Overexpression of *Oshsp16.9* Gene Encoding Small Heat Shock Protein Enhances Tolerance to Abiotic Stresses in Rice

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ABSTRACT Plants have adapted the ability to respond to various abiotic stresses such as high salinity, osmotic stress, high and low temperatures, and drought in order to survive. Small heat shock proteins (sHsps) play important and extensive roles in plant defenses against abiotic stresses. Herein, we cloned an sHsp gene from the rice, which we named *Oshsp16.9* based on the molecular weight of the protein. Real-time PCR analysis showed that expression of the *Oshsp16.9* gene was rapidly and strongly induced by stresses including high-salinity (250 mM NaCl), osmotic stress (300 mM mannitol), 100 μ M ABA, cold (4°C) and heat (45°C). Subcellular localization assay indicated that *Oshsp16.9* was localized specifically in the cytoplasm. In addition, overexpression of *Oshsp16.9* in rice conferred tolerance of transgenic plants to salt and drought stress. Taken together, these results suggest that the *Oshsp16.9* gene is an important determinant of stress response in plants.

Keywords Abiotic stress, Heat shock protein, Heat stress, Salt stress, Drought stress, ABA treatment

INTRODUCTION

Plants have adapted the ability to respond to various abiotic stresses such as high salinity, osmotic stress, high and low temperatures, and drought in order to survive. In response to high temperatures and other abiotic stresses, all organisms universally respond by accumulating heat shock proteins (HSPs) (Vierling, 1991). These HSPs are mainly involved in peptide-folding, protein assembly and transport, protection against irreversible protein denaturation, maintaining proteins in the normal folding state, and promoting the degradation of mis-folded proteins under various stresses, which can be summed as acting through “chaperone-like” activities (Krishna *et al.* 1995; Keeler *et al.* 2000; Sato and Yokoya 2008). Heat-shock proteins are classified on the basis of their approximate molecular weight into the following groups: Heat-shock proteins 100 kDa, i.e. Hsp100 (Clp), Hsp90, Hsp70 (DnaK), Hsp60 (GroEL), and small HSP (sHsps), where the molecular weight ranges

from 15 to 42 kDa (Trent 1996). These sHsps usually make a complex of small subunits wherein the molecular weight ranges from 200 to 800 kDa (Kim *et al.* 1998). The sHsps, the most abundant stress-induced proteins, have highly conserved C-terminals, where an α -crystallin domain (ACD) is found (Vierling 1991; Parsell and Lindquist 1993). Plants contain a wide array of sHsps that are divided into six classes based on their sequence alignments and immunological cross-reactivity. Three of these classes (CI, CII, and CIII) are located in the cytoplasm or nucleus (Scharf *et al.* 2001), while the others exist in the endoplasmic reticulum (Helm *et al.* 1993), mitochondria and plastids (Osteryoung and Vierling 1994). Plants vary greatly in the amount as well as type of Hsps expressed (Hamilton *et al.* 1996). *Arabidopsis thaliana* is the most highly studied species of plant, wherein the response to heat-shock treatment occurs through the participation of a number of different Hsps (Swindell *et al.* 2007). Higher plants are characterized by the presence of at least 20 types

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of sHsps, but one species could contain as many as 40 (Vierling 1991). These sHsps are usually undetectable in plant cells under physiological conditions and are induced upon stress and plant tolerance to stress, including drought, salinity, oxidized species, and low temperatures (Löw *et al.* 2000; Hamilton and Heckathorn 2001; Scharf *et al.* 2001; Zhang *et al.* 2008). It is believed that this diversification and abundance of the sHsps in plants is a reflection of the adaptation of the plant to heat stress (Waters *et al.* 1996). Earlier studies demonstrated that OsHsps can act as a protein chaperone, protecting pollen mother cells and tapetal cells against extreme heat (Murakami *et al.* 2004; Zou *et al.* 2009). To examine further its functioning and molecular mechanism under abiotic stresses, we transformed *Oshsp16.9* into rice as our model system. Our goal was to investigate how the overexpression of *Oshsp16.9* influences plant tolerance to stress, and to improve our overall understanding of the roles played by OsHsps during the stress response.

