



Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Heat shock factor gene family in rice: Genomic organization and transcript expression profiling in response to high temperature, low temperature and oxidative stresses

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ARTICLE INFO

Article history:

Received 24 February 2009

Accepted 19 May 2009

Available online xxx

Keywords:

Heat shock factor

High temperature

Low temperature

Oxidative stress

Transcript expression profile

ABSTRACT

Binding of heat shock factors (HSFs) with heat shock element sequence is critical for the transcriptional induction of heat shock genes. Rice genome sequence shows 26 *OsHsf* genes out of which 25 possess various important domains noted in HSFs i.e. DNA binding domain (DBD), oligomerization domain (OD), nuclear localization signal (NLS), nuclear export signal (NES) and AHA type activation domain. *OsHsf* entry LOC_Os06g226100 has the oligomerization domain but lacks the above other domains. Also, there are no ESTs or full-length cDNA noted for this entry in database. Expression profiling showed that 22 *OsHsf* genes are induced by high temperature. Induction of 10 and 14 *OsHsf* genes was also noted against low temperature stress and oxidative stress, respectively. All *OsHsf* genes induced by oxidative stress were also induced by high temperature. On the other hand, induction of 6 and 1 *OsHsf* genes was noted to be exclusive to high and low temperature stresses, respectively. Seven *OsHsf* genes showed induced expression in response to all the three stresses examined. While *in silico* promoter analysis showed that *OsHsf* genes contain upstream regulatory elements corresponding to different abiotic stresses, there was lack of correlation noted between the *in silico* profiling of the elements and their corresponding transcript expression patterns. Apart from stress inducibility, EST database suggests that various *OsHsf* genes are developmentally regulated in diverse tissue types.

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1. Introduction

Heat shock promoters are characterized by conserved palindromic element(s) with consensus motif 'nGAAnnTTCn'. This motif (also known as heat shock element HSE) or its different variants are important for interaction of heat shock factors (HSFs) with HSEs [1]. The binding of the HSFs to HSEs regulates transcription of heat shock genes (*Hsps*) under developmental as well as during stress conditions [2]. While significant variation has been noted in molecular weights of HSFs, these proteins in general have a common core structure comprising of an N-terminal DNA binding domain (DBD) characterized by a central helix-turn-helix (HTH) motif, an adjacent domain with a heptad hydrophobic repeat (HR-A/B) which is involved in oligomerization, short peptide motif essential for nuclear import [nuclear localization signal (NLS)] and export [nuclear export signal (NES)], and a C-terminal AHA type activation domain [3]. Based primarily on the structural features of oligomerization domain, plant HSFs are classified into three

evolutionary-conserved classes namely A, B and C [4]. The AHA type acidic activation domain is exclusively represented by class A members, in contrast C-terminal domains of class B and C are either neutral or basic in nature.

Abiotic stresses (drought, salinity, flooding, temperature, oxidative stress etc.) widely affect crop cultivation and productivity. Abiotic stress response in plants involves perception mechanisms, signal transduction networks and a large array of stress regulated genes. In nature, abiotic stresses occur in a complex way i.e. several different stresses may occur concomitantly or followed one after the other. Field-grown plants adopt diverse strategies to combat abiotic stresses depending on ecology, timing, severity and the stage of crop growth. Notably, transcription factors play a central role in regulation of plant abiotic stress responses. Certain common regulatory signaling components like reactive oxygen species (ROS) are also noted to have a role in stress-related gene activation mechanisms. There are ample evidences showing that stress tolerance of plants is controlled by a complex network of upstream regulatory and downstream target genes.

Extensive interactions have been reported in the molecular pathways between heat and oxidative stress [5]. Generation of ROS is considered to be one of the common denominators linking stress

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conditions like flooding, UV light exposure, wounding, pathogen attack etc [6,7]. Recently, it was postulated that ROS levels are probably sensed by redox responsive transcription factors such as HSF4a in *Arabidopsis*. These so-called sensors are proposed to act upstream in a cascade regulating other transcription factors (like Zat family members, WRKY transcription factor family etc.) and some stress responsive proteins [7]. Detailed work has shown that several HSFs (like HSF1b [8], HSF4a [9] and HSF8 [10]) are thought to be involved in ROS-regulated gene networks during abiotic stress conditions. It is suggested that the production of different types of ROS leads to HSF activation and consequently expression of other regulated genes and such events may represent the molecular link through which cellular response to heat and other forms of stress may be related.

Environmental stresses especially high and low temperatures are detrimental for rice productivity and growth [11,12]. It has been indicated that the production of rice will be severely affected with increases in mean global temperature [12]. The subject of cross-talk or overlap that underlies cellular, physiological, biochemical and molecular responses of plants to different stresses has therefore attracted a great deal of attention. Based on rice genomics studies, a large number of genes and their downstream/upstream target components linked to abiotic stress response have been unveiled.

The involvement of HSFs has been implicated in various abiotic stresses [13–16]. HSFs are thus of considerable interest to agriculturists. However, transcript expression profiling of rice *Oshsf* genes has been poorly examined as of now. We take a detailed look at this important gene family with the complete annotated rice genome sequence (TIGR Rice Annotation release 5). An extended account on expression data on *Oshsf* genes especially with reference to three major abiotic stress conditions (low temperature, high temperature and oxidative stress) is presented in this study.

2. Materials and methods

2.1. Rice database search for HSF genes and their cis-elements

The known sequences of *Arabidopsis thaliana* HSF genes were blasted against RGA (Rice genome Annotation, MSU; <http://rice.plantbiology.msu.edu>) database. In addition, search for the keywords “heat shock factor” or “heat shock transcription factor” was also performed. Various HSF domains were manually predicted using the known domains in *AtHsf*s for all classes. One kbp upstream regions of *Oshsf* genes were identified employing BLAST at NCBI (<http://blast.ncbi.nlm.nih.gov>) and were analyzed at PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) database. For the phylogenetic analysis, predicted protein sequences from the TIGR database were aligned using ClustalX (version 1.83) program. An unrooted neighbor joining (NJ; [17]) phylogenetic tree was constructed in ClustalX with default parameters (for pair wise alignment, the gap penalty was set to 35 and gap extension penalty to 0.75; multiple alignment parameters for making the phylogenetic tree were set to 15.00 for gap opening penalty and 0.3 for the gap extension penalty). The tree thus obtained was viewed using TREEVIEW software. For digital expression analysis of *Oshsf* genes, both full-length cDNAs (FL-cDNAs) and ESTs, were searched against rice HSF genes in TIGR for different tissues namely callus, flower, root, pistil, panicle, seed, leaf and shoot. The percentage frequency (number of ESTs found/total number of ESTs) values were deciphered for the gene family.

