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ORIGINAL PAPER

Functional analysis of Hsp70 superfamily proteins of rice (*Oryza sativa*)

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Abstract Heat stress results in misfolding and aggregation of cellular proteins. Heat shock proteins (Hsp) enable the cells to maintain proper folding of proteins, both in unstressed as well as stressed conditions. *Hsp70* genes encode for a group of highly conserved chaperone proteins across the living systems encompassing bacteria, plants, and animals. In the cellular chaperone network, Hsp70 family proteins interconnect other chaperones and play a dominant role in various cell processes. To assess the functionality of rice *Hsp70* genes, rice genome database was analyzed. Rice genome contains 32 *Hsp70* genes. Rice Hsp70 superfamily genes are represented by 24 Hsp70 family and 8 Hsp110 family members. Promoter and transcript expression analysis divulges that Hsp70 superfamily genes plays important role in heat stress. Ssc1 (mitochondrial Hsp70 protein in yeast) deleted yeast show compromised growth at 37 °C. Three mitochondrial rice Hsp70 sequences (i.e., mtHsp70-1, mtHsp70-2, and mtHsp70-3) complemented the Ssc1 mutation of yeast to differential extents. The information presented in this study provides detailed understanding of the Hsp70 protein family of rice, the crop species that is the major food for the world population.

Keywords Hsp70 superfamily · Hsp70 · Hsp110/SSE1 · Rice · Transcript expression · Yeast complementation

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Introduction

Temperature is a highly significant environmental factor in the growth and development of plants. All organisms respond to heat stress by synthesizing heat shock proteins (Hsps). Hsp70 family of proteins is present constitutively and upregulated in response to various stressors like heat, cold, anoxia, and metal (Li et al. 1999; Sung et al. 2001). Constitutively present Hsp70 proteins perform housekeeping functions of a cell and are called the "heat shock cognates (Hsc)." Hsp70 family proteins are involved in proper folding of nascent synthesized proteins, prevention of protein aggregation, translocation of proteins across membranes, and targeting proteins towards degradation; thus maintaining protein homeostasis (proteostasis) (Hartl et al. 2011). Under conditions of stress, Hsp70 maintain protein quality by preventing aggregation of stress-damaged proteins.

Hsp70 are ATP-dependent chaperones having a conserved ~44-kD N-terminal ATPase domain (also called nucleotide binding domain; NBD), a ~18-kD substrate binding domain (SBD) and a ~10-kD variable C-terminal "lid." The flexible C-terminal lid assists in holding the substrates at SBD. ATPase domain performs regulatory role and SBD associates to hydrophobic regions exposed in non-native substrates (Dragovic et al. 2006). The hydrophobic region of the client proteins binds transiently to the SBD and is regulated by NBD. Hsp70 proteins require two co-chaperones in the form of J-domain proteins and nucleotide exchange factors (NEFs) for their functions. Binding of J-domain proteins stimulates ATP hydrolysis that facilitates trapping of exposed hydrophobic region of substrates and closed conformation of SBD. NEFs participate in ATP–ADP exchange by catalyzing the release of ADP from

Hsp70, leading to conversion of open conformation Hsp70 and substrate release. Proteins of families like GrpE in *Escherichia coli*, Mge1 in yeast mitochondria, Hsp binding protein (Hsp-BP), or Fes1 and Hsp110 are reported to act as NEFs (Zhang et al. 2010; Liberek et al. 1991; Miao et al. 1997; Kabani et al. 2002). Hsp110 family proteins have high sequence and structural homology to Hsp70 and are therefore included in Hsp70 superfamily. Hsp110 have an N-terminal NBD similar to Hsp70 and C-terminal SBD. They are larger than Hsp70 in size due to either inserted acidic region in SBD or C-terminal extension (Liu and Hendrickson 2007).

Hsp70 is a multigenic family. Various members constituting this family are present in different cellular compartments. In *Saccharomyces cerevisiae*, 14 *Hsp70* genes representing nine cytosolic, three mitochondrial (Ssc, Ecm, and Ssq), and two ER (Kar2 and Lhs1) isoforms have been identified (Walsh et al. 2004). In *E. coli*, there are three *Hsp70* genes. *Arabidopsis* has 18 Hsp70 superfamily members, of which 14 belong to Hsp70 family and 4 belong to Hsp110/SSE family (Lin et al. 2001). In spinach, at least 12 *Hsp70* genes are identified (Guy and Li 1998). The C terminus of organellar Hsp70 proteins is highly conserved and unique for each organelle. The motif for the cytosolic group is EEVD, for the mitochondrion is PEAEYEEAKK and for the plastid is PEGDVIDADFTDSK (Guy and Li 1998). The C terminus EEVD motif of cytosolic Hsp70 proteins interacts with co-chaperones that contain several degenerate 34 amino acid repeats, called tetratricopeptide repeats.