MATERIALS AND METHODS

Isolation of full-length *Oshsp16.9* cDNA

The *Oshsp16.9* fragment was identified in the dbEST division of GenBank by the BLAST program using the α -crystallin domain of the molecular chaperone as the probe sequence. The full-length cDNA was isolated by RACE technology with the SMART RACE-PCR kit (Clontech, Palo Alto, CA, USA).

Subcellular localization analysis

The full open reading frame (ORF) of *Oshsp16.9* was amplified using the cDNA clone mentioned above as a template. The PCR product was ligated into the pGWB5-GFP vector, resulting in an in-frame fusion protein of the GFP (green fluorescent protein) gene and the *Oshsp16.9* ORF. The construct (p35S:GFP-*Oshsp16.9*) was transformed into onion epidermal cells by particle bombardment using a Biolistic PDS-1000/He gene gun system (BIO-RAD). After incubation of the transformed onion epidermal cells for 24h, the GFP signal was detected by confocal fluorescence

microscopy.

Gene expression analysis under abiotic stress

Seeds of the rice (*Oryza sativa*) var. Dongjin were grown under controlled conditions, with 28°C day / 25°C night temperatures, 14 h light / 10 h darkness in Yoshida solution (Yoshida *et al.* 1976) at 75% relative humidity. After 20 days of germination, seedlings were exposed to high-salinity (250 mM), osmotic (15% polyethylene glycol (PEG) 6000, 15% PEG6000 + 250 mM NaCl), dehydration (300mM mannitol), and 100 μ M ABA stresses. The plants were harvested after 0 h, 0.25 h, 0.5 h, 1 h, 5 h, 10 h, and 24 h of exposure to stress, frozen in liquid nitrogen, and stored at -80°C until further analysis (Rabbani *et al.* 2003). Total RNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer's protocol. Real-time PCR was conducted using a Bio-RAD I Cyclor IQ5 machine. The threshold cycle (Ct) values of triplicate PCR reactions were averaged, and relative quantification of the expression levels was performed using the comparative Ct method (Livak *et al.* 2001). The fold change in total RNA (target gene) relative to the reference gene (actin) was determined using the following formula: fold change = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t \text{ target gene}} - C_{t \text{ actin gene}})_{\text{transgenic plants}} - (C_{t \text{ target gene}} - C_{t \text{ actin gene}})_{\text{wild-type plants}}$. The following primers were designed through the website <http://primer3.ut.ee/> and employed for analysis: *OshspF1* 5'-GCGTCAAGAAGGAGGAGGT-3' and *OshspR1* 5'-GTGCCACTTGTCGTTCTTGT-3'; *ActinF* 5'-GCAGT-CCAAGAGAGGTATCC-3' and *ActinR* 5'-GTAGAA-GGTGTGATGCCAGA-3'. RT-PCR analysis of various tissues (root, stem, leaf, spike and seed) was conducted using 50ng of total RNA after treatment with *DNase I*. The amplification program consisted of an initial step at 42°C for 60 min, followed by 94°C for 5 min, 30 cycles of amplification (94°C for 1 min, 58°C for 1 min, 72°C for 1 min), and a final step at 72°C for 10 min. The amplified fragments were run on 1.2% agarose gels.

Transformation and molecular analysis

The *Oshsp16.9* gene was introduced into the pB2GW7.0 gateway vector for *Agrobacterium*-mediated plant trans-

formation. The construct including the *Oshsp16.9* gene was transformed into rice as described by Toki (2006). Transgenic rice plants were selected in MS medium containing 3 mg/L phosphinotricine (PPT) and analyzed (T_2 generation). For extraction of genomic DNA, rice seedlings were harvested, immediately frozen in liquid N_2 , and stored at $-80^{\circ}C$. Genomic DNA was extracted from leaves using Nucleon PhytoPure (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's protocol. For 35S::*Oshsp16.9* transgenic analysis, the PCR primer sets 35SF (5'-CGCACAAATCCCCTATCCTT-3'), *OshspR1* (5'-GTGCCACTTGTCGTCTTGT-3'), and barF (5'-GTCATCAGATTTTCGGTGACG-3'), barR (5'-TTATTGCGCGTTCAAAGTC-3') were designed and synthesized. The amplification program consisted of an initial denaturation step at $94^{\circ}C$ for 2 min, 30 cycles ($94^{\circ}C$ for 15 s, $53^{\circ}C$ 30 s, $72^{\circ}C$ for 1 min), and a final extension step at $72^{\circ}C$ 10 min. Total RNA from 14-day-old seedlings was isolated for RT-PCR analysis using the RBC plant RNA extraction kit. For southern blot, DNA aliquots (40 μ g) were digested overnight at $37^{\circ}C$ with *EcoRI*, a site that was not predicted in the *Oshsp16.9* cDNA. The transfer and hybridization were performed similar to that of the Southern blot analysis, according to the procedure of Yang *et al.* (2005).

