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ORIGINAL RESEARCH

Expression Profiles of Class A Rice Heat Shock Transcription Factor Genes Under Abiotic Stresses

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Abstract Expression profiles of 12 class A rice heat shock transcription factor genes (*OsHsfAs*) were analyzed by semi-quantitative reverse transcriptase polymerase chain reaction. The *OsHsfA* genes exhibited tissue-specific expressions under normal condition. *OsHsfA1a*, *A2d*, and *A9* were predominantly expressed in young spike. Expression responses of the 12 *OsHsfAs* under abiotic stresses were analyzed in the shoots of rice seedling. Most *OsHsfA* genes responded quickly to heat stress except for *OsHsfA1a*, *A3*, and *A9* which were almost unaffected. In particular, *OsHsfA2a* expression in response to heat stress was highest among the heat shock factors examined. However, the majority of the increased *OsHsfAs* expression responses to salt, polyethylene glycol (PEG), and cold treatments primarily occurred during the later stages (3 to 24 h) of stress exposure. Furthermore, most of *OsHsfA* gene expressions were little affected and only a few (*OsHsfA3*, *A4d*, *A7*, and *A9*) genes had slow responses to cold treatment. The results indicate that the transcript levels of *OsHsfAs* during heat stress exposure were distinct from those of plants subjected to salt, PEG, and cold stresses, suggesting that there might be different regulatory networks between heat and non-heat stress.

Keywords Abiotic stress · Expression profile · Heat shock transcription factor · *Oryza sativa*

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Introduction

Rice is one of the most important food crops supporting for almost half of world population. Global warming resulted in significant reductions in rice yields. It is reported that the yield would be reduced by up to 10% with an average daily temperature increase of 1°C (Peng et al. 2004). Furthermore, global temperatures are proposed to rise by 1.5°C to 4.5°C in the coming century (IPCC 2001). Consequently, rice production security under higher temperature has necessitated the importance to elaborate the molecular mechanism of heat stress and develop new rice cultivars with improved heat tolerance. As sessile organisms, plants have evolved a variety of mechanisms to rapidly respond to abiotic stresses by synthesizing increased amounts or new isoforms of heat stress proteins (Hsp), which function as molecular chaperones in repairing protein damage and maintaining homeostasis to grow and propagate under extreme environmental conditions (Hartl and Hayer-Hartl 2002). The central regulators of the expression of such heat-responsive genes are the heat stress transcription factors (Hsfs; Wu 1995).

During the initial stages of heat stress, Hsfs are the primary molecules responsible for relaying signals of cellular stress to the transcriptional apparatus and activating

the expression of the *Hsp* genes. Similar to other transcription factors, Hsfs have a modular structure with an N-terminal DNA binding domain, an adjacent domain with a heptad hydrophobic repeat (HR-A/B), short peptide motifs (nuclear localization signals), and a C-terminal activation domain (aromatic, hydrophobic and acidic amino acid, AHA motifs) (Nover et al. 2001). By structural peculiarities of the HR-A/B regions, plant Hsfs are assigned to three classes: A, B, and C. Furthermore, AHA motifs are essential for the activity of class A Hsfs. In contrast, class B and C Hsfs lack AHA motifs and have no activator function of their own (Kotak et al. 2004).

Recent studies have focused on plant Hsfs response to abiotic stresses. For example, transgenic *Arabidopsis*, soybean, and tomato overexpressing HsfA1 genes exhibited up-regulation of stress-associated genes and enhanced thermotolerance (Lohmann et al. 2004; Mishra et al. 2002; Zhu et al. 2006). In addition, plant Hsfs are also involved in response to oxidative stress (Li et al. 2005; Miller and Mittler 2006), high-salinity (Yokotani et al. 2008), and chilling stress (Li et al. 2003). However, most of the previous results focused on a single gene and the function of rice Hsfs remains largely unknown. We previously demonstrated that nine *OsHsps* were strongly induced by heat shock, and some can also be induced by other abiotic stresses (Zou et al. 2009). These brought our interests to further understand the expression characteristics of *OsHsf* genes in response to abiotic stresses. Although there were some reports on the expression analysis of *OsHsf* under abiotic stresses (Hu et al. 2009), the results did not well reflect the kinetics of gene expression because of less sampling time points. A detailed time-course analysis is thus of considerable interest. In the present study, we selected the 12 class A *OsHsf* genes to analyze for their spatial expression and response patterns under four different abiotic stresses including heat (42°C), salt (150 mmol l^{-1} NaCl), polyethylene glycol (PEG; 20%), and cold (4°C) treatments in a time course of 0, 15, 45 min, 1.5, 3, 5, 8, 12, and 24 h.

