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Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice

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ABSTRACT

Heat shock proteins (Hsps) are molecular chaperons, which function in protein folding and assembly, protein intracellular localization and secretion, and degradation of misfolded and truncated proteins. Heat shock factors (Hsfs) are the transcriptional activators of Hsps. It has been reported that Hsps and Hsfs are widely involved in response to various abiotic stresses such as heat, drought, salinity and cold. To elucidate the function and regulation of rice Hsp and Hsf genes, we examined a global expression profiling with heat stressed rice seedling, and then compared our results with the previous rice data under cold, drought and salt stresses. The comparison revealed that, while most Hsfs and Hsps had highly similar and overlapped response and regulation patterns under different stresses, some of those genes showed significantly specific response to distinct stress. We also found that heat-responsive gene profiling differed largely from those under cold/drought/salt stresses, and that drought treatment was more effective to up-regulate Hsf expression in rice than in *Arabidopsis*. Overall, our data suggests that Hsps and Hsfs might be important elements in cross-talk of different stress signal transduction networks.

1. Introduction

Abiotic stresses, such as drought, salinity, high/low temperature, represent seriously threat to agriculture and cause the huge loss of crop yield worldwide by more than 50% annually [1]. Because agriculturally crops are often under a combination of different types of stresses [2], understanding different pathways in response to abiotic stresses and the cross-talk among them is important to the improvement of crop production. Several studies suggested that while each abiotic stress can elicit unique response, there still is the existence of a substantial network of regulatory interactions and coordination of distinct pathways in plant response to different abiotic stresses. Such networks involved in rice response to cold, heat, drought and salt stresses, however, remain largely unknown.

Heat shock proteins (Hsps) are functionally linked to the large and diverse families of molecular chaperones that are defined by their capacity to recognize and to bind substrate proteins that are in an unstable, inactive state. During heat stress, the fraction of potential targets for molecular chaperones seems to dramatically increased. The molecular pathways leading to Hsp expression are not entirely understood, but involve temperature perception and multiple signal transduction pathways [3], which together lead to the activation of Hsfs that induce expression of heat shock genes by binding to heat shock element [4]. Plant Hsps and Hsfs have been well characterized in a few model plants such as tomato and *Arabidopsis*. Based on genome sequence data, 21, 27, 18, 7, and 8 genes have been identified for *Arabidopsis* Hsf, sHsp, Hsp70, Hsp90 and Hsp100 family, respectively [5–10]. A number of genome-wide microarray datasets provide a possible way to analyze the response of Hsps and Hsfs to different abiotic stresses. *Arabidopsis* Hsfs and Hsps are strongly induced by heat, cold, salt and osmotic stresses. Moreover, there is extensive overlapping response of Hsps and Hsfs to heat and other abiotic stresses, which indicates that Hsps and Hsfs are important elements in the cross-talk of different response pathways [10].

Since the completion of rice genome sequencing, several works have been reported on identification and functional elucidation of

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rice Hsfs and Hsps. Twenty-five rice Hsfs have been identified [11]. One base substitution in Spl7 (Os01g54550, HsfA4b) DNA-binding domain led to an amino acid change from tryptophan to cysteine, and caused the lesion-mimic phenotype of rice leaf under high temperature [12]. HsfA5 could bind to Spl7 and functioned as a repressor of HsfA4 in tomato [13]. Small Hsps (sHsps) have a relatively complex diversity at DNA sequence variation, copy numbers, and cellular localization. sHsps play a role as molecular chaperones in preventing aggregation of proteins and reactivating denatured proteins [14]. Over-expression of OsHsp17.7 (Os03g16040) enhanced rice tolerance to heat UV-B as well as to drought [15,16]. Hsp70 chaperones assist in various aspects of protein processing such as folding of new translated protein and protein translocation across organelle membrane [17]. Hsp90 can bind Hsp70 in many chaperone complexes and has important role in signal transduction [18]. Also Hsp90 is reported as an essential component of innate-immune response and pathogenic resistance in rice [19]. Hsp100 can prevent target proteins from aggregation and relate to proteolysis of target proteins. Rice Hsp101 (Os05g44340) could disaggregate of protein granules and complement the function of yeast Hsp104 [20].

Here we further identify rice Hsf and Hsp genes and analyze their expression profiles under different abiotic stresses. A whole-genome microarray analysis was carried out to investigate expression changes of rice Hsfs and Hsps in response to heat stress. Through comparing our experimental data with other expression data under salt, cold, and drought conditions (GEO accession: GSE6901) [21], we found that these rice Hsf and Hsp families responded to different stresses in an overlapping relationship. Our analysis also indicated that some Hsf and Hsp genes exhibited specific expression patterns in response to distinct stresses. And the responsive patterns of Hsps and Hsfs genes between *Arabidopsis* and rice were compared and difference was also discussed.

2. Result

2.1. Overview of genome-wide response to heat, cold, drought and salt

Rice genome microarray (Affymetrix) analysis was used to investigate the genome-wide expression changes in response to heat stress treatment. The rice expression data under cold, drought and salt were downloaded from GEO. Differential expression probe sets were detected by Limma as a Bioconductor package [22,23]. Genes that were up- or down-regulated by each stress were shown in Fig. 1A. The number of responsive probe sets under heat, cold, drought and salt treatments were 1054, 276, 2742 and 1200, respectively. Among them, the number of probe sets that were up-regulated by heat, cold, drought and salt were 631, 194, 1624 and 933, respectively, while the download-regulated probe sets were 423, 82, 1118 and 267, respectively.