2.2. Growth of rice seedlings, stress treatments

Rice [*Oryza sativa* L; cultivar Pusa Basmati (PB1), an indica type] seeds were washed with mild detergent and the detergent was

removed by washing the seeds thoroughly with running tap water. Seeds were subsequently rinsed with 70% ethanol at room temperature for 45 s and washed with sterile distilled water 5–6 times to remove traces of ethanol. The seeds were soaked overnight at RT in dark before placing on cotton bed in a tray for germination. Seedlings were grown at 28 ± 2 °C and 14 h light 10 h^{-1} dark cycle maintained in growth room (light intensity $\sim 250 \mu\text{mol m}^{-2} \text{ s}^{-1}$, humidity $\sim 40\%$) for 10 d. For temperature stress, uniform-sized seedlings were transferred to beakers, which contained distilled water at 42 ± 1 °C for heat stress (HS), at 5 ± 1 °C for cold stress (CS), and 10 mM H_2O_2 at 28 ± 2 °C for oxidative stress, and maintained at the requisite temperatures in BOD (for different time intervals as shown). Subsequent to completion of the stress intervals, tissues were harvested, frozen in liquid nitrogen and kept at -80 °C. The above stress temperatures and conditions were optimized by analyzing expression of several known stress-related genes (considered as marker genes in this work) by RT-PCR. These genes included *Oshsp 26* and *Oshsp 17.3* (for heat stress and oxidative stress; [18,19]); *Oshsp101* (for heat stress; [20]); *OsDREB1a*, *OsDREB2*, *OsCOIN*, *cbf1*, (for cold stress; [21–23]) and *OsAFX8*, *OsSodCc1*, *OsSodCc2* (for oxidative stress; [24,25]). Primers sequences for amplifying the above marker genes are provided in Supplementary Table 1. From this analysis, stress parameters showing clear up-regulation in the levels of the requisite transcripts were worked out. RNA samples from tissues were then processed for microarray and real-time PCR (Q-PCR) analysis.

2.3. Microarray analysis

60-mer rice 44 k oligo DNA array kits (AMADID: No: 015241, Agilent Technologies, USA) which contains 45 018 features/microarray and ~ 40 000 transcripts were used. Total RNA was isolated from ~ 100 mg tissue using Tri-reagent (Sigma, USA) as per the manufacturer's instructions, and further purified using RNA easy mini elute kit (Qiagen, USA). The yield and RNA purity were determined spectrophotometrically. Integrity of the RNA was checked using Agilent Bioanalyzer (Agilent Technologies, USA). 200 ng total RNA was labeled with Cy3 using an Agilent low RNA input fluorescent linear amplification kit (Agilent Technologies, USA). Hybridization and wash processes were performed according to the manufacturer's instructions and hybridized microarrays were scanned using Agilent microarray scanner (G2505B, Agilent Technologies, USA). Feature extraction software (version 9.5.1 Agilent Technologies, USA) was employed for the image analysis and data extraction process. The normalization was done using GeneSpring GX version 7.3.1 (Agilent Technologies, USA) using the recommended per chip and per gene data transformation: set measurements less than 0.01–0.01, per chip: normalize to 50th percentile per gene; normalize to specific samples (treated vs control). Data analysis was done using GeneSpring GX version 7.3.1 (Agilent Technologies, USA) and Microsoft Excel. Three biological replicates were used for the microarray analysis.

2.4. Q-PCR analysis

Total RNA was isolated from ~ 100 mg tissue using Tri-reagent (Sigma, USA) as of above. RNA samples were treated with DNase (RNase-free DNase from Qiagen, USA) and further purified using RNA easy mini elute kit (Qiagen, USA). The quality and quantity of RNA yield was analyzed spectrophotometrically as well as by gel analysis as per the standard protocols [26]. For Q-PCR analysis, primers were designed for all the genes preferentially from 3' end of the gene using PRIMER EXPRESS version 2.0 (PE Applied Biosystems, USA) with default parameters. Each primer was checked using BLAST tool of NCBI database with filter off for its

specificity for respective gene, which was further confirmed by dissociation curve analysis obtained after the PCR reaction (see list of primers used in [Supplementary Table 1](#)). First strand cDNA was synthesized by reverse transcription using 4 µg of total RNA of 10-d-old whole seedlings of rice (after DNase treatment and column purification using RNA easy mini elute kit, Qiagen) in 100 µl of reaction volume using high-capacity cDNA archive kit (Applied Biosystems, USA). Diluted cDNA samples were used for Q-PCR analysis with 200 nM of each primer mixed with SYBR green PCR master mix as per manufacturer's instructions. The reaction was carried out in 96-well optical reaction plates (Applied Biosystems, USA), using ABI Prism 7000 sequence detection system and software (PE Applied Biosystems, USA). To normalize the variance among samples, *actin* was used as an endogenous control. Relative expression values were calculated after normalizing against the maximum expression value. In total, two biological replicates and three technical replicates were used for the Q-PCR analysis.

3. Results

3.1. Rice HSF gene family

With the complete genome sequence (Rice Annotation release 5), we noted 25 clear hit genes in *OsHsf* gene family. Of these, 13 genes belong to class A, 8 genes to class B and the rest 4 to class C. We noted one more entry for *OsHsf* namely LOC_Os06g22610 when searched by keyword "heat shock transcription factor" at RGA database. The DNA binding domain, NLS, NES or AHA motif were found to be lacking in LOC_Os06g22610 in the predicted 190 amino acids long protein. However, domain search did indicate the presence of oligomerization domain in this gene. The domain architecture of different HSF classes is shown schematically in [Fig. 1a](#). Considering this entry also as HSF (though a variant type), we infer that there are overall 26 genes encoding for *OsHsf* family. The characteristic features of all 26 *OsHsf* entries are shown in [Supplementary Table 2](#). Phylogenetic analysis showed that distinct