Materials and Methods

Plants Materials, Growth Condition, and Treatments

For *OsHsfAs* organ expression analysis, the planting and sample collections of roots, stems, leaves, and spikes were performed according to Zou et al. (Zou et al. 2009). Three replications were done in this study.

Three-week-old rice seedlings were used for stress treatments. Rice seeds (*Oryza sativa* L. ssp. *japonica*) were imbibed in water in the dark at 28°C for 3 days. Germinated seeds were grown on sandy soil (paddy soil mixed with two thirds of river sand) in a climate chamber (28°C, 80% RH, and a 12-h-light [>3,000 lx]/12-h-dark cycle) and irrigated with 1/2 MS liquid culture. After 3 weeks, the seedlings were used for the abiotic stress treatments. For heat shock and cold treatments, the vigorous seedlings were transferred to a growth chamber at 42°C and 4°C, respectively. Salt and PEG stresses were conducted by submerging roots of the seedlings with 1/2 MS liquid culture containing 150 mmol l^{-1} sodium chloride and 20% (*w*/*v*) PEG 6000, respectively. For each treatment, samples were harvested at 0, 15, 45 min, 1.5, 3, 5, 8, 12, and 24 h, separately. A pool of shoots from ten rice seedlings were collected as one biological replicate and each stress treatment was repeated three times.

After collection, all samples were quickly frozen in liquid nitrogen and stored at −80°C for further RNA extraction.

RNA Isolation and Semi-Quantitative RT-PCR Analysis

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, then treated with DNase I (Fermentas, Vilnius, Lithuania) to remove the genomic DNA contamination for 30 min at 37°C. The first strand cDNA was reverse transcribed from 5 μg of total RNA using Oligo$(dT)_{18}$ primers with ReverTra Ace (Toyobo, Osaka, Japan) in a 20-μL reaction volume for 60 min at 42°C. PCR was carried out using Taq DNA polymerase (Fermentas, Vilnius, Lithuania). The amplification reaction mixture consisted of 1× buffer, 0.2 mmol l^{-1} dNTPs of dATP, dGTP, dCTP and dTTP, 2.5 U Taq DNA polymerase, 2 μl cDNA, and sense and antisense primers at 0.2 mmol l^{-1} in a final volume of 25 μl. The PCR amplification was programmed as initially denatured at 94°C for 5 min, followed by 26–42 cycles of 94°C for 30 s, 55–60°C for 30 s, 72°C for 30 s (Table 1), and ended with 8 min of extension at 72°C. Table 1 lists the primer sequences and the sizes of PCR products for each gene. A rice *Actin* gene (*RAc1*, accession number AB047313) expression level was used as a positive internal control. The PCR products were separated on 2% agarose gel. Gel images were recorded using a Gel Doc 1000 analyzer (Bio-Rad, Richmond, CA, USA). The band intensities were scanned by gel quantitative analysis software Glyko Bandscan 5.0 and the band optical density values of *OsHsfAs* and *Actin* genes under different stresses were obtained, respectively.

The relative expression levels were obtained by normalizing optical density values of *OsHsfAs* against those of their corresponding *Actin* at different time points. For the summary of expression response of the 12 *OsHsfA* genes under four abiotic stresses, the expression responses were categorized according to the fold change of each gene in response to stresses comparing to the control at 0 time point