The extent of overlapped genes response to two or more stresses was examined. Venn diagram showed the probe sets which were shared under different stresses (Fig. 1B). There were only 10 probe sets (genes) that responded to all four stresses (Supplementary Table 1, the top 10 probe sets). Among them, only two genes had putative functions, while the functions of others were still unknown. The number of overlapping responsive probe sets between heat stress and each of cold, drought and salt stresses were 33, 240 and 127, respectively. The number of overlapping responsive probe sets between drought/cold, salt/cold and drought/salt were 197, 141 and 985, respectively (Supplementary Table 1). Totally, 3926 probe sets showed differentially expression in response to at least one stress. To further compare expression profiles under different stresses, these 3926 genes were used to perform cluster analysis. They showed similar expression pattern under drought and salt stresses, but exhibited minimal expression similarity between heat and other three stresses (Fig. 1C).

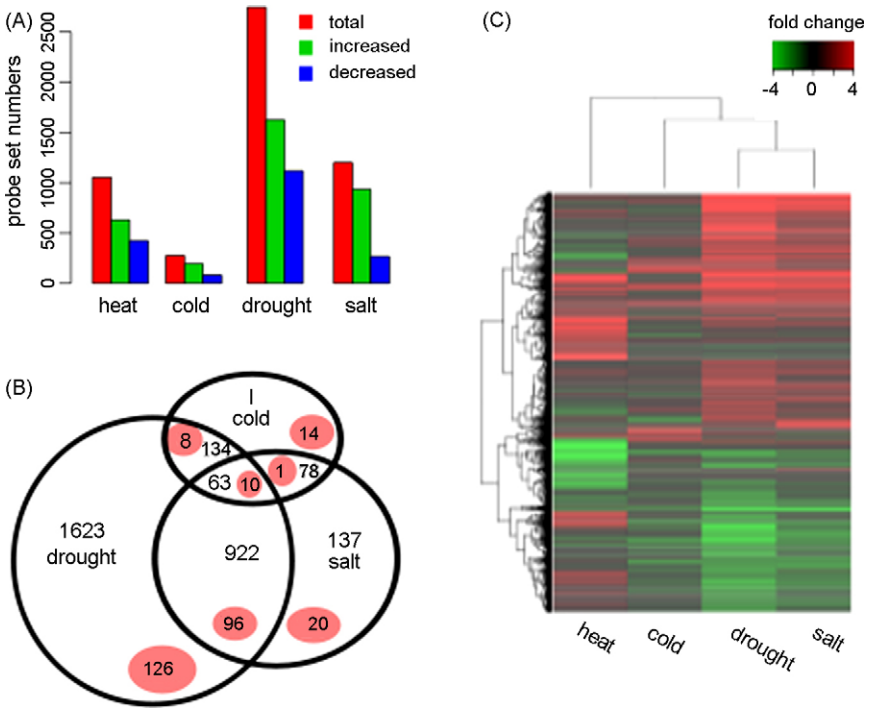

Fig. 1. A summary of response probe sets of rice seedlings under heat, cold, drought and salt stresses. The significant response probe sets were selected using Limma package and extracted by adjusted $P < 0.05$. (A) The number of increased and decreased probe sets under four abiotic stresses. Vertical axis indicated the response probe set numbers. Horizontal axis indicates four stresses. Red, green, and blue bars represented the total, increased, and decreased probe sets, respectively. (B) Venn diagram of response probe sets under four stresses. Three circles indicate the response probe sets under cold, drought and salt stresses. The 1054 response probe sets under heat treatment were not shown. Numbers in red circles indicated probe set numbers co-regulated by heat stress and other stresses where red circles positioned. (C) Cluster analysis of 3926 probe sets that responded at least to one of these four abiotic stresses. Response expression level was measured by $\log_2$ fold change.

Among genes induced by heat stress, many of them were Hsp and Hsf genes that would be analyzed later. For other genes that were previously reported to have functions in cold, drought and salt stresses, our results showed that those genes were also affected by heat stress, including Myb-like DNA-binding domain protein, BRASSINOSTEROID INSENSITIVE 1-associated receptor like kinase 1, CBL-interacting serine/threonine-protein kinase 15 and putative two-component response regulator-like proteins. However, there were no genes that strikingly responded to drought and/or salt stresses, such as DREB1a, LEA, WSI76, MAP65, that were found to respond to heat stress. To gain an overall view of responsive genes under heat stress, gene ontology analysis was performed. Annotation of these responsive genes showed that they could be assigned into different functional groups, such as stress stimulus, lipid metabolism, biosynthesis, and so forth (Supplementary Table 2).