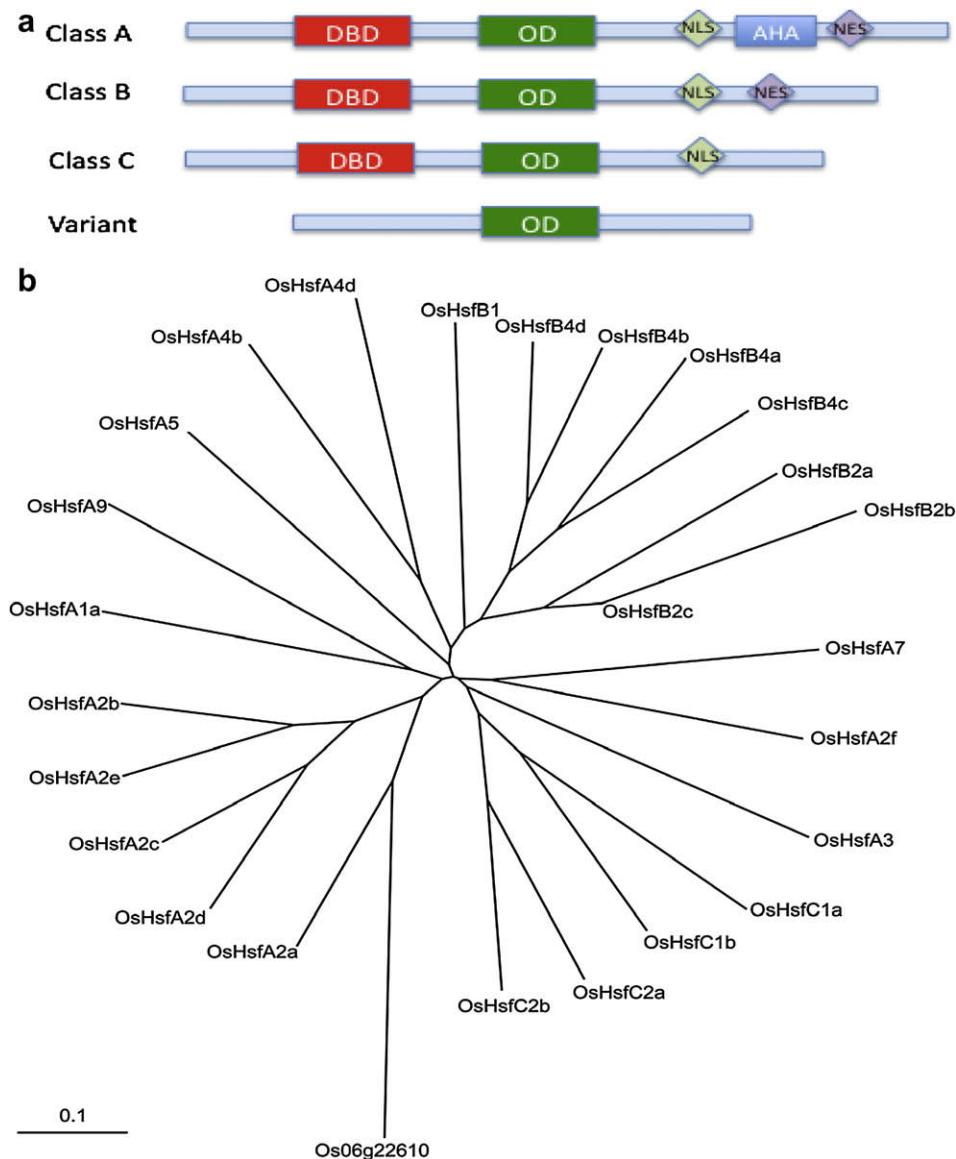


Fig. 1. (a) Schematic representation of the different HSF classes. DNA binding domain (DBD), oligomerization domain (OD), nuclear localization signal (NLS), nuclear export signal (NES) and AHA motifs are shown. Variant represents the *OsHsf* entry LOC_Os06g22610. (b) Phylogenetic tree based on the amino acid sequence of the *OsHsf* genes. The new *OsHsf* entry is also shown. The scale bar depicts 0.1 amino acid substitution per site.

groups of *OsHsfs* are class-wise distributed (Fig. 1b). All class A2 *OsHsfs* (*OsHsfA2a*, *OsHsfA2b*, *OsHsfA2c*, *OsHsfA2d* and *OsHsfA2e*), *OsHsfA1a*, *OsHsfA9* and *Os06g22610* (the new *OsHsf* entry) were grouped in a single major clade. All class B *OsHsfs* showed divergence from a common point and were grouped with *OsHsfA5*, *OsHsfA4d* and *OsHsfA4b*. All class C members showed a common divergence point in the evolutionary lineage and appeared much closer to *OsHsfA3*. *OsHsfA7* and *OsHsfA2f* appeared to have diverged much early from this clade.

Phylogenetic analysis showed that a significant divergence appears to have taken place in class A *OsHsf* members during the course of evolution. All duplicated *OsHsf* gene pairs were seen as sister clade with a distinct dichotomy, suggesting their recent evolutionary divergence.

3.2. Tissue-based expression of *OsHsf* family genes

Further, expression profiling of all the 26 *OsHsfs* was analyzed by the scrutiny of digital expression profile of ESTs (TIGR gene expression evidence), FL-cDNA (KOME database) and database at RiceGE (<http://signal.salk.edu/cgi-bin/RiceGE>) and Geneinvestigator [27] (www.geneinvestigator.ethz.ch). The latter two sources facilitated the analysis of whole genome microarray based expression data for vegetative stage, developmental stages of inflorescence and seed as well as various environmental stresses. Geneinvestigator analysis was carried out employing diverse tissue types. Further, this analysis was done at different developmental stages. Strikingly, four class A members i.e. *OsHsfA1a*, *OsHsfA2e*, *OsHsfA4b* and *OsHsfA5* showed higher transcript levels in nearly all tissue types

(Fig. 2a). This was not the case with other class A members or with class B and class C members, indicating that constitutive expression of different *OsHsfs* is differential in diverse tissues. It was also noted that these four class A members show higher level expression at different developmental stages (Fig. 2b). Another significant observation is that seed tissues (like embryo, endosperm) as well as different developmental stages of seed development (like milk stage, dough stage) was associated with higher level expression of all the three classes of *OsHsfs* (Fig. 2). Unlike seed, expression of class B and class C members of *OsHsfs* was not very significant in different tissues of the panicle except like *OsHsfB4b* showing high expression levels in ovary and *OsHsfC1b* showing higher expression in stigma. *OsHsfC1a* transcript levels were significantly higher at germination. Three B members namely *OsHsfB1*, *OsHsfB2b* and *OsHsfB2a* were higher at milk stage. At dough stage, several members of class A (*OsHsfA1a*, *OsHsfA2e*, *OsHsfA4b*, *OsHsfA5*, *OsHsfA7*), class B (*OsHsfB1*, *OsHsfB2b*, *OsHsfB4a*) and class C (*OsHsfC2a*, *OsHsfC1b*) were noted to be high. For *Os06g22610*, levels of expression remained nearly unaffected by tissue types or developmental stages.

ESTs counts were used for examining the tissue-based expression of *OsHsf* genes (Supplementary Table 3). As this analysis is carried on expression levels under unstressed conditions, it represents constitutive expression of the *OsHsf* genes. *OsHsfA1a*, *OsHsfA2e*, *OsHsfB1* and *OsHsfB4b* transcripts were the major *OsHsf* transcripts noted in seeds. However, it appears that the expression of these transcripts was not seed-exclusive. *OsHsfB1* gene transcripts showed highest level of expression in root and seed tissues. Highest expression in leaf and panicle tissues was for *OsHsfA4d* and *OsHsfA2e* transcripts,

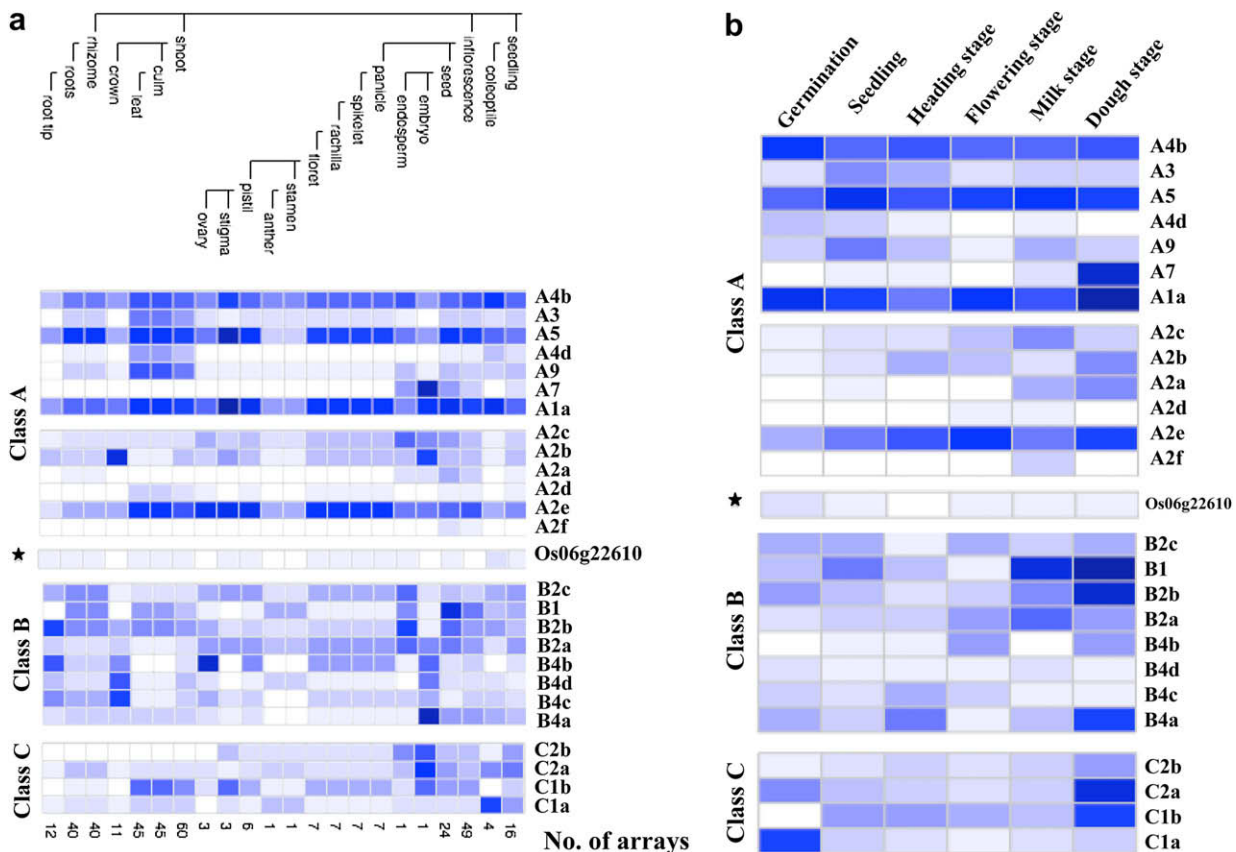


Fig. 2. *In silico* expression analysis of *Hsf* genes in rice. The expression analysis was performed using the Geneinvestigator database. The analysis included data from 166 arrays reported for different tissues and developmental stages in rice.