Hsp70 proteins perform diverse biological functions either with their co-chaperones or in collaboration with other chaperones. Whereas DnaK system efficiently solubilized and refolded small protein aggregates (Diamant et al. 2000), large protein aggregates are solubilized in concert with Hsp100/ClpB (Diamant et al. 2000; Acebron et al. 2009). Expression of cytosolic *Hsp70* genes is linked to acquired thermotolerance because of their property to reactivate the protein aggregates formed under various stress conditions (Lee and Schoffl 1996; Yang et al. 2009; Qi et al. 2011; Zhou et al. 2012; Sung and Guy 2003). It is noted that Hsp70 may function as a negative feedback regulator of HSF activity (Shi et al. 1998; Kim and Schoffl 2002). In this process, Hsp70 binds to denatured proteins and releases HSF leading to its activation during heat stress (Shi et al. 1998). Hsp70 proteins in plants are involved in additional specific functions. Chloroplast Hsp70 (cpHsp70) in *Arabidopsis* are implicated in chloroplast development (Latijnhouwers et al. 2010). Mutants of cpHsp70 develop variegated cotyledons, malformed leaves, growth retardation and impaired root growth in addition to being heat sensitive (Latijnhouwers et al. 2010). Hsp70 shows complex developmental regulation during the vegetative and reproductive phases of growth. In tomato, Hsc70 transcripts were detected in mature anthers (Duck and Folk 1994). Several plant cytosolic *Hsp70* genes are expressed during seed development, maturation, and/or germination (DeRocher and Vierling 1995; Sung et al. 2001). While levels of Hsp70 proteins are noted to be abundant in dry seeds, their levels drastically decline within 72 h after the onset of imbibitions (Sung et al. 2001).

Plant Hsp70s are also responsive to varied environmental signals. Several members of the Hsp70 family in spinach are regulated by a light/dark signal independent of circadian rhythm (Li and Guy 2001). Hsp70 members are involved in post-translational translocation of proteins across membranes in case of mitochondria and chloroplasts (Su and Li 2010). In rice, ER Hsp70 (BiP) is found to associate with nascent polypeptides that emerge into the ER lumen during prolamin translocation and facilitate peptide folding and assembly (Muench et al. 1997). Over-expression of BiP relieves "ER stress" (Leborgne-Castel et al. 1999).

We analyzed Hsp70 family of rice to understand the functional network of chaperones in this important food species. Rice mitochondrial Hsp70 has been proposed as suppressor of heat and H_2O_2-induced programmed cell death (Qi et al. 2011). We identified 32 *Hsp70* genes in rice genome. Transcript expression analysis revealed that various Hsp70 are regulated under different stress conditions and the genes are expressed constitutively or regulated developmentally. Yeast mitochondrial Hsp70 mutant transformed with rice mitochondrial Hsp70 proteins suggested an evolutionary conserved mechanism of action of these proteins. This study provides the functional relevance of Hsp70 component of the Hsp70/J proteins bichaperone machine of rice.

Materials and methods

Identification of Hsp70 proteins in rice genome

For Hsp70 retrieval in rice genome annotation project (RGAP; http://rice.plantbiology.msu.edu/), putative function search using "Hsp70" as query returned no results. Replacing Hsp70 with "DnaK" showed 31 entries in rice genome. Using protein sequence of one of the genes (i.e., Os01g62290) as query in blast search resulted in 34 genes with maximum *E* value of 9.6e−39. The protein sequences of these genes subjected to domain search in SMART (http://smart.embl-heidelberg.de/smart) showed Hsp70 domain in 32 proteins. Same 32 genes were recovered following blast search and subsequent SMART analysis when database was queried with protein sequence of *Arabidopsis* Hsp70, At1g16030. The exon–intron organization of *Hsp70* genes was analyzed in Spidey (www.ncbi.nlm.nih.gov/spidey/) taking genomic sequence from RGAP and cDNA sequences from KOME (http://cdna01.dna.affrc.go.jp/cDNA/) and from

RGAP if the full-length (FL) cDNA was not present in KOME. Multiple sequence alignment of amino acid sequences of Hsp70 proteins was performed using the ClustalX 2.0 (Larkin et al. 2007) with default parameters. The NJ tree with 1,000 bootstrap was constructed in ClustalX 2.0 and viewed using Treeview1.6.6. Subcellular localization of proteins was analyzed at WoLFPSORT, Predotar, PSORT, Softberry, and TargetP database. Subcellular compartment was decided based on consensus localization in two algorithms and Hsp70-specific C-terminal sequence.

