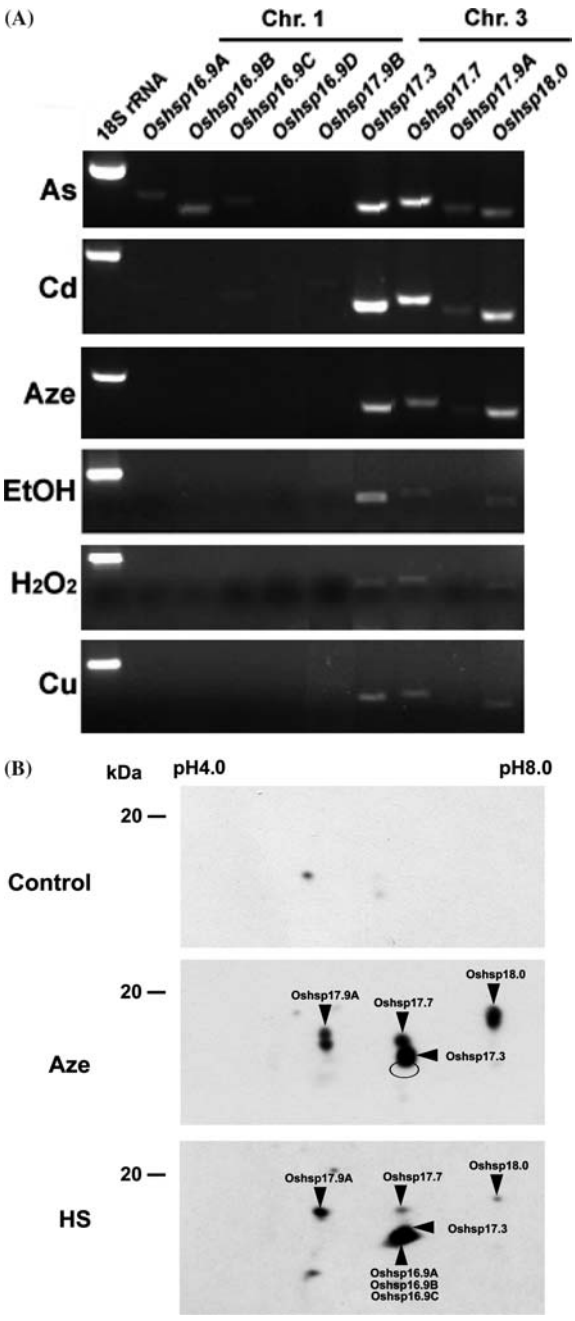

Figure 5. Selective expression patterns of the rice sHSP-CI genes in response to chemical inducers and protein profiles of sHSP-CIs induced by Aze or HS. (A) Three-day-old rice seedlings were subject to 250 μM $NaAsO_2$, 1 mM $CdCl_2$, 5 mM Aze, 0.03% (v/v) H_2O_2, 500 μM $CuCl_2$, for 2 h, or 5% (v/v) ethanol for 6 h. The RT-PCR products were shown by ethidium bromide staining, and the RT-PCR product of the 18S rRNA was used as an internal PCR control. (B) Identification of the sHSP-CIs by 2-DE and western blotting analysis of total proteins from seedlings. Three-day-old rice seedlings were incubated in shaking buffer at 28 °C for 18 h (control), 5 mM Aze for 18 h (Aze), or HS at 41 °C (HS) for 2 h. The circle indicates the protein spots corresponding to Oshsp16.9A, Oshsp16.9B, and Oshsp16.9C are absent in the Aze treatment. For each treatment, 100 μg of total proteins were loaded for analysis. The chemiluminescent reagents were used for immunological detection. Exposure time to chemiluminescent film was 60 s.

Arabidopsis, considerable evidence indicates an interlinking between responses to heat stress and oxidative stress (Panchuk *et al*., 2002). Thus, we determined the relationship between heat- and chemical-induced sHSP gene expressions. We treated the seedlings with 0.03% (v/v) H_2O_2 and examined the responses of the rice sHSP-CI genes. H_2O_2 specifically induced *Oshsp17.3*, *Oshsp17.7*, and *Oshsp18.0* (Figure 5A). The expression kinetics of these genes in response to H_2O_2 were similar to those of Cd, Aze, and ethanol treatments (data not shown). Copper is a redox-active metal that generates ROS. We treated the rice seedlings with $CuCl_2$ for further whether ROS was involved in the selective induction of sHSP genes. We obtained the same results as shown in Figure 5A, These findings suggest that ROS are involved in the induction of rice sHSP-CI genes as other various stresses.