respectively. In flower highest expression was for *OsHsfA1a*. In pistil, transcripts of *OsHsfA4b*, *OsHsfB4b*, *OsHsfC1b* and *OsHsfC2b* were detected with *OsHsfB4b* showing the highest expression among the four. Notably, *OsHsfC2b* transcript appears to be pistil-specific. *OsHsfA1a* transcripts showed equally high expression in callus, flower, root and shoot tissues. Transcripts for most *OsHsf* genes (except *OsHsfA2a*, *OsHsfA2c*, *OsHsfA7*, *OsHsfB2a*, *OsHsfB4a*, *OsHsfB4c*, *OsHsfB4d*, *OsHsfC1a* and *OsHsfC2b*) were present in shoot. In callus, transcript expression was seen for nearly all *OsHsf* genes except for *OsHsfA2d*, *OsHsfB4a*, *OsHsfC1a* and *OsHsfC2b*.

3.3. Expression profile of *OsHsf* family genes under stress conditions

Microarray profiling of 10-d-old rice seedlings subjected to different stress treatments [heat shock for 10 min and 30 min (HS10min and HS30min); cold shock for 1 h and 5 h (CS1h and CS5h) and oxidative stress (by H₂O₂ application) for 1 h and 4 h

(OS1h and OS4h)] was carried out employing 44 k rice whole genome array (Agilent technologies, USA, unpublished data). Probes for 23 *OsHsf* genes were present on the microarray slide used (those not present on microarray slide included *OsHsfB4d*, *OsHsfB4a* and Os06g22610). Of the 23 genes, 16 *OsHsfs* were up-regulated by ≥ 2 folds (log 2 value) in response to HS. Out of these 16, 8 were found to be up-regulated by ≥ 2 folds only during early heat shock (HS10min); the rest of the 8 genes were up-regulated at both HS10min and HS30min time points (Fig. 3; for detailed log ratio transformed values refer to Supplementary Table 4). *OsHsfC1a* was the only HSF gene noted to be down-regulated by more than 1 fold at HS10min. Based on microarray profiling, *OsHsfA4d* and *OsHsfC2a* transcripts were up-regulated by ≥ 2 folds in all the three stresses (HS, CS and OS) at one or the other time points of the stress treatment. *OsHsfA2a*, *OsHsfA2f*, *OsHsfB2a* and *OsHsfB2b* genes were up-regulated both during HS and OS treatments. *OsHsfA3*, *OsHsfA4d* and *OsHsfA9* genes were up-regulated by ≥ 2 folds during both CS

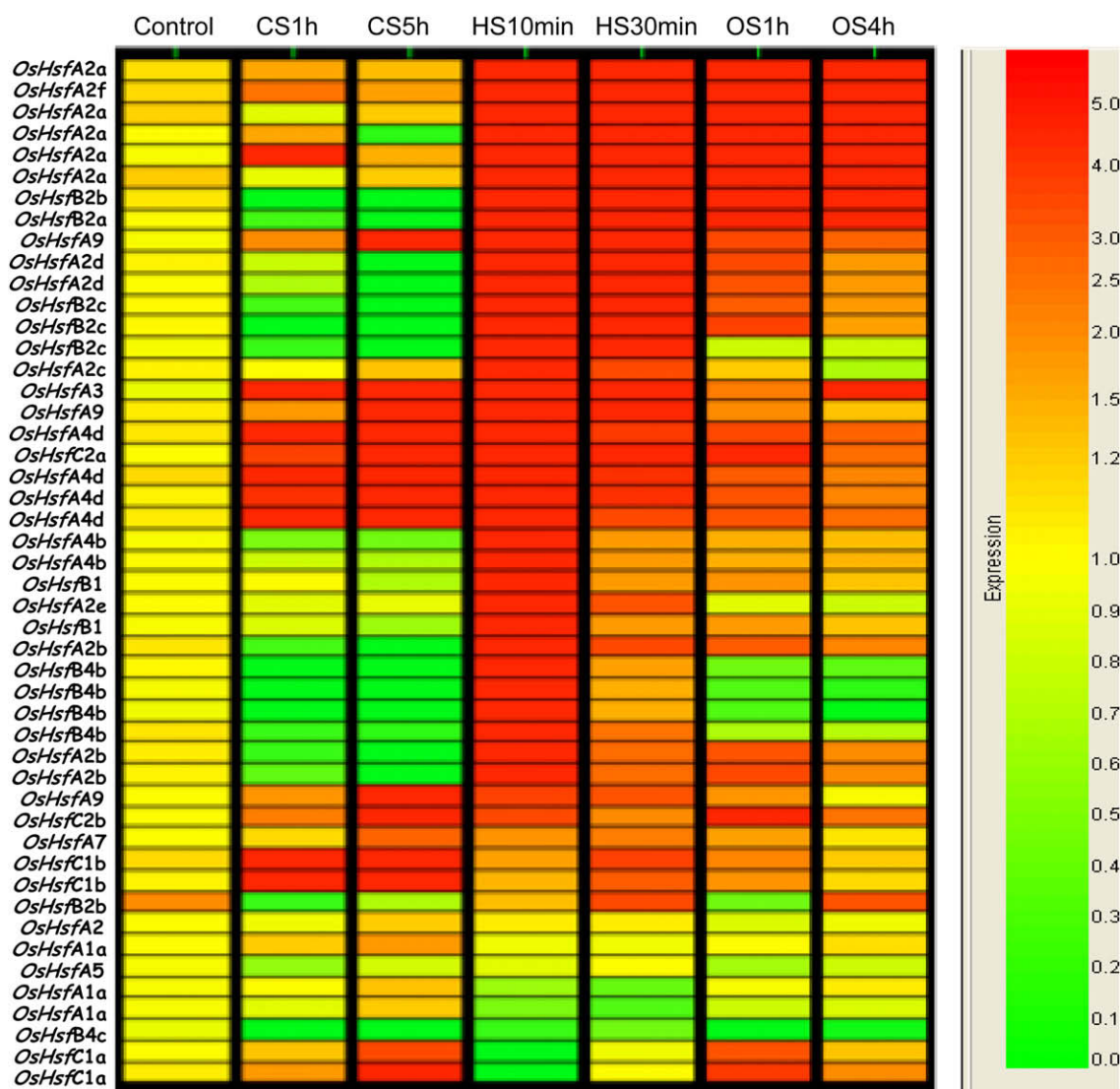


Fig. 3. Expression profile of *OsHsfs*. Hierarchical cluster display of expression profile for twenty-three *OsHsf* genes showing differential expression in rice (color bar represents the log₂ expression values; green color representing low level expression, yellow shows medium level expression and red signifies the high level expression). CS1h: 5 °C, 1 h; CS5h: 5 °C, 5 h; HS10min: 42 °C, 10 min; HS30min: 42 °C, 30 min; OS1h: 10 mM H₂O₂ at 28 °C, 1 h; OS4h: 10 mM H₂O₂ at 28 °C, 4 h. The repeated IDs were kept to signify the reproducibility with different probes on the microarray, which in some cases represent the splice variants/different cDNA clones (see Supplementary Table 4 for details). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Transcript abundance (TA) of *OsHsfs* during different stress treatments (HS10min: heat shock for 10 min, HS30min: heat shock for 30 min, CS1h: cold shock for 1 h, CS5h: cold shock for 5 h, OS1h: oxidative stress for 1 h, OS4h: oxidative stress for 4 h). TA values >2 and <0.5 are considered non-significant change with respect to the control. *refers to the LOC_Os06g22610, **represents no significant expression detected by Q-PCR and NA refers to the case where the probe was not present on the microarray for the gene.