Table 1 Primer sequences and RT-PCR product sizes of the 12 *OsHsfA* genes

Gene	Primer Sequence (5′→3′)	Product Size (bp)
OsHsfA1a	Forward: ACAGCAACTTCTCCTCCTT Reverse: CAGAGTCCAACGAACCAT	510
OsHsfA2a	Forward: TTCGTAGGGTGACGTAATCG Reverse: TCGAAGCCACCGTCCTAG	288
OsHsfA2b	Forward: CTCAAGAACATCAAGCGTAGA Reverse: GGTTCCTTGCAGCCTATC	219
OsHsfA2c	Forward: CTGATAGGTGGGAGTTTGC Reverse: TATAGGCCGCCTTCTTTT	413
OsHsfA2d	Forward: GCAGCAAAGCACGAAAGC Reverse: GGACAAATCGGAGCCACT	578
OsHsfA2e	Forward: TCAAGCACAACAACTTCTCCAG Reverse: TTTGCTGCTCCTGCCTCAG	288
OsHsfA3	Forward: CCCACCACTTCAAGCACA Reverse: GGACGTAGGGGAGAAATCTA	551
OsHsfA4b	Forward: CGTGCGACAGCTCAACAC Reverse: GAAGAAGGGCCTGGGACA	636
OsHsfA4d	Forward: GCTCAACACCTACGGTTTCC Reverse: GCCACTAGCCTGCCTTCC	300
OsHsfA5	Forward: CCGCCTACTTCAAGCACA Reverse: AAAGCATCCGTGAAAATC	451
OsHsfA7	Forward: TTCGCCAGCTCAACACCTA Reverse: TCCATCAGCCGTTGTCTT	445
OsHsfA9	Forward: CTCCCACCTGTCCCAGAAAC Reverse: GAACTGCGAAAAGGCGTCCC	598
Actin	Forward: CTTCAACACCCCTGCTATG Reverse: TCCATCAGGAAGCTCGTAG	358

after being normalized against corresponding *Actin* band optical density values.

Results and Discussion

To investigate the accumulation patterns of the 12 *OsHsfA* transcripts in different tissues, total RNA was extracted from young roots, stems, leaves, and spikes 2 days before booting for semi-quantitative RT-PCR analysis, respectively. Our study revealed that the 12 *OsHsfA* genes exhibited diverse expressions in different organs under normal condition, and their expression could be detected in almost all organs we checked (Fig. 1). *OsHsfA2a* were detected slightly higher in roots, *OsHsfA1a*, *A2d*, *A4d*, and *A7* were expressed at high level in leaves. It was shown previously that mutation of *OsHsfA4d* led to a lesion mimic phenotype in mature leaves (Yamanouchi et al. 2002). In addition, *OsHsfA1a*, *A2d*, and *A9* were predominantly expressed in young spikes. The abundant expression of *OsHsfA1a* in spikes and our recent observation of obviously low seed-setting rate in *OsHsfA1a* RNAi transgenic rice (data not shown) suggest the important role of OsHsfA1a in the spike development. *AtHsfA9* was exclusively expressed in the late stage of seed development and not during other stages of plant growth (Kotak et al. 2007) and *HaHsfA9* was specifically expressed during embryogenesis under normal condition in sunflower (Almoguera et al. 2002). These results indicated that *HsfA9* might be involved in the normal embryogenesis and/or seed development. Usually reproductive organs were much more sensitive to heat stress than vegetable organs. Heat stress resulting in yield reduction in rice production happened during the pollen development. Thus, further investigation is required to clarify the role of OsHsfA1a, A2d, and A9 in the reproductive developmental programs.

Expressions of *Hsf* genes have been shown to be enhanced by elevated temperature (Liu et al. 2005; Liu et al. 2009; Swindell et al. 2007). In *Arabidopsis*, Hsfs were strongly up-regulated in response to heat stress exposure (Swindell et al. 2007). In our study, nine *OsHsfAs* were up-regulated in the shoots of rice seedling under heat treatment with different response pattern (Fig. 2). The transcript levels of *OsHsfA4b*, *A5*, *A7*, and five *OsHsfA2s* were up-regulated in the early stages of heat stress exposure, with the effect gradually diminishing during prolonged heat stress. While *OsHsfA4d* transcripts increased and maintained at relatively high level until 24 h. Hsfs and Hsps are central components of heat shock regulatory network. It has long been recognized that Hsps are enhanced by elevated temperature in plants (Hu et al. 2009; Koo et al. 2003; Sung et al. 2001; Zou et al. 2009) and the expression of Hsps are controlled by