2.2. Identification of rice Hsps and Hsfs and their distribution on chromosomes

Rice Hsps and Hsfs were identified by similarity searching against the rice genome sequence data using *Arabidopsis* Hsf and Hsp genes followed by manual check [10]. The number of identified gene of Hsf, sHsp, Hsp70, Hsp90, and Hsp100 families in rice was 25, 29, 26, 9, and 10, respectively. The gene names associated to 25 rice Hsf genes were indicated in Supplementary Table 3 according to von Koskull-Döring et al. [11]. To analyze the phylogenetic relationship of these heat shock proteins in rice and *Arabidopsis*, phylogenetic trees of these five families were constructed by Phylip [24]. Most of genes from rice and *Arabidopsis* could be grouped into the same sub-clusters, which indicated that they were highly conserved throughout five Hsp and Hsf families (Supplementary Figs. 1–5).

To further investigate the expansion of these gene families, Hsp and Hsf genes were plotted on chromosomes (Fig. 2). The total of 99 genes of Hsfs and Hsps were distributed on 12 chromosomes. There were two clusters of tandem duplicated sHsp genes belonging to cytosolic class I [6]. One cluster was found on chromosome 1 and included five members. It seemed that these tandem clustered genes were highly expressed at normal seedlings and four of these five genes had similar expression patterns that specifically induced by heat stress (Supplementary Table 3). Another tandem repeat sHsp cluster locating on chromosome 3 included four members, and all of these four members responded to heat stress significantly. The expression pattern of all of these nine sHsp genes under heat stress coincided with another study [25]. According to rice chromosome duplication analysis, 14 gene pairs were located at duplicated segments on chromosomes (Supplementary Table 4). It showed that tandem duplication and chromosome segmental duplication played an important role in the expansion of rice Hsfs and Hsps.

2.3. Overlapping induction of rice Hsps and Hsfs under heat, cold, drought and salt stresses

To detect the overlapping expression of Hsfs and Hsps under heat, cold, drought and salt stresses, an overview statistic variant T value was calculated. The T value was a median of response level for each gene family under each of four stresses. To test whether the medians were different from genome-wide response, a resampling procedure was carried out to test the significance of T value. As described in Table 1, Hsf gene family was most strongly up-regulated by drought and salt, with T values being 0.91, 0.86 respectively. sHsp and Hsp90 families were most significantly induced by heat stress, with T values being 2.73 and 1.02, respectively (Table 1). The highest expression induction of Hsp100 family was caused by both heat and salt stresses. Hsf and sHsp gene families exhibited widely induction by heat, drought and salt treatments ($P < 0.0015$). In contrast, Hsp70, Hsp90 and Hsp100 families showed more specific response under those stresses.

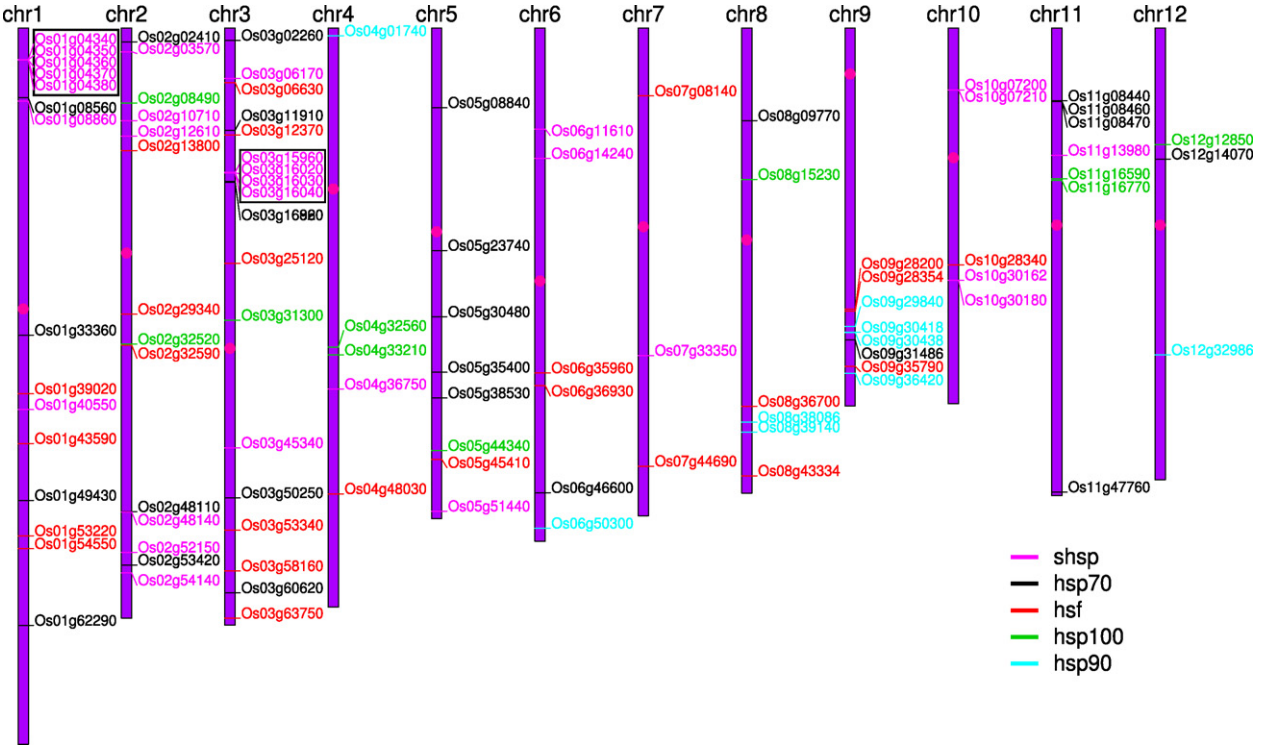

Fig. 2. Chromosome distribution of heat stress transcription factors and heat shock proteins. The 99 heat stress genes were marked on 12 rice chromosomes (vertical bar). Five different heat shock gene families were indicated by different colored lines on chromosomes. The magenta ovals on chromosomes indicated the position of centromeres. Chromosome numbers are indicated at the top of each bar. Tow tandem clustered genes of sHsps were indicated by black boxes at chromosomes 1 and 3.