<i>OsHsf</i>	TA during HS10min		TA during HS30min		TA during CS1h		TA during CS5h		TA during OS1h		Ta during OS4h	
	Real time	Microarray ratio	Real time	Microarray ratio	Real time	Microarray ratio	Real time	Microarray ratio	Real time	Microarray ratio	Real time	Microarray ratio
<i>OsHsfA7</i>	1.89	1.77	5.62	2.07	1.15	1.11	3.21	2.40	1.47	1.54	127.68	1.04
<i>OsHsfC1a</i>	0.46	0.37	1.69	1.00	1.67	1.60	3.04	4.05	1.31	3.19	1.93	1.84
<i>OsHsfC1b</i>	2.03	1.54	5.29	2.92	35.42	19.91	80.12	53.56	0.80	1.96	1.95	1.19
<i>OsHsfA4b</i>	4.85	4.60	3.19	1.62	1.13	0.88	1.68	0.82	1.67	1.40	2.39	1.36
<i>OsHsfC2a</i>	4.50	5.67	13.07	7.21	5.36	2.90	14.43	10.70	11.36	11.65	3.82	2.27
<i>OsHsfA5</i>	0.83	1.02	1.75	1.02	1.11	0.96	1.06	1.16	0.67	0.92	1.34	0.95
<i>OsHsfA3</i>	4.37	7.12	9.68	12.89	7.30	7.67	9.83	13.07	0.93	2.02	1.85	3.41
<i>OsHsfA2d</i>	48.08	45.38	7.71	5.53	0.89	0.87	0.52	0.46	1.77	2.81	1.79	1.67
<i>OsHsfA9</i>	2.07	2.93	3.83	2.61	1.93	1.74	3.39	3.74	1.31	1.74	2.13	0.98
<i>OsHsfB4d</i>	1.28	NA	3.01	NA	0.94	NA	0.30	NA	0.75	NA	1.08	NA
<i>OsHsfA2a</i>	12 630.96	15 620.34	3689.16	5973.38	1.02	1.45	1.03	1.27	41.53	77.14	56.12	101.89
<i>OsHsfA2e</i>	5.30	4.57	5.55	2.61	1.57	0.93	1.16	0.95	1.25	0.92	1.62	0.88
<i>OsHsfA1a</i>	2.19	0.96	1.80	0.97	0.71	1.19	0.69	1.66	0.81	0.99	1.96	1.06
<i>OsHsfB2a</i>	46.70	123.69	9.38	16.39	1.03	0.60	0.62	0.52	2.90	9.65	2.50	3.41
<i>OsHsfA4d</i>	3.60	5.94	8.49	3.06	5.92	4.52	5.10	9.08	2.10	2.82	1.88	2.38
<i>OsHsfC2b</i>	3.11	2.84	2.99	1.81	3.88	2.06	3.35	3.44	4.94	8.02	3.05	2.20
<i>OsHsfA2f</i>	6901.60	13 971.25	365.00	7236.05	1.01	2.17	1.41	1.54	3.31	5.66	19.13	44.72
<i>OsHsfA2b</i>	3.30	4.02	3.32	2.76	0.72	0.06	0.42	0.41	1.58	2.57	1.83	1.88
<i>OsHsfB4b</i>	52.25	3.65	42.42	1.54	1.91	0.42	33.07	0.35	30.51	0.71	46.17	0.68
<i>OsHsfB4a</i>	5.52	NA	7.97	NA	2.68	NA	4.50	NA	6.36	NA	5.41	NA
<i>OsHsfB2b</i>	3.79	1.27	4.41	2.88	0.48	0.63	0.67	0.81	0.41	0.70	2.97	2.67
<i>OsHsfB4c</i>	0.44	0.62	1.46	0.71	0.56	0.37	0.33	0.24	0.34	0.56	1.31	0.60
<i>OsHsfB1</i>	3.44	4.52	3.28	1.64	0.04	0.91	0.07	0.78	2.24	1.64	3.12	1.21
<i>OsHsfB2c</i>	13.91	27.19	32.85	10.88	0.78	0.64	0.37	0.44	0.55	2.70	1.06	1.62
<i>OsHsfA2c</i>	255.18	9.12	40.04	2.84	0.68	0.99	0.76	1.21	4.13	1.18	3.31	0.82
<i>OsHsfA2*</i>	**NA											

and HS treatments. *OsHsfC1b* and *OsHsfC2b* genes were up-regulated by ≥ 2 folds during both CS and OS stresses. *OsHsfA2a* showed highest transcript levels both during HS and OS treatments (Fig. 3). During CS treatment, *OsHsfC1b* was noted to be maximally expressed (Fig. 3).

To further validate the microarray observations, Q-PCR analysis was carried out for all the 26 *OsHsf* genes. The transcript abundance (TA) values obtained for various *OsHsf* genes from this analysis are shown in Table 1. Table 1 also reflects that by and large a good correlation existed between the microarray and the Q-PCR profilings with respect to expression patterns of most of the *OsHsf* genes. TA values of *OsHsfA4d*, *OsHsfB4a*, *OsHsfB4b*, *OsHsfC2a* and *OsHsfC2b* transcripts were noted to be up-regulated by ≥ 1 folds under all the three stress conditions as per the Q-PCR data (Fig. 4). *OsHsfA3* and *OsHsfC1b* transcripts were seen to be up-regulated during CS and HS treatments (Fig. 4). *OsHsfA2a*, *OsHsfA2c*, *OsHsfA2d*, *OsHsfA7*, *OsHsfA2f*, *OsHsfB1*, *OsHsfB2a*, and *OsHsfB4c*, genes showed high TA values during HS and OS treatments in Q-PCR (Fig. 4). During HS treatment, transcript levels of *OsHsfA1a*, *OsHsfA2a*, *OsHsfA2c*, *OsHsfA2d*, *OsHsfA2f*, *OsHsfA4b*, *OsHsfB2a* and *OsHsfB4b* genes were higher at HS10min than at HS30min time points. Based on the Q-PCR results, the numbers of up-regulated *OsHsf* genes in individual stresses and in common to two or all the three stresses are diagrammatically depicted in Fig. 5. From this analysis, it is amply clear that all *OsHsf* genes induced by oxidative stress were as well induced by high temperature stress. On the other hand, expression of 6 (*OsHsfA1a*, *OsHsfA2b*, *OsHsfA2d*, *OsHsfA2e*, *OsHsfB2c* and *OsHsfB4d*) and 1 (*OsHsfC1a*) *OsHsf* genes were exclusive to high and low temperature stresses, respectively. Amongst the various *OsHsf* genes, (1) transcript levels corresponding to *OsHsfA5* did not show much change under any of the conditions analyzed, (2) *OsHsfC1a* transcript showed an accumulation in CS5h only and (3) *OsHsfB4c* transcript was repressed at HS10min, CS (both time points) and at OS1h treatments (Table 1). We didn't observe any significant expression in case of Os06g22610 gene in Q-PCR.