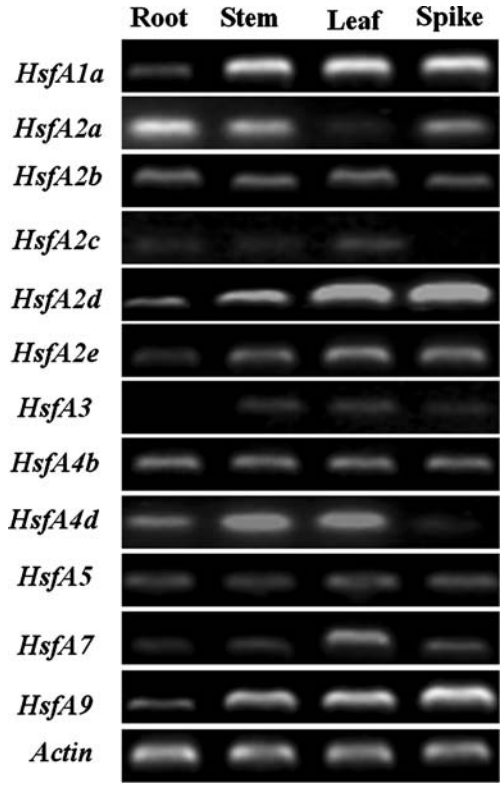

Fig. 1 Tissue-specific expression patterns of 12 *OsHsfA* genes. Transcript levels were analyzed by RT-PCR. A rice *Actin* gene was included as a control for constitutive expression in the assays

Hsfs. On the other hand, enough Hsp accumulations could feed back to repress Hsfs activity. This may explain the declined expressions of some *OsHsfAs* during prolonged heat stress. Furthermore, three A2-type *OsHsf* (*A2a*, *A2c*, and *A2d*) responded more quickly than other *OsHsfAs* under heat stress in our experiment. Liu et al. (Liu et al. 2005; Liu et al. 2009) also reported that the accumulation of *OsHsf6* (*OsHsfA2c*) and *OsHsf7* (*OsHsfA2d*) mRNA was quickly elevated within 10 min of HS treatment. These results may imply that three A2-type *OsHsfs* were involved in primary heat stress response. However, in tomato, HsfA2 accumulated to high level after exposure to prolonged heat stress (Port et al. 2004; Scharf et al. 1998). In contrast to tomato containing only one *HsfA2*, rice has HsfA2 group composed of five different *HsfA2s*. Therefore, there might be a more complicated heat shock regulation system in rice than that in tomato. Interestingly, *OsHsfA2a* showed highest transcript levels among the heat shock factors examined during the time course of heat treatment, our data is consistent with Ogawa et al. (Ogawa et al. 2007) who proposed that the induction of *HsfA2* expression in response to heat stress is highest among all 21 *Arabidopsis Hsf*s. It may suggest that this gene plays an important role in rice heat stress response, further functional characterization is necessary to elucidate the possible role of *OsHsfA2a* in heat stress tolerance. The unaffected expression of *OsHsfA1a*, *A3*, and *A9* under heat stress may be due to the lack of heat shock element in the promoter by searching the PLACE database. In contrast, the tomato *HsfA1* is the master regulator of the