To find segmental duplication events, genome duplications of rice in MSU database were identified with a maximum permitted distance between collinear gene pairs of 100 kB (http://rice.plantbiology.msu.edu/segmental_dup/index.shtml).

Motif analysis

In order to find conserved motifs in rice *Hsp70* gene family members, "Multiple EM for Motif Elicitation" (MEME) version 3.5.4 (Bailey et al. 2006) was used. The parameters used for the analysis were: number of repetitions—any; maximum number of motifs—12 and optimum width of motif—≥2 and ≤300.

Isolation of RNA, RT-PCR analysis, and cloning of FL-cDNA

Total RNA was isolated from stressed and non-stressed (control) 3-week-old rice seedlings (*Oryza sativa* L; cultivar Pusa basmati 1 (PB1)) using TRI reagent (Sigma, USA) as per the manufacturer's instructions. Concentration of RNA was quantified spectrophotometrically and quality was analyzed by gel electrophoresis. For cDNA synthesis, 5 μg of total RNA of each sample was reverse transcribed using reverse transcriptase (RevertAid H Minus Reverse transcriptase, MBI, Fermentas). RT-PCR amplification was performed for *Hsp70* genes using gene-specific primers listed in Supplementary Table 1.

For yeast mutant complementation, *Hsp70* genes were amplified using FL-cDNA clones procured from Rice Genome Resource Centre, Japan (primers used are listed in Supplementary Table 1). PCR was carried out using Phusion™ Hi-Fi DNA polymerase in presence of 1 % DMSO in a 50-μl reaction. The amplified products after digestion with requisite enzymes (Supplementary Table 1) were cloned in p426 vector (Mumberg et al. 1995) under the control of glyceraldehydes-3-phosphate dehydrogenase (GPD) promoter and transformed in mutant yeast cells. Empty vector was also transformed in yeast cells as vector control. The phenotype of the yeast was scored by incubating the plates spotted with tenfold serial dilutions of yeast cells. Prior to spotting the OD_{600} of yeast cells was normalized to 0.2.

Results

Hsp70 superfamily has Hsp70 and Hsp110 proteins

In rice genome, 32 sequences showed Hsp70 domain. Except for chromosomes 4, 7, and 10, *Hsp70* genes were scattered over all chromosomes in rice genome (Table 1). Analysis of phylogenetic tree generated from aligned amino acid sequences of rice Hsp70 proteins showed four well-supported lineages (Fig. 1). While *Hsp70* genes clustered on clades A, B, and C, clade D contained Hsp110 family genes. Hsp110 family is a subfamily of Hsp70 superfamily, structurally very similar to Hsp70 having N-terminal ATPase domain and C-terminal peptide binding domain and functions as NEFs for Hsp70 family proteins. Analysis of C-terminal Hsp70 sequences and in silico localization ascertained that the clades A and B genes are cytoplasmic and ER, respectively (Table 1 and Supplementary Table 2). Mitochondrial and chloroplastic *Hsp70* genes cluster on clade C. Transmembrane domain (TMD) and ER retention signal HDEL were noticed in ER sequences of clade B except in *BiP6* gene that appears diverged from other members of clade B. Six of 13 genes in clade A are classical cytoplasmic (Hsp70 having characteristic C-terminal EEVD sequence) while two lineages (uHsp70-1 and uHsp70-2) appeared evolutionary distant (Fig. 1). On clade A, four genes present on chromosome 11 are arranged in tandem (Supplementary Fig. 1) suggesting localized gene duplication event. In addition, three pairs of genes are located on segmental duplicated regions of rice chromosomes (Fig. 1). Two of the segmental duplicates are Hsp70 (cHsp70-1 and cHsp70-6 and cHsp70-7 and uHsp70-2) and one pair (Hsp110-1 and Hsp110-4) is Hsp110 family. Except for one pair (cHsp70-7 and uHsp70-2), the duplicated genes have close phylogenetic relationship (Fig. 1). Motif analysis showed that C terminus of Hsp110 family is more diverged from Hsp70 family while the ATP binding domain has high motif similarity (Fig. 2). Closely related Hsp70 proteins in the phylogenetic tree have similar motif composition (Fig. 2). All the diverged genes in their respective clades differed in the domain structure, mass or intron–exon arrangement (Fig. 1; Table 1). Examination of the intron–exon organization showed that nine genes in clade A contain one intron, three genes have two introns while one gene is intron-less. The position of the intron is conserved in all one intron containing clade A genes except for cHsp70-11. Intron numbers are highly variable in clade B genes. Among the six genes in this clade, three genes are intron-less while other three genes contain two, five, and seven introns. The number and position of introns in two chloroplast *Hsp70* genes in clade C is highly conserved. However, out of three mitochondrial *Hsp70* genes in clade C, one gene has four introns and two genes have five introns each. Hsp110 family clade D was bifurcated into two subclades: subclade D2 with four genes having multiple