3.4. Regulatory elements in rice HSF gene promoters

In silico survey of the putative *cis*-elements in the 1 kbp region upstream of the translation initiation codon of various *OsHsf* genes showed the presence of HSEs in 12 of the 26 *OsHsf* encoding genes (Fig. 6; Supplementary Table 5). ABA responsive elements (ABREs, CGTCA motif, CE3 motif; [28]) were seen in most of the genes (exceptions being *OsHsfA1a*, *OsHsfA5*, *OsHsfA7* and *OsHsfB2b*). LTR, a *cis*-element involved in low temperature response [29], was found to be present only in class A *Hsfs* members (*OsHsfA2a*, *OsHsfA2b*, *OsHsfA2c*, *OsHsfA2d*, *OsHsfA3*, *OsHsfA4d* and in the entry Os06g22610). Of these, only *OsHsfA3* and *OsHsfA4d* showed induction during cold stress. Desiccation responsive element (DRE) was noted only in case of *OsHsfB4c* gene. Anoxia responsive element (ARE) and Myb binding site were also observed in most of the *OsHsf* genes.

4. Discussion

Based on the draft genome sequence of rice, Baniwal et al. [30] proposed existence of 23 HSF encoding genes in rice genome. Guo et al. [31] have recently reported that the number of genes encoding rice HSF family is 25. With the finished genome sequence (Rice Annotation release 5), we confirm that 25 genes as reported by Guo et al. [31] constitute *OsHsf* gene family. Our analyses further shows that entries made by Guo et al. [31] as LOC_Os08g43340 and LOC_Os09g28200 need to be corrected as LOC_Os08g43334 and LOC_Os09g28354, respectively. We found an additional entry for HSF namely LOC_Os06g22610 when searched by keyword 'heat shock transcription factor' at RGA database. LOC_Os06g22610 gene was noted to contain oligomerization domain but lacked DNA binding domain. Expression analysis by Q-PCR suggested that LOC_Os06g22610 gene did not respond to the stress conditions. We also could not find full-length cDNA or ESTs for the LOC_Os06g22610 gene. The functionality of this gene therefore

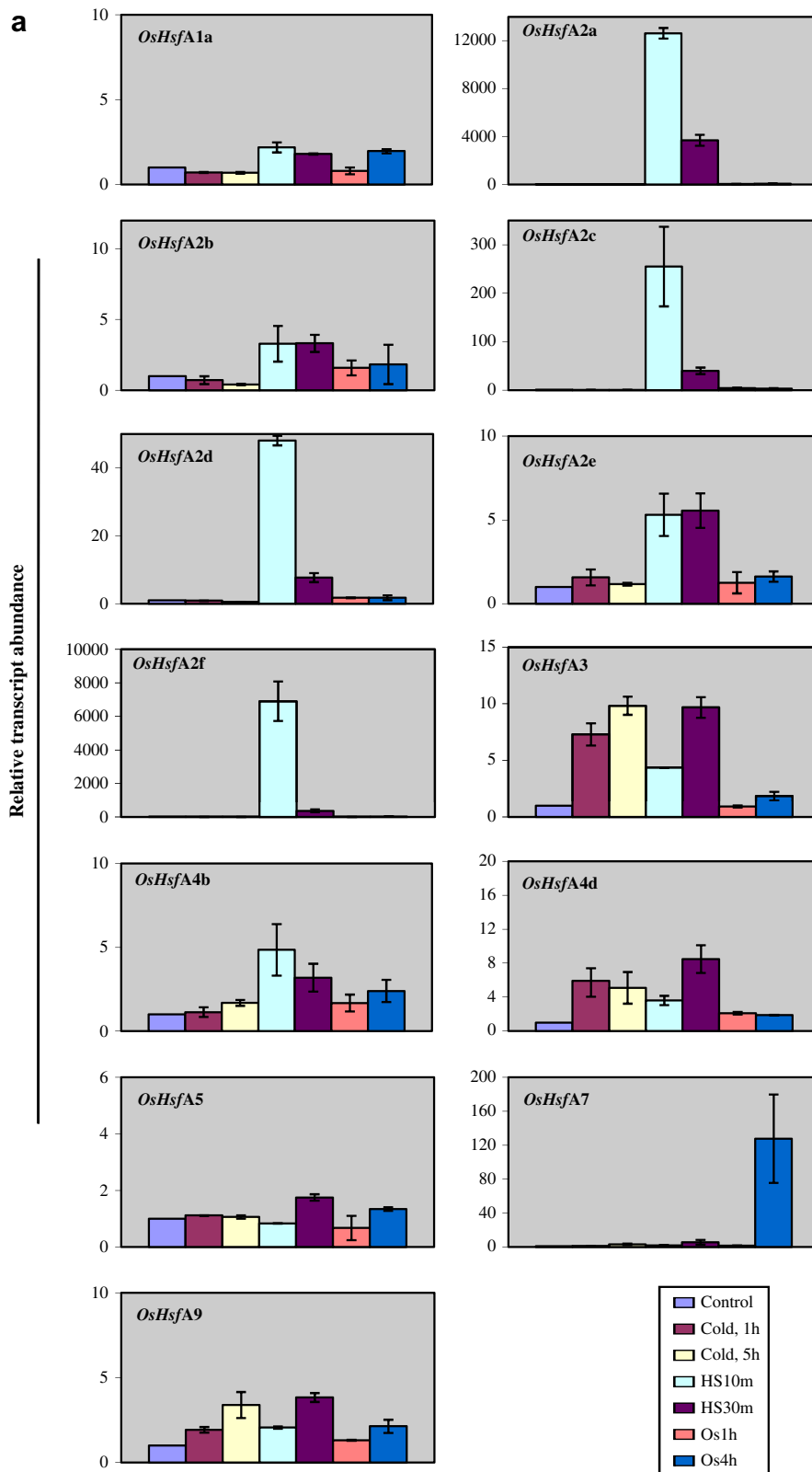


Fig. 4. Relative transcript abundance of various *OsHsfs* in different stress conditions. a. Class A *OsHsfs*, b. Class B *OsHsfs*, c. Class C *OsHsfs*. Relative transcript abundance values represent the expression values obtained after normalizing against maximum expression value. For exact values see Table 1.