Analysis of transgenic plants for abiotic stress tolerance

Two homozygous T_2 generation transgenic rice plants lines were used in all subsequent abiotic stress assays, with application of 20-30 samples in 2 independent biological experimental replicates, unless otherwise noted. WT and transgenic line seeds were germinated and grown on 1/2 x MS medium in a culture room under a 16:8 h light:dark photoperiod at $24^{\circ}C$. For the drought and salt stress treatments, each of the transgenic seedlings were grown as stated above for 14d. The plants for salt treatment were then transferred to 1/2 x MS media containing 250 mM NaCl, while another treatment plot was exposed to drought conditions. After 1 day of exposure to the stress conditions, plants were photographed and the survival rates were measured after recovery for 20 days. Statistical analyses were performed using the Student's *t*-test in Microsoft Excel. Significance was determined for *P*-values below 0.05.

RESULTS

Cloning and sequence analysis of *Oshsp16.9*

A partial cDNA of *Oshsp16.9* was identified by a database search of the dbEST division of GenBank using the heat shock protein 16.9 motif as the probe sequence. The full-length cDNA of *Oshsp16.9* was isolated using RACE-PCR, which revealed the gene to be 754 bp in length, consisting of a 117 bp 5'-untranslated region (UTR), a complete ORF of 453 bp encoding a polypeptide of 150 amino acids, and a 3'-UTR of 184 bp (Fig. 1). BLAST protein sequence analysis showed that *Oshsp16.9* exhibited high identity with GU120339 (*Oryza sativa* Japonica group) (98%) over the entire protein length, and a similarly high sequence identity, though just over the α -crystallin domain (ACD), with HQ156231 (*Oryza sativa* Indica group) (98%), X94192 (*Cenchrus americanus*) (98%), EU952538 (*Zea mays*) (96%) and X13431 (*Triticum aestivum*) (94%) (Fig. 1A). That the ACD shared more than 94% identity suggested origin from a single ancestral gene that most likely underwent recent events of tandem duplication. The conserved α -crystallin domain of sHSPs was used as a query to search the genome of *O. sativa*, revealing the existence of 23 genes encoding sHSPs. The phylogenetic relationship of the deduced amino acid sequence of *Oshsp16.9* to sHSP proteins from other organisms was determined using ClustalW analysis from the BioEdit program (Fig. 1B). In the phylogenetic tree, the 23 sHSPs were divided into three main groups, which showed close relationships with the hsp 70 sequences of other plant species.

Subcellular localization, copy number and expression of *Oshsp16.9* gene

The subcellular localization of *Oshsp16.9* was also analyzed by particle bombardment for transient expression analysis of the *Oshsp16.9*-GFP fusion gene in onion epidermal cells. As shown in Fig. 2A, cells transformed with *Oshsp16.9*-GFP displayed fluorescence specifically in the cytoplasm, suggesting that *Oshsp16.9* is a cytoplasmically-localized protein. Southern blot analysis was also carried out using probes specific to the C-terminal region. The probes revealed a single band, suggesting that each

Fig. 1. A, Sequence alignment of molecular chaperones, including the α -crystallin domains (ACDs) from different proteins. The α -crystallin domains (ACD) were marked by underline. B, Sequence alignment of the known members of the HSP family. Accession numbers are as follows: *OsHSP16.9*, AK105464, AK072830 and AK061896 (*Oryza sativa* Japonica group); HQ156231 (*Oryza sativa* Indica group); EU952538 (*Zea mays*); GU120339 (*Oryza sativa* Japonica group); X13431 (*Triticum aestivum*); X94192 (*Cenchrus americanus*); BAB02269 and NP200076 (*Arabidopsis thaliana*).