Table 1
Overview of response of heat shock proteins and heat shock transcription factors to heat, cold, drought and salt stresses.

Family	Heat	Cold	Drought	Salt
Hsf	0.28(<0.001)	0.03(0.165)	0.91(<0.001)	0.86(<0.001)
sHsp	2.73(<0.001)	0.07(0.022)	0.20(0.001)	0.25(<0.001)
Hsp70	0.07(0.067)	0.03(0.130)	0.02(0.132)	−0.02(0.810)
Hsp90	1.02(<0.001)	−0.16(0.036)	−0.09(0.321)	0.01(0.205)
Hsp100	−0.26(0.024)	−0.08(0.164)	−0.15(0.035)	0.23(0.006)

T statistic values associated with Hsf, sHsp, Hsp70, Hsp90 and Hsp100 gene families under heat, cold, drought and salt stresses were calculated as the median levels of $\log_2$ fold change of *n* genes within a gene family. The significance (*P*) of associated *T* value was shown in parentheses. The *P* value was obtained by randomly selecting *n* genes from all genes represented on the rice genome array 10,000 times. *P* value exceeding 0.0015 were not significant by Benjamini–Hochberg adjustment (with nominal type I error rate of alpha = 0.05).

As shown in Fig. 3, many genes were strongly induced by more than one stress, except for three genes, two from sHsp family and one from Hsf family, which had reduced expression. The response levels and significances of each probe sets associated with Hsf and Hsp genes were described in Supplementary Table 3. Eight of 25 Hsf genes were significantly responsive to at least 1 stress, including 7 genes responsive to drought stress, 5 to salt, 3 to heat, and 1 to cold. Among those eight responsive Hsf genes, Os01g39020 (HsfA6b) and Os02g13800 (HsfC2a) were induced by heat, drought and salt, while Os01g53220 (HsfC1b) responded to cold, drought and salt. Sixteen of 29 sHsp genes were significantly induced by at least 1 stress. For Hsp70 family, seven genes were up-regulated by heat, drought and salt. Os04g01740 (OsHsp90-1) however, was strongly induced under heat stress, but not induced by cold, drought or salt. There were six probe sets associated with Os04g01740, and the mean fold change of these six probe sets was 7.41. This may indicate that Os04g01740 play an important role in heat response. For Hsp100 gene family, Os05g44340 was induced by heat and drought, while Os02g32520 was induced by drought and salt.

We further compared gene expression patterns from four stresses (Fig. 4). Pearson correlation coefficient (*R*) represented the degree of co-regulation between two stresses. For the six stress pairs (Fig. 4), all five Hsp and Hsf gene families exhibited the similar response pattern under drought and salt treatments (Fig. 4, the sixth row). This suggested that the similar response network existed between drought and salt stresses in seedling. Although Hsp70 family had low induced expression level under all four stresses studied (Table 1), they had the similar expression pattern under five of six stress pairs, except under heat/cold treatments (Fig. 4, the third column). Other co-regulated response patterns were sHsp family under heat/salt stresses, Hsp100 family under cold/drought, cold/salt stresses.

2.4. Confirmation of microarray expression results of Hsps and Hsfs by quantitative RT-PCR

To validate microarray datasets and investigate the kinetics of gene expression, we analyzed expression of some Hsps and Hsfs by quantitative RT-PCR. Nine sHsp genes were selected to confirm the microarray results. They were strongly induced by heat stress after 1 h, and maintained at a relatively high level at 3 and 10 h (Fig. 5A). The gene numbers validated by quantitative RT-PCR for Hsf, Hsp90, Hsp100 and Hsp70 family were 6, 1, 1 and 4, respectively. The expression patterns of all these genes were consistent with the microarray results (Fig. 5, Supplement Table 3).