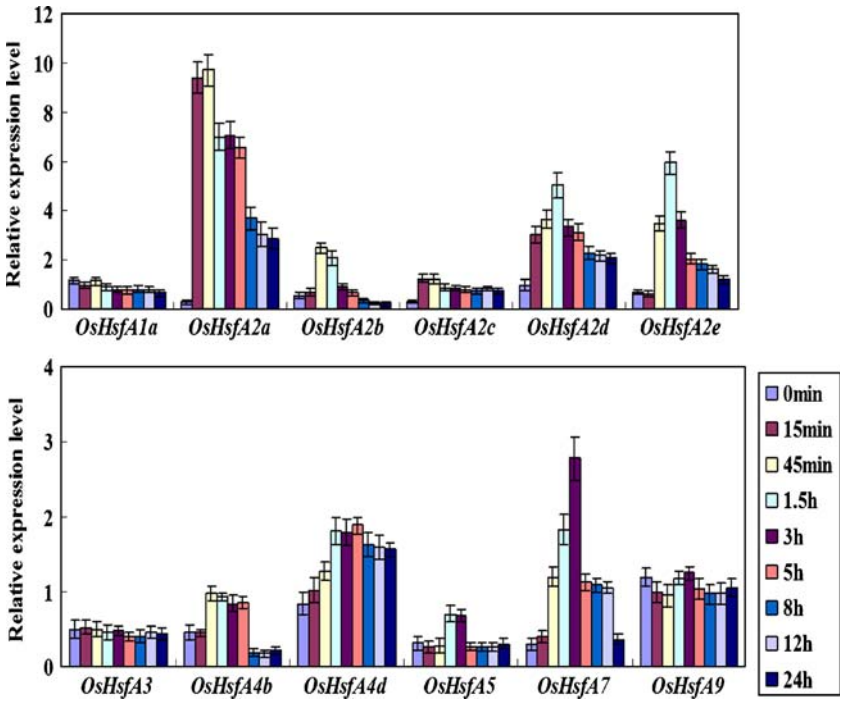

Fig. 2 Expression profiles of 12 *OsHsfA* genes in response to heat shock treatment. Three-week-old rice seedlings were subject to 42°C. The RT-PCR products of 12 *OsHsfA* genes were separated by agarose gel electrophoresis and shown by ethidium bromide staining. The band optical density values were obtained by gel quantitative analysis software Glyko Bandscan 5.0 software. The relative expression levels were obtained by normalizing optical density values of *OsHsfAs* against those of their corresponding *Actin* at different time points

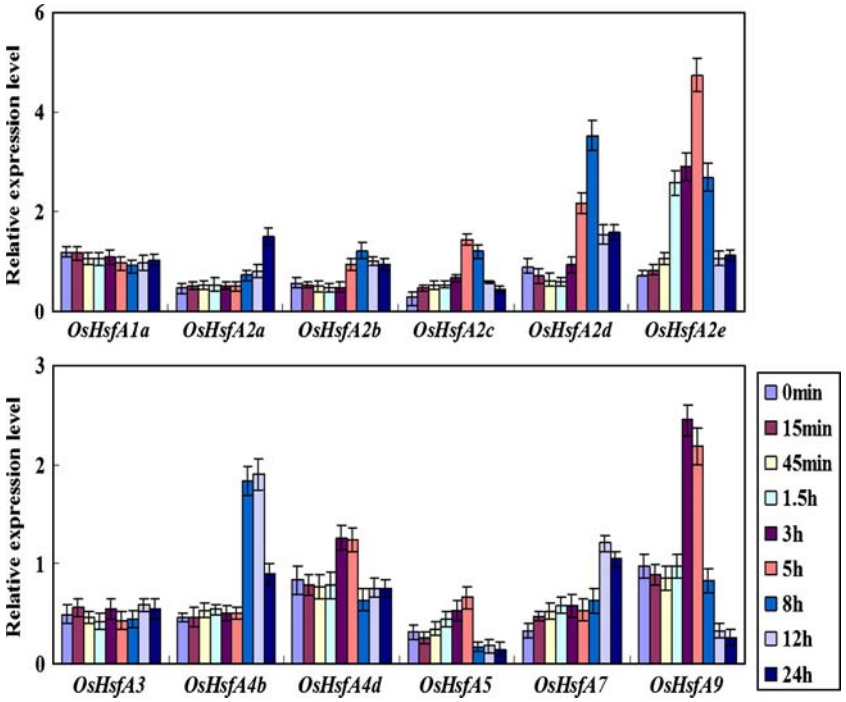

Fig. 3 Expression profiles of 12 *OsHsfA* genes in response to high salt treatment. Three-week-old rice seedlings were subject to 150 mmol l^{-1} NaCl. The expression data were processed as described in Fig. 2 legend

heat response and is essential for thermotolerance (Mishra et al. 2002).

OsHsfA genes in the rice seedling exhibited remarkable diverse expression profiles in response to some other abiotic stresses besides heat stress. Expression of *OsHsfA2c* and *A2d* at 5 to 8 h, *OsHsfA2e* at 1.5 to 8 h, and *OsHsfA4b* at 8 to 12 h were obviously enhanced by high salt. Meanwhile, *OsHsfA2a* transcripts came to peak at 24 h while *OsHsfA2b* at 5 to 24 h, *OsHsfA5*, *A9* at 3 to 5 h, and *OsHsfA7* at 12 to 24 h were induced in our experiment (Fig. 3). The expressions of *OsHsfA1a* at 5 to12 h, *OsHsfA2a* and *A7* at 3 to 24 h, *OsHsfA3* at 45 min to12 h, *OsHsfA5* at 8 to 24 h, and *OsHsfA9* at 45 min to 24 h were visibly enhanced and the mRNA accumulations of *OsHsfA2d*, *A2e*, and *A4b* were more abundant at 5 to 24 h by PEG treatment (Fig. 4). Notably, the majority of *OsHsfA* gene expression was little affected to cold treatment and only four *OsHsfA* genes (*OsHsfA3*, *A4d*,