Fig. 2. A, Cytoplasmic localization of the *Oshsp16.9* gene. p35S:GFP-*Oshsp16.9* was transiently expressed in onion epidermal cells. The photograph was taken in the dark field for green fluorescence (a), and under bright light for the morphology of the cell (b). B, Southern blot analysis. The specific region of *Oshsp16.9* was used as a probe. Restriction enzymes used were as follows; M: molecular marker, B: *Bam*HI, E: *Eco*RI, H: *Hind*III, P: *Pst* I. C, Relative expression of *Oshsp16.9* in organs collected from rice. Data presented are means and standard deviations of 3 independent replicated experiments.

member of the *Oshsp16.9* genes existed as a single copy in the rice genome (Fig. 2B). Expression analysis of the *Oshsp16.9* gene transcript was carried out on various tissues (root, stem, leaf, spike and seed). Semi-quantitative RT-PCR analysis showed broad expression of the *Oshsp16.9* transcript across tissues, with the highest expression in the seed and spike, among reproductive tissues, and in the leaves, among the vegetative tissues (Fig. 2C).

Expression of *Oshsp16.9* is induced by various abiotic stresses

Changes of expression in the root and leaf tissues were examined separately to gain an understanding of the role or contribution each made to the stress response. The results

from real-time PCR analysis showed that expression of *Oshsp16.9* was induced under various abiotic stresses in seedlings. In the presence of salt stress (250 mM NaCl), the transcript levels were rapidly induced by several fold after 0.5 hour in the root, reaching a peak after 2 hours, then returning to pre-treatment levels (Fig. 3). The *Oshsp16.9* transcript also showed rapid induction in leaf tissue, peaking at 14 fold and 22 fold after 0.25 hour of exposure to heat (45°C) and cold (4°C) shock, followed by a gradual decrease in expression to the pre-treatment levels during 24 hours of exposure. *Oshsp16.9* was also examined after treatment with ABA to determine if this stress hormone might be involved in regulating the transcript levels. Exogenous ABA treatment (100 µM) resulted in strong

Fig. 3. Expression profiles of *Oshsp16.9* in shoots and roots of rice after abiotic stress treatments. Expression levels were examined via real-time PCR analysis at various times after treatment with salt (250 mM NaCl), heat (45°C), cold (4°C), 100 µM abscisic acid (ABA), 300mM mannitol and osmotic stress (15% PEG6000) in tissue from 20-day-old plants. Actin was used as a positive control for relative expression for each treatment. Data presented are means $\pm$ SD of three independent replicated experiments.

Oshsp16.9 induction within 0.5 hours, which gradually decreased to the pre-treatment levels over 24 hours. Finally, the roles of dehydration and osmotic stress in the induction of *Oshsp16.9* transcript were also examined. In the presence of dehydration stress (300 mM mannitol), transcript levels were rapidly induced by 5–12 fold after 0.25–0.5 hours in the root, reaching peak induction after 2 hours, then returning to pre-treatment levels. In the case of osmotic stress (15% PEG), increase in the transcript levels in the root by 8 fold after 0.5 hours was observed, which gradually increasing during treatment until 5 hours, then continued to increase, reaching a peak of induction after 24 hours (Fig. 3).

Drought and salt stress tolerance increases in *Oshsp16.9* transgenic plants

To study the physiological functions of *Oshsp16.9*, several *Oshsp16.9*-overexpressing transgenic rice plant lines were generating by fusing the full-length cDNA sequence to the CaMV 35S promoter in the pB2GW7.0 gateway vector. Five positive transgenic plant lines, noted as TG #3, #5, #6, #8 and #9, were confirmed by genomic PCR, Southern blot, and RT-PCR to contain a single and highly expressed copy of *Oshsp16.9*, not normally found in the rice cultivar (Fig. 4A–C). No significant gross morph-