Transient expression assays of the promoter activity supported the in vivo *selective expression of rice sHSP-CI genes by Aze treatment*

To test whether the selective induction of sHSP genes by Aze treatment observed *in vivo* was evoked by the differences related to promoter activity, we prepared three promoter::GUS constructs for transient expression assays by bombarded to rice coleoptiles. One construct contained a 631-bp promoter region of *Oshsp16.9A* on chromosome 1 and the other two constructs contained the 567-bp promoter region of *Oshsp17.3* and *Oshsp18.0* on chromosome 3 (Figure 6A). The *Oshsp16.9A* promoter was induced 6.2-fold by HS, but not by Aze treatment. In contrast, *Oshsp17.3* or *Oshsp18.0* promoter was induced over 14-fold by HS and at least 7-fold by Aze treatment (Figure 6B). The results of transient expression assays supported the *in vivo* selective expression of sHSP-CI genes by Aze treatment indicating that the promoter activity is involved in differential transcription.

Specific members of rice sHSP-CIs are induced during seed development

Members of the sHSP gene families are developmentally regulated in seeds (Almoguera *et al*., 1998; Wehmeyer and Vierling, 2000). We analyzed the transcript levels of sHSP-CI genes in grains that were turning brown (15 days after pollination [DAP]). As shown in Figure 7A, *Oshsp16.9A* showed the greatest induction of the family during seed maturation. Transcript levels of *Oshsp16.9B*,

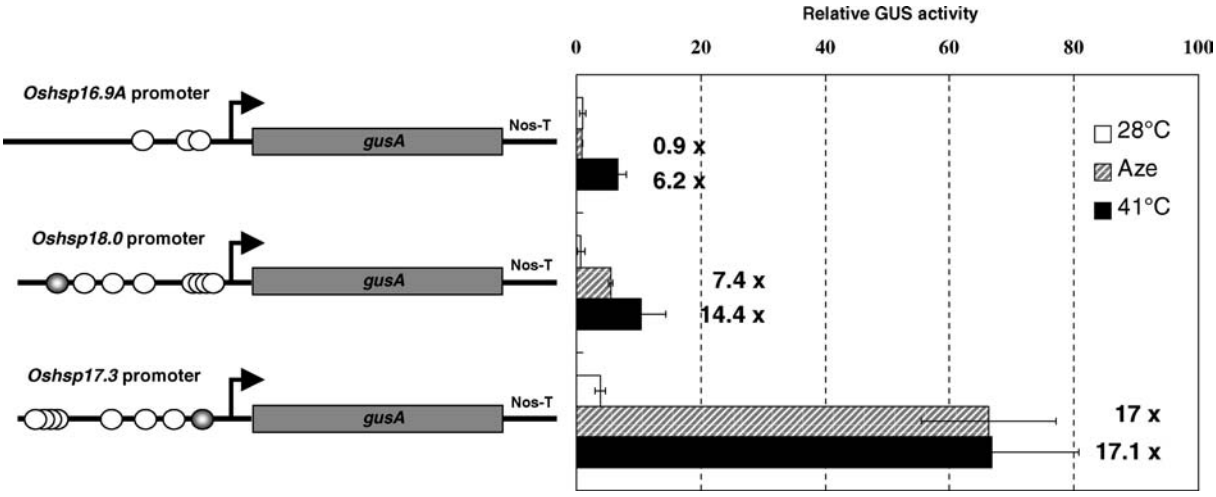

Figure 6. Transient expression assays of *Oshsp16.9A*, *Oshsp17.3* and *Oshsp18.0* promoter activity by HS and Aze treatments. Schematic diagrams of the promoter::GUS reporter constructs used in the bombardment experiments (left panel). The 631-bp promoter region of *Oshsp16.9A* and 567-bp promoter regions of *Oshsp17.3* and *Oshsp18.0* were used for assays. The arrangements of HSEs were as indicated in Figure 1F. The GUS coding region is represented by a shade box. Nos-T is the terminator of a nopaline synthase gene. The GUS activities of all samples were normalized against those of a luciferase internal control. Bombarded coleoptiles were incubated for at least 6 h at 28 °C, and then the samples were transferred to shaking buffer for 2-h HS treatment or 4-h Aze treatment; the samples were kept at 28 °C for overnight before GUS analysis. The magnitude of induction relative to control (28 °C) was indicated in the right side of bar. The data represent results of four independent transformations in an experiment. Each experiment was repeated at least two times and similar results were obtained. Bars indicate relative GUS activities ±SE from four independent transformations.