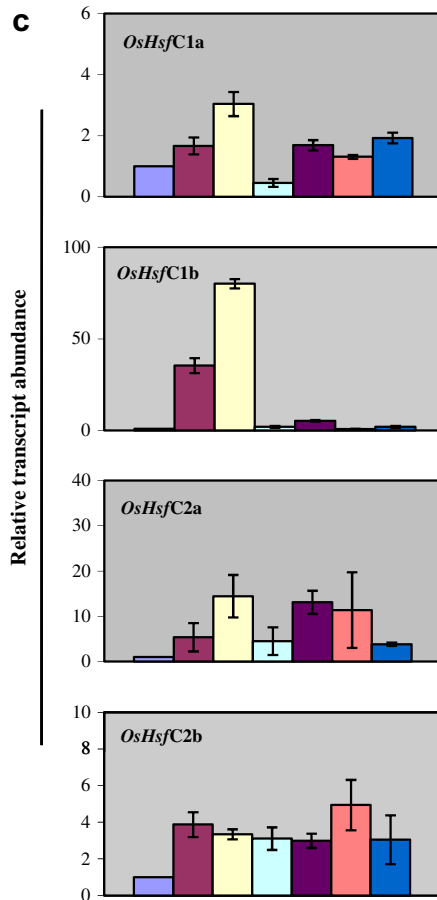
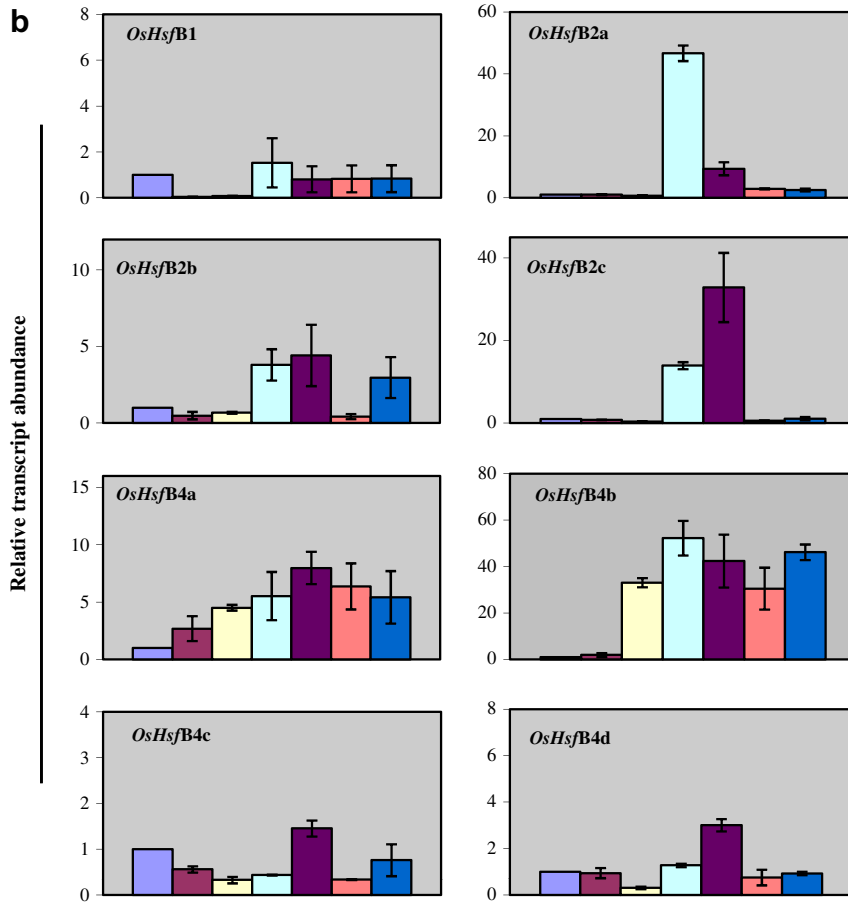


Fig. 4. (continued).

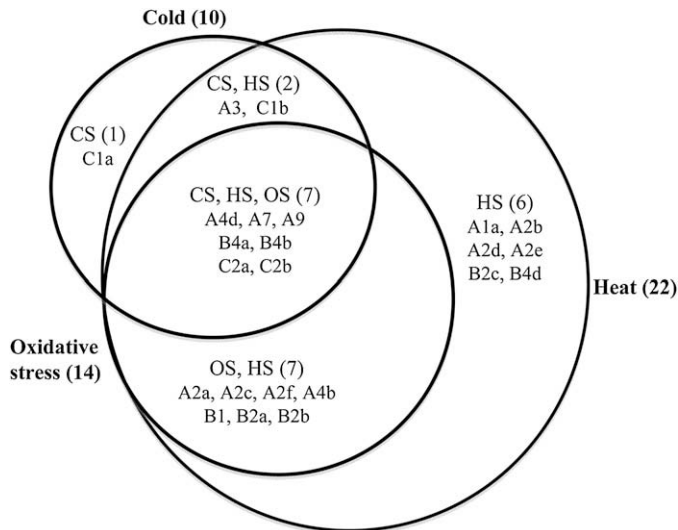


Fig. 5. Venn diagram showing differentially up-regulated *OsHsf* genes in heat stress (HS10min/HS30min), oxidative stress (OS1h/OS4h) and cold stress (CS1h/CS5h). The genes, which show more than 1 fold change with respect to the control, were considered differentially expressed in this analysis. Numbers in the parentheses represent the total number of *OsHsfs*, which are denoted by their class nomenclature as well.

remains an enigma: the incomplete structure of this HSF gene may represent a non-functional *OsHsf* gene. Alternatively there is a possibility that LOC_Os06g22610 gene is transcribed and translated and may have a regulatory role in a way that its interaction with other *OsHsfs* due to the oligomerization domain, may lead to activation or repression of the HS response. This aspect merits further detailed analysis.

In *Arabidopsis*, *Hsfs* are noted to be differentially expressed in tissue and stage specific manner [32]. Based on the EST counts in various tissues (under unstressed condition), *OsHsfs* genes are also noted to be expressed to variable extents in tissue specific manner (Supplementary Table 3). In *Arabidopsis* and sunflower, *HsfA9* is exclusively expressed during seed development [33,34]. *OsHsfA9* (rice homologue of *Arabidopsis* and sunflower *HsfA9*) was not expressed during seed development. In this study, we didn't note any *OsHsf* which was expressed only in a particular tissue type but selected *OsHsf* genes showed higher levels of expression in specified tissue types. *OsHsfB1* in root and seed tissues, *OsHsfA4d* in leaf, *OsHsfA2e* in panicle, *OsHsfA1a* in flower and *OsHsfB4b* in pistil represent the noteworthy cases in this regard. The constitutively expressed *OsHsfs* may have a role in housekeeping function or in regulating the expression of stress induced HSFs as proposed for *HsfA1a/A1b* in *Arabidopsis* and *HsfA1a* in tomato [35,36].

In *Arabidopsis*, expression of *Hsfs* is noted to be strongly induced by heat, cold, salt and osmotic stress. The inducibility pattern of *Hsf* genes in response to varied abiotic stresses has not been analyzed in other plant species. Expression profiling analysis carried out in this study reflected that *OsHsfs* also respond to varied abiotic stresses. Microarray and Q-PCR results showed that *OsHsfs* (except *OsHsfA5*, *OsHsfB4c*, *OsHsfC1a* and LOC_Os06g22610) were induced by heat shock to variable extents. Q-PCR showed that *OsHsfC1a* was initially slightly down-regulated in the heat shock response. It is possible that the *Hsf* genes which remain unaltered or down-regulated in expression may lie downstream in the hierarchy of the events involved in heat shock response or are repressed by other members of the family or by other interacting and regulating components of the pathway. This argument warrants further analysis. From the limited nature of this study, it is not possible for us to comment on which *OsHsf* is the primary trigger of the heat

shock response in rice. Based on the early as well as higher inducibility during heat shock, we however assume that, *OsHsfA2a*, *OsHsfA2c*, *OsHsfA2d*, *OsHsfA2f* and *OsHsfB4b* may be regarded as the sensors of the heat shock response in rice. Cold stress resulted in down-regulation of all class B *OsHsfs* except for *OsHsfB4a* and *OsHsfB4b* genes. *OsHsfB4b* showed high expression levels after 5 h of cold treatment. Among the class A members, *OsHsfA3*, *OsHsfA4d* and *OsHsfA9* showed up-regulation in the transcript levels after CS. In contrast, all class C *OsHsfs* showed inducible expression in response to low temperature with *OsHsfC1b* showing the strongest expression. Importantly, this may suggest that class C HSF members play important role in cold stress response. *OsHsfC1b* appears to be a primary sensor of the extreme low temperature in case of rice. Oxidative stress caused enhanced transcript expression to variable extents of selected *OsHsfs*. Based on this study, *OsHsfA2a*, *OsHsfB4b* and *OsHsfC2a* appear to be the primary players in the pathways that involve ROS accumulation and sensing. Our data is in concurrence with Miller et al. [7] who proposed that HSFs can be the sensors of the changes in the ROS levels. Among all the *OsHsfs*, *OsHsfA2a* showed the strongest expression at both the time points of the oxidative stress. We also propose that *OsHsfA2f* and *OsHsfA7* may be involved in the late response to the oxidative stress. It is noteworthy that *OsHsfA2a* showed highest transcript induction in both HS and OS. *OsHsfA5* appeared to be the least responsive to the three stress conditions. *OsHsf* genes showing co-induction (such as *OsHsfA2a* and *OsHsfA2f* in HS and OS and *OsHsfB4b* and *OsHsfC2a* in all the three stress conditions) may represent pivotal points in possible cross-talk among the stress responsive pathways.