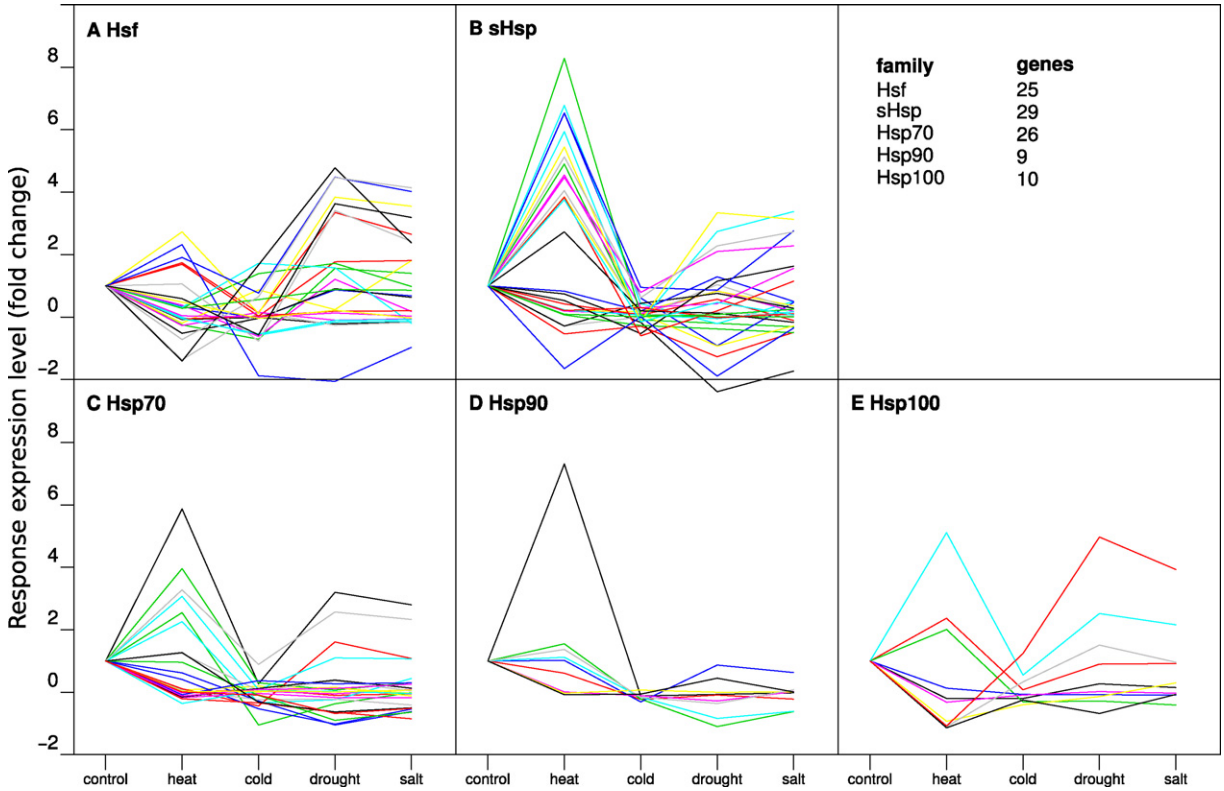

Fig. 3. The expression pattern of Hsp and Hsf genes under four abiotic stresses. The horizontal axis of each sub-figure corresponded to four stresses. Expression levels of control seedlings assumed as one. The vertical axis for each sub-figure indicated response expression (fold change) of each gene under a given stress. Five sub-figures represent to (A) Hsf, (B) sHsp, (C) Hsp70, (D) Hsp90, and (E) Hsp100 sub-families. Different colored lines represented Hsp or Hsf genes of these five sub-families. Gene numbers for each heat shock gene family were shown at the top right sub-figure.