Table 1 Hsp70 domain containing genes of rice

Gene ID	Protein name	Localization	Amino acids	Molecular weight (kD)	FL-cDNA
Hsp70 family					
Os01g62290	cHsp70-1	Nuc/cytoplasm	648	71	AK243277
Os03g16860	cHsp70-2	Nuc/cytoplasm	650	71	AK072830, AK099275
Os03g16880	cHsp70-3	Nuc/cytoplasm	560	61	AK106371
Os03g16920	cHsp70-4	Nuc/cytoplasm	653	72	AK287481
Os03g60620	cHsp70-5	Nuc/cytoplasm	649	71	AK069740, AK102784
Os05g38530	cHsp70-6	Nuc/cytoplasm	646	71	AK243004
Os11g47760	cHsp70-7	Nuc/cytoplasm	649	71	AK065431
Os11g08440	cHsp70-8	Nuc/cytoplasm	577	63	AK063977, AK103083
Os11g08445	cHsp70-9	Nuc/cytoplasm	658	71	N/A
Os11g08460	cHsp70-10	Nuc/cytoplasm	562	62	AK106272
Os11g08470	cHsp70-11	Nuc/cytoplasm	467	61	N/A
Os12g38180	uHsp70-2	unpredicted	215	24	AK099797
Os02g02410	BiP1	ER	665	73	AK119653
Os03g50250	BiP2	ER	669	73	N/A
Os05g30480	BiP3	ER	669	72	N/A
Os05g35400	BiP4	ER	687	74	AK106696
Os08g09770	BiP5	ER	676	74	N/A
Os01g33360	BiP6	ER	608	66	N/A
Os02g53420	mtHsp70-1	Mitochondria	670	73	AK065228
Os03g02260	mtHsp70-2	Mitochondria	676	73	AK103835
Os09g31486	mtHsp70-3	Mitochondria	684	73	AK069787
Os05g23740	cpHsp70-1	Chloroplast	689	74	AK060410
Os12g14070	cpHsp70-2	Chloroplast	698	74	AK121949
Os01g49430	uHsp70-1	unpredicted	539	58	N/A
Hsp110/SSE family					
Os01g08560	Hsp110-1	Cytoplasm	845	93	AK100676
Os02g48110	Hsp110-2	ER	902	99	AK100997
Os03g11910	Hsp110-3	Cytoplasm	578	62	AK102685
Os05g08840	Hsp110-4	Cytoplasm	853	94	AK122040
Os05g51360	Hsp110-5	ER	437	47	AK120015
Os06g10990	Hsp110-6	Cytoplasm	470	50	AK071518, AK104048
Os06g46600	Hsp110-7	Cytoplasm	753	83	N/A
Os12g05760	Hsp110-8	ER	461	48	N/A

introns and molecular weight matched with Hsp110 family genes. In D2, one gene (Hsp110-2) is ER protein having HDEL sequence and a TMD and other three genes are nuclear/cytoplasmic. D1 clade differed from D2 clade in having lesser or no introns and lower molecular weight (47–62 kD) proteins because of the absence of C-terminal extension. Three proteins in this clade have TMD and ER localization whereas one protein (Hsp110-3) is nuclear/cytoplasmic. Orthologs of Hsp70 superfamily genes with features different from the conserved C-terminal sequence were searched in other sequenced genomes and a tree was generated with the retrieved protein sequences (Supplementary Fig. 2). The tree indicated that genes with similar characteristics are present in other genomes as well with the exception of uHsp70-1. Most of the eukaryotic genomes contain multiple members of Hsp70 family existing in same sub-cellular compartment. Consistent with this, Hsp70 family of rice putatively encode for 11 cytosolic, 5 ER luminal, 3 mitochondrial, and 2 chloroplast proteins. The cytoplasmic Hsp70 clade is much complex in rice. Five genes in this group of rice are smaller in size and lack the conserved C-terminal EEVD sequence, a situation similar to yeast. In yeast, four cytosolic *Hsp70* genes (Ssa) with EEVD and three genes (two Ssb and one Ssz1) without EEVD sequence have distinct non-overlapping functions. Yeast *Ssb* genes are associated with ribosomes and function in translation (Nelson et al. 1992). However, it is