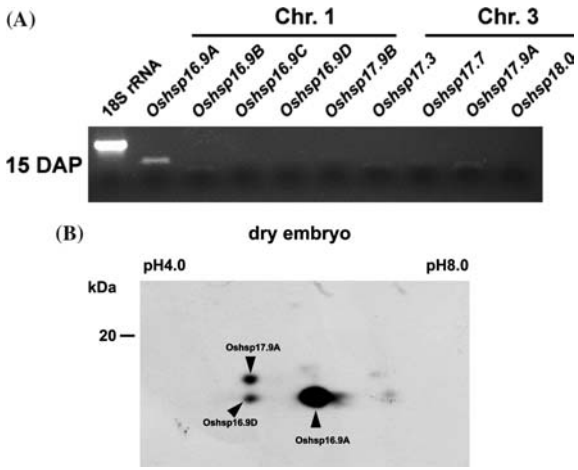

Figure 7. Expression of rice sHSP-CI gene family members during seed maturation. (A) Seed samples were harvested at 15 DAP for RT-PCR analysis. The grains began to turn brown. (B) Identification of the sHSP-CIs by 2-DE and western blotting analysis of total proteins from dry embryos separated from mature seeds. One hundred micrograms of total proteins were loaded for analysis. The chemiluminescent reagents were used for immunological detection. Exposure time to chemiluminescent film was 30 s.

Oshsp17.3, *Oshsp17.7*, and *Oshsp17.9A* were also detected during this period but to a lesser extent. Western blotting analyses of the dry embryo proteins indicated the prominent accumulation of Oshsp16.9A protein (Figure 7B).

Discussion

Rice sHSP-CI gene family

This study presents genomic organization information of a nine members of the sHSP-CI gene family in rice. Compared to *Arabidopsis*, which has six members of sHSP-CI family (Scharf *et al*., 2001); rice sHSP-CI family has nine members. Whether all members of the rice family exist in the HSC is not clear. In our previous study, mixture of HSCs from rice and soybean after dissociation with urea could reform HSCs with an intermediate molecular mass (Jinn *et al*., 1995). We proposed that hetero-oligomers could be formed in plants. Our current study indicates that at least seven members of sHSP-CI family are present in the HSCs in rice (Figure 2B).

In *Arabidopsis*, sHSP-CI and CI-related genes are dispersed among all five chromosomes (Scharf *et al*., 2001). In contrast, rice sHSP-CI genes are divided into two groups clustered on two loci on chromosome 1 and 3. Phylogenetic and expression analyses indicate that sHSP-CI genes clustered on a chromosome more resemble to each other than to those on other chromosome in rice. This suggests that genes on individual chromosomes may have been duplicated independently. The two groups of rice sHSP-CIs belong to two of the three clades of sHSPs found in monocots. The amino acid alignment implies the wheat sHSP-CIs are very similar to Oshsp16.9A. Oshsp16.9B, and Oshsp16.9C (data not shown). The reason for the absence of rice members in the third clades may be resulted from an inserted motif between the residues 33 and 37 in the wheat clade, which is different from the rice clades.

Heat shock response

In *Arabidopsis* and spinach, the HSP70 genes respond rapidly to heat shock (Sung *et al*., 2001). In our current study, some of the rice sHSP-CI genes have relatively faster response kinetics than the other gene family members (Figures 3 and 4). It has been reported that the regulation of HSP gene expression is at the transcriptional level and in an autoregulatory manner (Schöffl *et al*., 1998). Thus, after reaching a peak induction during prolonged exposure to heat shock, the levels of HSP gene transcripts were reduced (Figure 5). The expression of heat shock genes is mainly attributed to activation of Hsf under heat stress. Clusters of HSE modules, which mediate efficient binding of Hsf, require at least two modules (5′-nGAAnnTTCn-3′) (Schöffl *et al*., 1998; Nover *et al*., 2001). The numbers of HSEs in the promoters were correlated with the expression levels of rice sHSP-CI genes. At least three clusters of HSE modules were found in the promoter regions of sHSP-CI genes, which were strongly induced by HS treatment (Figure 1F). In contrast, low expression levels of *Oshsp16.9D* and *Oshsp17.9B* could be contributed to lacking efficient HSEs in these two genes (Figure 1F). *Oshsp17.3* and *Oshsp18.0* share a 356 bp bi-directional promoter with one perfect and seven imperfect HSEs predicted between the two TATA boxes (Figure 1D and F). We noticed that these elements were functional in both directions and resulted in transient expression of GUS activity in response to HS treatment (Figure 6).