In silico survey of the putative *cis*-elements reflected that 12 *OsHsfs* have HSEs in the 1 kbp upstream region. This indicates that the expression of HSE-containing these 12 *OsHsf* genes might be regulated by HSFs themselves, via formation of a regulatory network as proposed by Nover et al. [4]. Notably, all *OsHsf* genes containing HSEs in their upstream region were inducible by heat shock treatment. *OsHsfA2a*, which has maximum number of HSEs (i.e. 3) showed the highest expression during heat shock. However, it is also to be noted that 1 kbp upstream region of certain *OsHsf* genes (for instance *OsHsfA2c*) lacked any distinct HSE but still showed heat shock inducibility. Further, class C members, which were noted to be clearly responsive to cold stress and other genes for instance *OsHsfA9*, *OsHsfB4a* and *OsHsfB4b* which showed CS inducibility, didn't contain distinct LTRs in their 1 kbp upstream region. Even though *OsHsfA2a*, *OsHsfA2b*, *OsHsfA2c*, *OsHsfA2d* possess LTRs in their upstream region, these genes remained uninduced under CS. It thus accrues that for more detailed understanding of the upstream regulators of HSFs, it is important that the conclusions drawn from the *in silico* data are reconfirmed experimentally.

The co-expression profiling of *Hsfs* in response to varied abiotic stresses as noted by Swindell et al. [32] in *Arabidopsis* and in this study on rice indicates that these transcription factors might be regulating multiple mechanisms. In yeast, binding of HSF to a multitude of promoters (including genes which do not encode the heat shock proteins) is shown [37]. There is no data on which all non-HSP genes are regulated by the plant HSFs. Genome-wide chromatin immunoprecipitation (ChIP) microarray analysis for deciphering *in vivo* targets could uncover entirely novel gene clusters, pathways and functions of HSFs in future studies. Plants encounter a combination of different stresses under field conditions, and it is of prime importance to generate crop varieties that are tolerant to a range of stress conditions. The potential of HSFs in stress responses can be tapped for the generation of abiotic stress tolerant transgenics [38]. Recently, it has been shown that *OsHsfA2e* gene confers thermotolerance in transgenic *Arabidopsis* plants [39]. Possible involvement of other

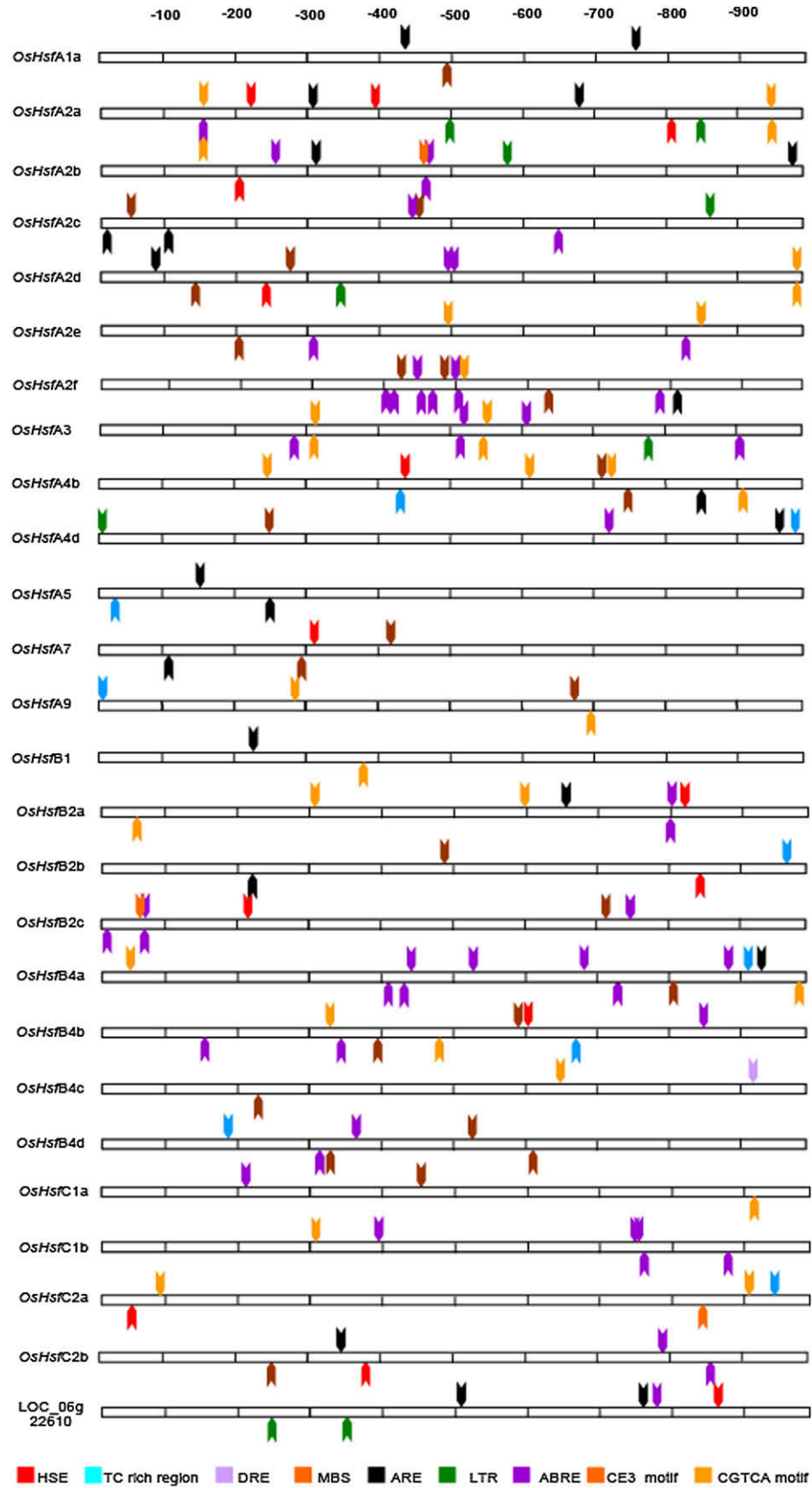


Fig. 6. cis-elements present in the 1 kb region upstream of translation start site in rice Hsf genes. The analysis was performed using PlantCARE database. The arrows mark the relative position of the different elements.

Oshsf genes in modulating high temperature tolerance in transgenic plants remains to be analyzed.

Acknowledgements

AG is thankful to research funding obtained through CPMB and Indo-Finland projects from Department of Biotechnology, Government of India. DM and AS acknowledge the fellowship awards from CSIR, India. We acknowledge the technical support from Genotypic Pvt. Ltd., India for the microarray services. We thank Prof. A. K. Tyagi for the use of Q-PCR facility.

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plaphy.2009.05.003.

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