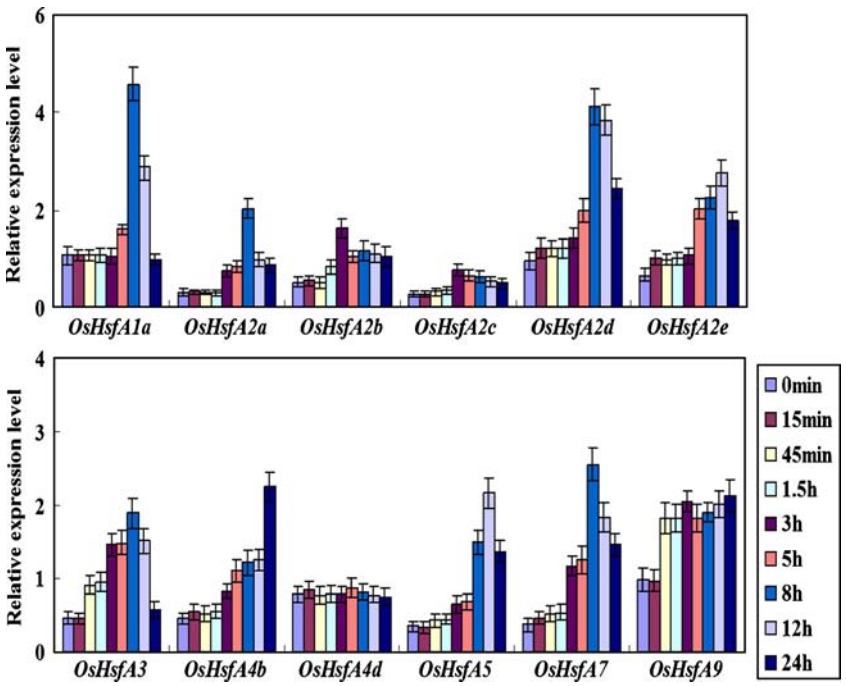

Fig. 4 Expression profiles of 12 *OsHsfA* genes in response to PEG treatment. Three-week-old rice seedlings were subject to 20% PEG. The expression data were processed as described in Fig. 2 legend