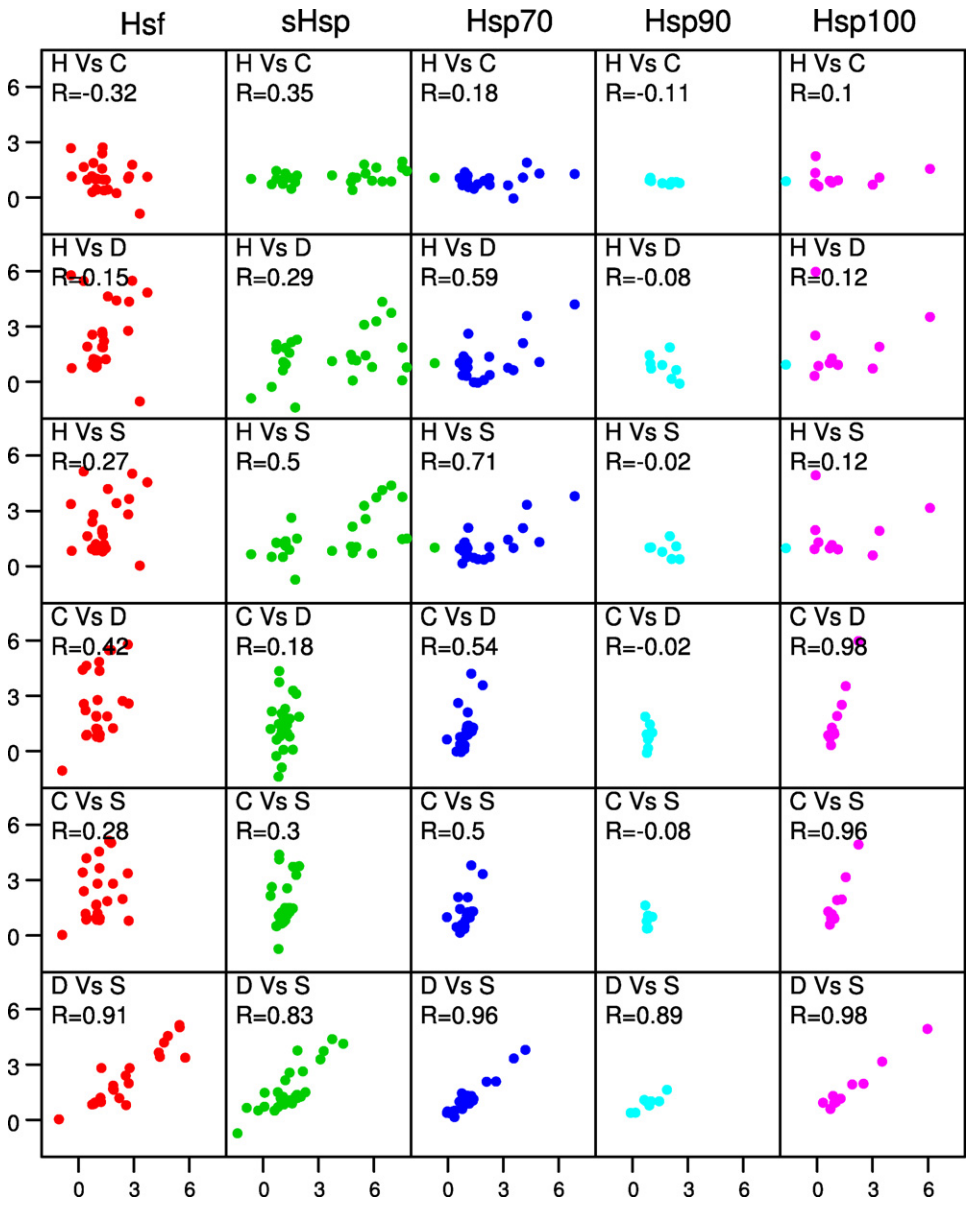

Fig. 4. Expression correlation of Hsp and Hsf genes between any two of heat, cold, drought and salt stresses. For each family of Hsf, sHsp, Hsp70, Hsp90 and Hsp100 (the five columns), responsive expression levels of gene members under any two stress were plotted. At each top right of sub-figure, H stands for heat stress, C for cold stress, D for drought stress, S for salt stress. For example, 'H Vs C' meant that *x*-axis and *y*-axis represented the response fold change under heat and cold stresses, respectively. Pearson correlation coefficient (*R*) represents the expression pattern similarity of a given gene family under two stresses.

3. Discussion

In this study, we used a genome-wide approach to identify genes that potentially participate in rice response to heat stress. To our knowledge, it was the first attempt to investigate an overall perspective of rice heat shock transcription factors and heat shock proteins by identifying these genes in rice genome and exploring the expression profiles of these genes under heat, cold, drought and salt stresses.

Although there were some reports on the identification of genome-wide expression changes under cold, drought and salt treatments and their signal networks in *Arabidopsis* and rice [26,27], there were few reports on the characterization of the response network between heat and other abiotic stresses in rice. In this research we analyzed rice gene expression patterns under heat stress, and compared with that under cold, drought and salt stresses from GEO database. The numbers of genes in response to heat, cold, drought and salt stresses were quite different; they were 276, 1054, 2742 and 1200, respectively. Till now, there was no systematic research reporting that rice was more vulnerable to water deficiency or less sensitive to low temperature than other abiotic stresses since it is hard to weigh the intensity of different treatments. Another research on genome-wide profile analysis of rice organs, reported that there were 2090 and 2957 genes responding to drought and salinity treatments, respectively [27]. This indicated that different stress effects resulted from specific stress types, time duration, different tissues and development stages. Although there were 240 genes responding to both heat and drought, clustering analysis showed genome-wide response patterns under heat stress were rather different from cold, drought and salt. This result is consistent with previous studies in *Arabidopsis* [28]. It is urgent to develop multiple stresses resistance crops while global warming may lead to a variety of climate changes [29]. Therefore it will be helpful to identify co-regulators from the 240 genes that responded to both heat and drought stresses.