ological defects were observed between the transgenic and non-transgenic plants under standard growth conditions. Transgenic lines also showed similar patterns of growth to the WT under non-stressed conditions, although slight overall increases in fresh weight (FW) could be observed for some of the lines (Fig. 5). The transgenic lines were then examined for responses to various abiotic stresses and compared with the WT, as detailed below. For examination of the survival rate, the seedlings were subjected to stress through exposure to salt (250 mM NaCl) and drought (Fig. 5A and B). After salt and drought stresses, all seedlings (WT, TG #3, and TG #9) showed significant reduction in the measured survival rate; however, the transgenic lines (TG #3 and TG #9) showed greater tolerance to salt and drought stresses than WT, with significant increases in response to the stress conditions for 1 day. Plants were also examined after given a recovery period of 20 days. After recovery for 20 days, the survival rates of the *Oshsp16.9* transgenic plants (about 20–60%) were much higher than those of the control plants (only 5–10%) (Fig. 5B). These results suggest that the overexpression of *Oshsp16.9* in transgenic rice enhanced drought tolerance (*P*-values of 0.05, respectively, by Student's *t*-test). In addition, the transgenic lines showed high relative expression levels of candidate genes (CBL9, protein kinase, Ubiquitin) under

Fig. 4. Expression levels of *Oshsp16.9*-overexpressing lines. A: PCR amplification of transferred *Oshsp16.9* genes in transgenic rice lines. The amplification products were separated using a 1% agarose gel. Lane M: 1-kb DNA ladder; Lane WT: wild-type plant; Lanes T1–T9: PCR products generated from the DNA template of independent transgenic lines; and Lane P: PCR product of the vector plasmid that contained *Oshsp16.9*. B: Southern blot analysis for detection of the *Oshsp16.9* gene in transgenic plants. Genomic DNA of five transgenic lines were digested with *Eco*RI, separated with 0.8% agarose gel electrophoresis and hybridized with a *Oshsp16.9* fragment probe. C: Analysis of *Oshsp16.9* gene expression in the transgenic rice lines by RT-PCR. As a loading control, the samples were also amplified with specific primers for the rice actin gene.

drought and salt stress (250 mM NaCl) (Fig. 6). In conclusion, this study showed that transgenic rice plants overexpressing *Oshsp16.9* had significant improvement of the tolerance to drought and salinity. Together, these results suggest that *Oshsp16.9* plays a strong role in several stress-related

processes, and that its overexpression may lead to the creation of transgenic plants which are tolerant to salt, cold, heat and dehydration stresses. These results suggest that this gene may be useful for the genetic improvement of stress tolerance in rice.

Fig. 5. A, Drought and salt tolerance assays of *Oshsp16.9* transgenic rice. Three-week-old seedlings were exposed to air conditions for 10 h, and then transferred to normal hydroponic conditions and allowed to recover for 20 days. In addition, three-week-old seedlings were watered with 250 mM NaCl solution for 24 h, and then allowed to recover for 20 days. B, Survival rates of the WT and transgenic rice lines. The data presented are the means $\pm$ SE of three independent experiments. The values with significant differences according to *t*-tests are at the level of $P < 0.05$.

Fig. 6. Relative expression levels of candidate genes in *Oshsp16.9*-overexpressing lines under drought (exposed to air) and salt stresses (250 mM NaCl). Total RNA was extracted from three-week-old rice seedlings.