Plants contain more Hsf genes than animals, which have only one to four genes encoding Hsf. The *Arabidopsis* genome contains 21 copies of Hsf, whereas more than 23 copies are found in the rice genome (Nover *et al*., 2001; Kotak *et al*., 2004). A distinct feature of the plant Hsf family is that the expression of several but not all of its members is heat-induced, and this feature suggests a multi-step mechanism of Hsf involvement in the HS response. Accumulated evidences indicate that the formation of heterocomplexes among Hsfs synergistically activates the HSP promoters (Rojas *et al*., 2002; He *et al*., 2003). Genetic evidences indicated that AtHsfA1a and AtHsfA1b are fast response regulators that may be important for coordination of stress gene expressions and generation of stress tolerance under rapidly changing environmental conditions in natural habitats (Lohmann *et al*., 2004). Thus, it is interesting to study whether rice Hsfs are involved in the differential expression of the sHSP-CI gene family members under heat shock response.

HS-like response induced by chemical inducers

Despite of the extensive studies of cross-tolerance conferred by HSPs in yeast and animals, little information is available for sHSPs in plants (Kuo *et al*., 2000). In the current study, most of the sHSP-CI genes were expressed in response to As treatment (Figure 5A). In contrast, Aze, Cd, and ethanol treatments induced only the genes clustered on chromosome 3 (Figure 5A). This suggests that the induction mechanisms may be different among As and Cd, Aze, or ethanol treatments. In addition, the expression kinetics following Aze treatment is different from that of Cd treatment (data not shown). This indicates that the inducing, sustaining, and repressing mechanisms are also different from that of Cd and As treatment. The regulatory mechanisms controlling transcriptional induction, translational preference, and repression of the HS-like response in soybean seedlings are affected differently between As and Cd treatments (Edelman *et al*., 1988). Although downstream events in HS signal transduction pathways have been investigated in plants, little is known about how the HS signal is perceived and transduced for the activation of HSP genes (Sung *et al*., 2003). ABI3, MAP kinases, Ca^{2+} and calmodulin have been proposed to be important components upstream in the expression of some sHSP genes in plants (Kovtun *et al*., 2000; Wehmeyer and Vierling, 2000; Agrawal *et al*., 2002; Liu *et al*., 2003; Li *et al*., 2004). So, it is possible that there are several signal transduction pathways responsible for sHSP induction in plant cells, and these pathways may be interlinked with other stress responses. Alternatively, plant Hsf and HSE could be involved in gene regulation under different stresses and perhaps play an interlinking role between heat and other stresses. The specific sequence of HSE in *Ha hsp17.6 G1* and preferential transcriptional activation by HaHsfA9 were shown to be crucial for Hsf promoter selectivity in developmental regulation and stress response (Almoguera *et al*., 2002; Rojas *et al*., 2002). HSEs were also shown to contribute partially to the induction of the genes by oxidative stress (Storozhenko *et al*., 1998). Nevertheless, clusters of canonical HSE modules were found in the promoters of *Oshsp16.9A* and *Oshsp17.3* or *Oshp18.0*; however, results of transient expression assays indicated that the canonical HSEs might not be sufficient for the selective induction by Aze or other stress agents (Figure 6). It is conceivable that plant Hsfs have diversified during evolution in parallel with the sequences of the HSEs. Promoter discrimination by different Hsfs has been proposed to explain the differential transcription of sHSP genes during plant development (Rojas *et al*., 2002). Thus, discrimination may also be crucial for Aze treatment, and might require additional transcription factors.

Role of H_2O_2 in the selective induction of rice sHSP-CI genes

Abiotic stresses such as drought, salinity, extreme temperatures, chemical toxicity, and oxidative stress are often interconnected and may result in similar cellular damages. As a consequence, these diverse environmental stresses often activate similar cell signaling pathways (Sung *et al*., 2003; Wang *et al*., 2003). Recent evidence has demonstrated that H_2O_2 serves as a cellular signaling molecule in plants and acts through the MAPK cascades to turn on stress-related genes including sHSP genes (Kovtun *et al*., 2000; Rentel *et al*., 2004; Shou *et al*., 2004). It was not known whether

all members of the sHSP gene family were induced by ROS-generated stress in plants. The current study shows that heat stress evokes the accumulation of ROS in plants; however, H_2O_2 treatment selectively induced the expression of rice sHSP-CI genes in plants.

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