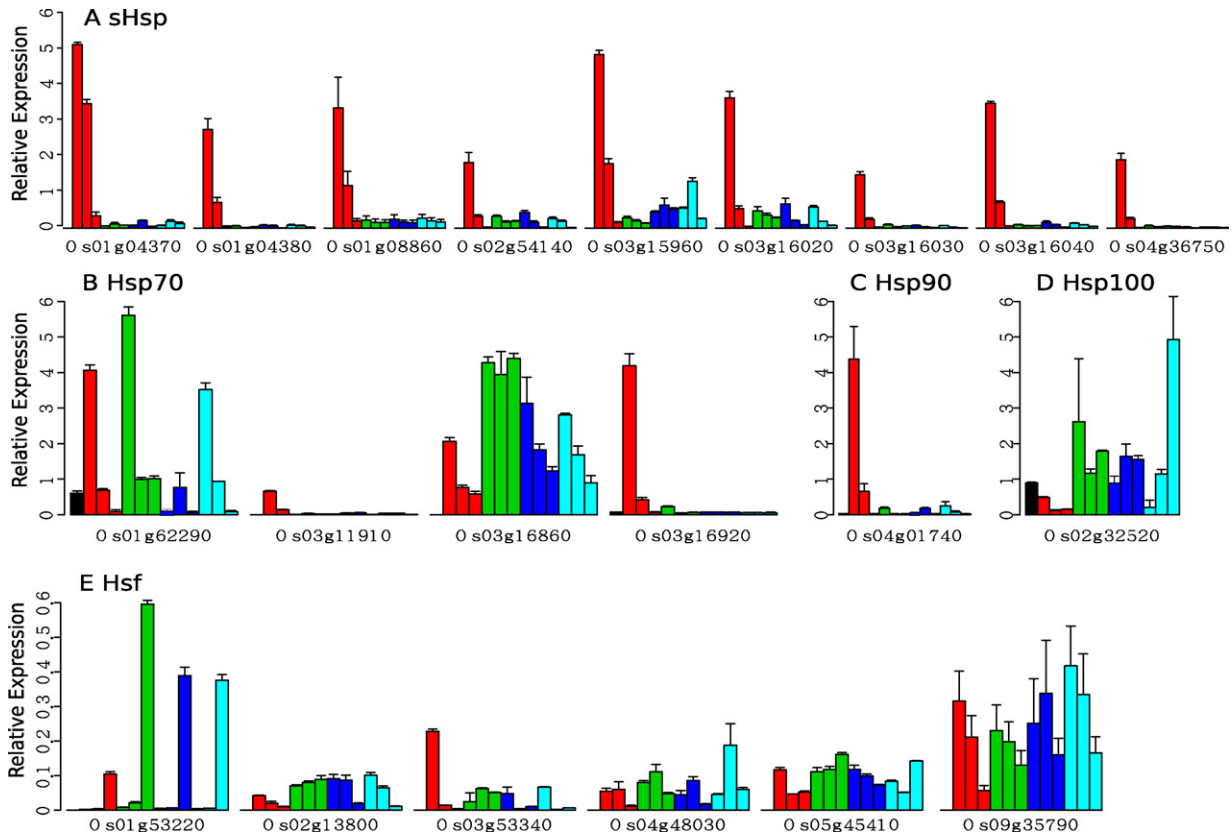

Fig. 5. The relative expression level of heat related genes in rice. Genes were grouped by gene family. Gene expression levels were evaluated by comparison to two internal reference genes (UBQ5 and eEF-1α). For each gene, the leading black bar was the expression level of control seedlings, and the red, green, blue and aqua bars represented the responded expression under heat, cold, drought and salt, respectively. For each stress treatment, the three bars that in the same color represented the expression level in a time course of 1, 3, and 10 h.

Hsfs and Hsps are important components of heat shock regulatory network. There are only 1, 1, 4 Hsf genes in yeast, *Drosophila* and vertebrates, respectively. But there were 25 Hsf genes identified in rice, many of which were produced by chromosome segmental duplication. It was believed that at least several Hsf and Hsp genes have non-redundant roles in signal network [3,10]. The similar expression patterns of Hsfs and Hsps under these abiotic stresses indicated that the response of these genes might be evoked by the same cis-regulatory elements in their promoters, similar to the results of several other studies [27,30]. The induction mechanisms of this response may include the accumulation of denatured proteins in the cytoplasm [3], changes in membrane lipid composition and fluidity [31] or generation of reactive oxygen species [32]. For example, members of Hsp70 were induced by the similar levels under different stresses, although Hsp70 family had low responsive median *T* values (Table 1). This might be because many of Hsp70 genes had persistent expression at normal conditions and only a few genes were co-regulated by heat, drought, and salt treatments. The highly overlapping response and similar regulation patterns of Hsfs and Hsps among different stresses indicated that they were important elements in cross-talk of responsive signal transduction networks.

Abiotic stresses that most strongly induced the expression of Hsfs were osmotic, cold, salt, and heat in *Arabidopsis* [10]. In this study, we showed that drought and salt induced the expression of rice Hsfs more strongly than heat and cold. This suggests a possible difference in response strength between OsHsf and AtHsf families. We found that the expression levels of rice sHsp family were up-regulated significantly by all four abiotic stresses, especially by heat stress. Over-expression of *HsfA2* and *HSP17.6A* could improve both heat and osmotic tolerance in *Arabidopsis* [33,34]. But the up-regulation of the expression levels of sHsps under heat treatment might indicate the difference of regulation pattern between heat and other stresses. We conducted detailed analysis of the Hsp100 gene (Os02g32520) which was up-regulated by drought and salt stresses but not by heat and cold stresses. Its expression patterns were confirmed by quantitative RT-PCR. The *Arabidopsis* ortholog of this rice gene was not induced by heat stress in shoots either [10]. This gene (Os02g32520) was also predicted to be ERD1 protein which responded to dehydration at early time in *Arabidopsis* and rice [35,36]. So it indicates that this gene has specific regulation module under abiotic stresses. A rice Hsp90 gene (OsHsp90-1) was greatly induced by high temperature but not by other stresses, which was also confirmed by quantitative RT-PCR. The *Arabidopsis* ortholog Hsp90-1 of this rice gene also showed similar expression pattern [10]. These results demonstrated divergent functions of Hsp and Hsf genes in response to distinct abiotic stresses.

4. Materials and methods

4.1. Plant material and stress treatments

The Zhonghua 11 seedlings (ZH11, *Oryza sativa* subsp. *japonica*) were grown under the condition of 14 h light/10 h dark at 28–30 °C. For heat stress, 2-week-old seedlings were transferred to the temperature-controlled growth chamber and maintained at 42 °C under light. For salt treatment, 2-week-old seedlings were cultured in water supplied with 200 mM NaCl. For drought treatment, roots of seedlings were dried and then placed upon tissue paper in culture cabinet under normal growth conditions. For cold

treatment, seedlings were kept at 4 °C without light. Seedling shoots were harvested at 0, 1, 3, 10 h after each treatment for quantitative PCR analysis, while samples at 0 and 10 h after heat treatment were used to perform microarray hybridization.