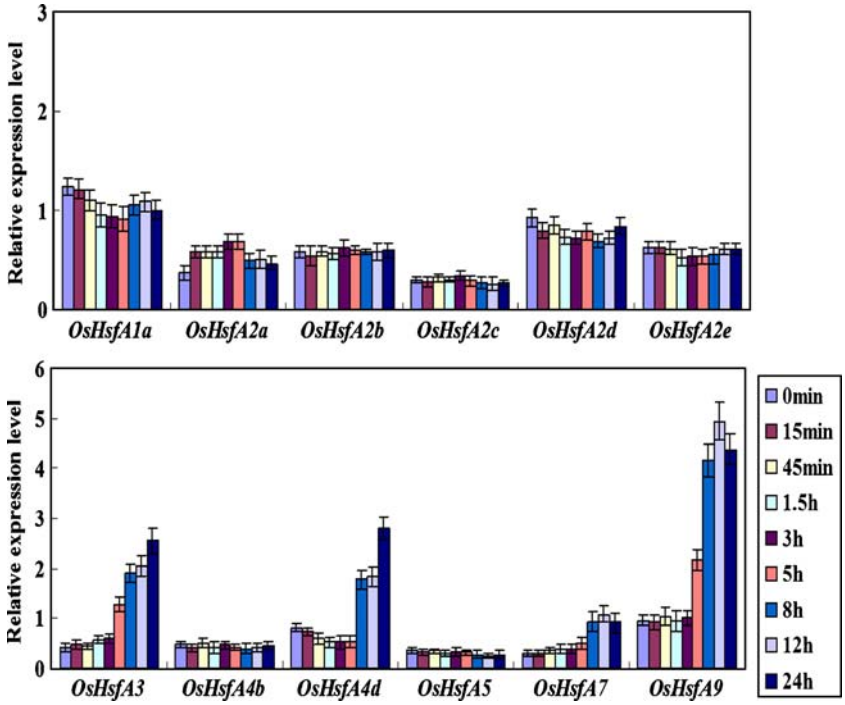

Fig. 5 Expression profiles of 12 *OsHsfA* genes in response to cold treatment. Three-week-old rice seedlings were subject to 4°C. The expression data were processed as described in Fig. 2 legend

A7, and *A9*) were obviously up-regulated during the late treatment stage (Fig. 5). Zou et al. (Zou et al. 2009) reported transcript levels of nine *OsHsp* genes were almost unaffected by cold stress. These observations indicated that the majority of *OsHsfA* expression responses to salt, PEG, and cold treatment primarily occur in the late stages of stress exposure. Our results accorded with the Swindell's findings in *Arabidopsis* in responses to salt, osmotic, and cold stress (Swindell et al. 2007), suggesting that this mode of defense response is conserved among different plants. A possible explanation for the results is PEG, salt, and cold stress treatments are each believed to have a deleterious impact on cellular water potential (Verslues et al. 2006).

Recent researches reported heat shock transcription factors play important roles in linking heat shock with other stress signals. The transcripts of *AtHsfA2* increased under several types of environmental stress, including heat and oxidative stress (Li et al. 2005), heat and osmotic stress (Ogawa et al. 2007), and high light plus heat stress (Nishizawa et al. 2006). This indicates that AtHsfA2 is a key regulator in the induction of the defense system under several stress conditions. The transcripts of *AtHsfA6a* increased in response to drought stress (Rizhsky et al. 2004). Overexpression of *OsHsfA2e* resulted in enhanced thermo and salt tolerance in transgenic *Arabidopsis* (Yokotani et al. 2008). Expression of *AtHsfA7a* was reported to be elevated in cells during light stress (Pnueli et al. 2003). Our results showed five *OsHsfA2s*, *OsHsfA4b*, and *A5* were up-regulated under heat, salt, and PEG stresses. It is noteworthy that *OsHsfA7* responded to all the four treatments that we examined (Table 2). These provided further evidence that Hsfs are widely involved in response to various abiotic stresses. However, it should be noted that our analysis was based upon separate single stress, not stress combination, whether these genes may represent co-regulators among the stress responsive pathways warrant further investigation.

In conclusion, the transcript levels and time-course responses of *OsHsfAs* under heat stress exposure were distinct

Table 2 Expression responses of the 12 *OsHsfA* genes under four abiotic stresses

Gene	Heat	Salt	PEG	Cold
OsHsfA1a	-	-	++	-
OsHsfA2a	+++	+	+++	-
OsHsfA2b	++	+	+	-
OsHsfA2c	+	++	+	-
OsHsfA2d	++	++	++	-
OsHsfA2e	+++	+++	++	-
OsHsfA3	-	-	++	+++
OsHsfA4b	+	++	++	-
OsHsfA4d	+	-	-	+
OsHsfA5	+	+	+++	-
OsHsfA7	+++	+	+++	+
OsHsfA9	-	+	+	++

The expression responses were categorized according to the fold change of each gene in response to stresses comparing to the control at 0 time point after being normalized against corresponding *Actin* band optical density values

"-" decreased little or increased less than twofold, "+" increased two- to fourfold, "++" increased four- to sixfold, "+++" increased more than sixfold

from those under salt, PEG, and cold stress in the shoots of rice seedling, suggesting that there might be different regulatory networks for heat and non-heat stresses. Little is known about the overlapping elements and cross-talk among diverse forms of stress response networks in rice. Plants encounter different stress combination under field conditions, further investigation to identify the co-regulators from the 12 *OsHsfAs* may have the potential for the development of transgenic rice with improved multiple stresses resistance. Our preliminary data provided a very useful reference as well as a starting point for revealing the individual function of *OsHsfAs*. Transgenic rice plants expressing *OsHsfAs* is under way in our laboratory and will yield more information on the functional specialization of *OsHsfA* family genes.

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