DISCUSSION

Heat shock proteins (HSPs) are conserved in prokaryotes and eukaryotes, and are especially abundant in plants (Trent, 1996). They are one of the main products expressed in cells in response to stress. HSPs can be divided into six families, including HSP100, HSP90, HSP70, HSP60, small heat shock proteins (sHSPs) and ubiquitin (8.5 kDa) (Scharf *et al.* 2001; Hu *et al.* 2009). The group of sHSPs, with molecular masses ranging from 15 to 42 kDa, are conserved intra-cellular chaperones for other proteins (Trent, 1996). They are associated with the nuclei, cytoskeleton, membranes, and both localized and protein-protein interactions, such as folding in organelles. Based on the sequence of the flanking region, we obtained the 453 bp full-length cDNA of a putative sHSP gene in rice by RACE, tentatively designated as *Oshsp16.9* (Fig. 1). This gene was annotated by the RGAD Rice Genome Annotation Database (<http://rgp.dna.affrc.go.jp/RiceHMM/>). Despite the significant amount of data concerning the structure, gene regulation and function of the genes in the plant sHsp family, attributing specific roles to individual proteins in stress tolerance has been difficult (Mu *et al.* 2011). Southern blot analysis revealed that the *Oshsp16.9* gene yielded a single band, suggesting that each member of the *Oshsp16.9* genes existed as a single copy in the rice genome (Fig. 2). Previous studies on *Oryza sativa* heat shock protein (OsHSP) genes showed that they exhibited diverse expression in different organs and enhanced the tolerance of transgenic plants to various stresses, including abiotic stress (Sun *et al.* 2002; Zou *et al.* 2009). In addition, sHSPs are known to function in pollen development and seed germination, as well as resistance to UV damage, enhancement of cell survival against both thermal extremes (50°C and 4°C) and seed and root development (Sung *et al.* 2001; Murakami *et al.* 2004; Zou *et al.* 2009). Expression of the *Oshsp16.9* gene transcript was observed in different parts of the plant, including the root, stem, leaf, spike and seed. Higher expression in the seeds, leaves and spikes was detected than in other tissues of the plant (Fig. 2). Some sHsps are also activated in response to osmotic and salt stress. The expression of *OsHSP23.7* in rice was reported to increase during salt stress treatment, and the *OsHSP24.1*

gene was enhanced following treatment with 10 % PEG (Zou *et al.* 2009). *At-HSP17.6A* and *At-HSP17.6-II* were induced by 0.2 M NaCl and 20 % PEG with similar kinetics in Arabidopsis (Sun *et al.* 2002). *OsHsfC1b* was induced in *O. sativa* roots after 30 min of salt stress, while expression was down regulated in the roots after mannitol treatment (Ruibal *et al.* 2013). In the present study, after the seedlings were faced with various stresses, the expression of the *Oshsp16.9* gene was increased by many fold until the maximum time limit, whereat the induction capability reached a peak (Fig. 3). Many molecular chaperones are stress proteins, and many were originally identified as heat-shock proteins. Molecular chaperones are key components contributing to cellular homeostasis in both optimal and adverse growth conditions. Exogenous ABA treatment demonstrated that the levels of *Oshsp16.9* transcript were higher at the initial stage of induction, but eventually decreased to the pretreatment levels. Exogenous ABA, heavy metals and dehydration stress induces heat tolerance in maize seedlings (Bonham-Smith *et al.* 1987). Numerous studies have shown that the plant hormone abscisic acid (ABA) plays a crucial role in controlling the downstream responses which are essential for adaptation to abiotic stress. As part of the osmotic stress response, regulation of gene expression occurs in both an ABA-dependent and ABA-independent manner (Shinozaki Yamaguchi-Shinozaki, 2000; Ruibal *et al.* 2013). Interestingly, the gene *Oshsp16.9* was activated in response to both osmotic and dehydration stresses (Fig. 3). Individual members of each class of HSPs/chaperones have particular functions, but the co-operation between different HSP/chaperone networks appears to be a central principle of the integrated HSP/chaperone machinery. The transgenic plants, which were transformed to overexpress *Oshsp16.9*, showed similar performance in most of the growth parameters under non-stress conditions (Fig. 4). However, after exposure to salt and drought stresses, the transgenic rice plants showed significant growth differences compared to the WT, confirming that overexpression of *Oshsp16.9* in transgenic rice enhanced tolerance to drought (Fig. 5). The response of plants under drought and heat stress is composed of suppression of photosynthesis, enhancement of respiration, induction of a large number of defense genes, including genes related to

pathogen defense, and changes in genes related to sugar metabolism (Rizhsky *et al.* 2002, Guo *et al.* 2007). This study showed that transgenic rice overexpressing *Oshsp16.9* had significant improvement of the tolerance to drought and salinity. Together, these results suggest that *Oshsp16.9* plays a strong role in several stress-related processes, and that its overexpression may lead to the generation of transgenic plants with tolerance to salt, cold, heat and dehydration stresses. Our results suggest that this gene is an important determinant of stress response in plants.

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