4.2. Microarray hybridization and data analysis

The Affymetrix GeneChip Rice Genome Array (Gene Expression Omnibus platform accession no. GPL2025) was employed for microarray hybridization. This array contains 51,279 probe sets that representing two rice cultivars, 48,564 probe sets for *japonica* transcripts and 1260 probe sets for *indica* transcripts. Total RNA of heat stressed sample (42 °C 10 h) and control sample (42 °C 0 h) were isolated using Trizol reagent (Invitrogen). Preparation of labeled target cRNA and subsequent purification and fragmentation were carried out using one-cycle target labeling. Hybridization, washing, and staining were performed as described in the supplier's protocol. All arrays were scanned on GeneChip Scanner 3000 using GeneChip Operating Software Version 1.4. The data sets have been deposited on GEO (accession number GSE14275). The expression data under cold, drought and salt treatments were downloaded from GEO database (accession number GSE6901; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6901). One-week-old rice seedlings (IR64 subspecies) was used in the experiments of GSE6901 datasets, and the growth conditions and stress treatments were similar as ours. For cold treatment, the seedlings were kept at 4 °C for 3 h. For drought treatment, rice seedlings were dried for 3 h at 28 °C using tissue paper. For salt treatment, rice seedlings were transferred to 200 mM NaCl solution for 3 h at 28 °C. And seedlings kept in water at 28 °C for 3 h served as control. Heat treatment (42 °C 10 h) was compared with control (42 °C 0 h) and cold, drought, while cold, drought and salt treatments were compared with the control samples from GSE6901 at GEO. The original CEL data were normalized using RMA. To identify genes that respond to salt, drought, cold and heat, *R* implementation of Limma as a Bioconductor package was used to perform hypothesis tests by fitting a linear model [21,23,37]. A *P* value adjustment was used in false discovery rate control for multiple testing [38]. Significantly responded genes were selected by adjusted *P* values less than 0.05. Genes that responded to at least one treatment were subjected to expression pattern analysis. Cluster analysis of those response genes under different stresses were performed using gplots package.

4.3. Identification and expression analysis of Hsps and Hsfs

Protein sequences of *Arabidopsis* Hsfs and Hsps were used as queries to search against the rice protein database release 5 using BlastP [10,39]. Totally, there were 25, 29, 26, 9 and 10 genes found for OsHsf, OssHsp, OsHsp70, OsHsp90 and OsHsp100 sub-families, respectively.

Distribution of these Hsps and Hsfs was plotted on 12 chromosomes. Supposed transcription start position of these Hsp and Hsf genes were identified by spidey (http://www.ncbi.nlm.nih.gov/spidey/index.html) using full length cDNA. Hsp and Hsf genes that located at chromosome duplication segments were extracted from rice genome annotation website (http://rice.plantbiology.msu.edu/segmental_dup/index.shtml). Tandem positioned Hsf or Hsp genes were determined when there were no other genes between them. Two clusters of sHsp genes were found at rice chromosomes 1 and 3. Phylogenetic analysis was performed using Phylip [24]. Initial alignments were done with ClustalW using rice and *Arabidopsis* Hsps and Hsfs protein sequences.

There were 131 probe sets found for 98 Hsp and Hsf genes, while no probe set for Os11g08470 had been found. Among those probes, 82 probe sets corresponded to single genes, while the remaining probe sets were associated with 17 genes. No single probe set was found to represent more than one gene. For these 17 genes associated with more than one probe set, the mean fold change of these probe sets for each gene was calculated and used in later analysis.

A *T* value was calculated for each heat shock gene family under given stress treatment, and used to represent the median level of response expression [10]. This value reflects the degrees of responses of a Hsp or Hsf gene family under stress. The significance of *T* is evaluated by a resample procedure under the hypothesis that *n* genes in a Hsf or Hsp family are a random sample of the total genes from the rice genome array (51,279). Otherwise, the alternative is that *n* genes are a non-random result that produced a *T* statistic larger than expected by a random sample. The resample procedures are repeated for 10,000 times, each time randomly select *n* from 51,279 genes for a given gene family, and the *T* value is calculated for each of these 10,000 samples. The *T* value of this given family is significant while the proportion of resampling yields a larger or equal *T* statistic less than 0.05. The significant *T* means that the responsive expression of this heat shock gene family is larger than expected by chance. *P* values exceeding 0.0015 in table were not significant by Benjamini–Hochberg adjustment [38].

4.4. Quantitative real-time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen), followed by Dnase I digestion before quantitative PCR. Reverse transcription and quantitative PCR were performed on Applied Biosystems 7500 real-time PCR System using One Step SYBR PrimeScript RT-PCR Kit (TaKaRa) according to manufacturer's instructions. The relative expression of 21 genes in response to heat, cold, drought and salt treatments after 0, 3, and 10 h was measured. The 21 genes and their primers are listed in Supplementary Table 5. Expression measurements were repeated for three times for each control and stress–time treatment samples. Two rice genes used as internal reference genes for calculating relative transcript levels were UBQ5 (AK061988) and eEF-1α (AK061464) [40].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2009.01.016.

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