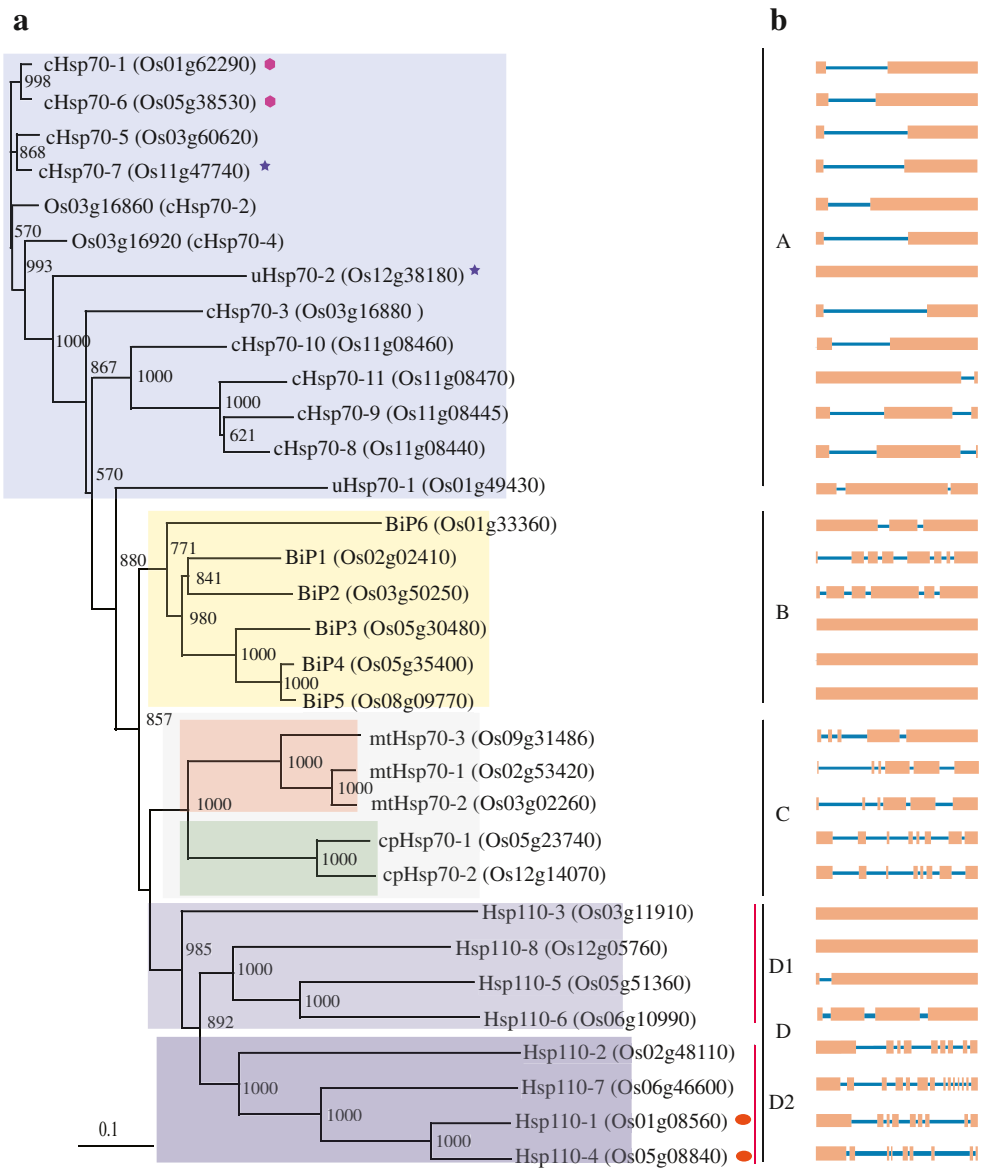


Fig. 1 Phylogenetic analysis of Hsp70 superfamily of rice. **a** Amino acid sequences of rice Hsp70 superfamily genes aligned in clustalX (2.0) were used for generating NJ tree with bootstrap. Phylogenetic tree was visualized in Treeview 1.6.6. The numbers for the interior nodes indicate the bootstrap values for 1,000 replications. The scale at the bottom is the number of amino acid substitutions per site. Segmental duplicated pairs are marked with similar color symbols. **b** Exon–intron arrangement of Hsp70 superfamily genes. The exons and introns are represented by box and lines, respectively. The order of genes in (**b**) match with the order shown in (**a**)

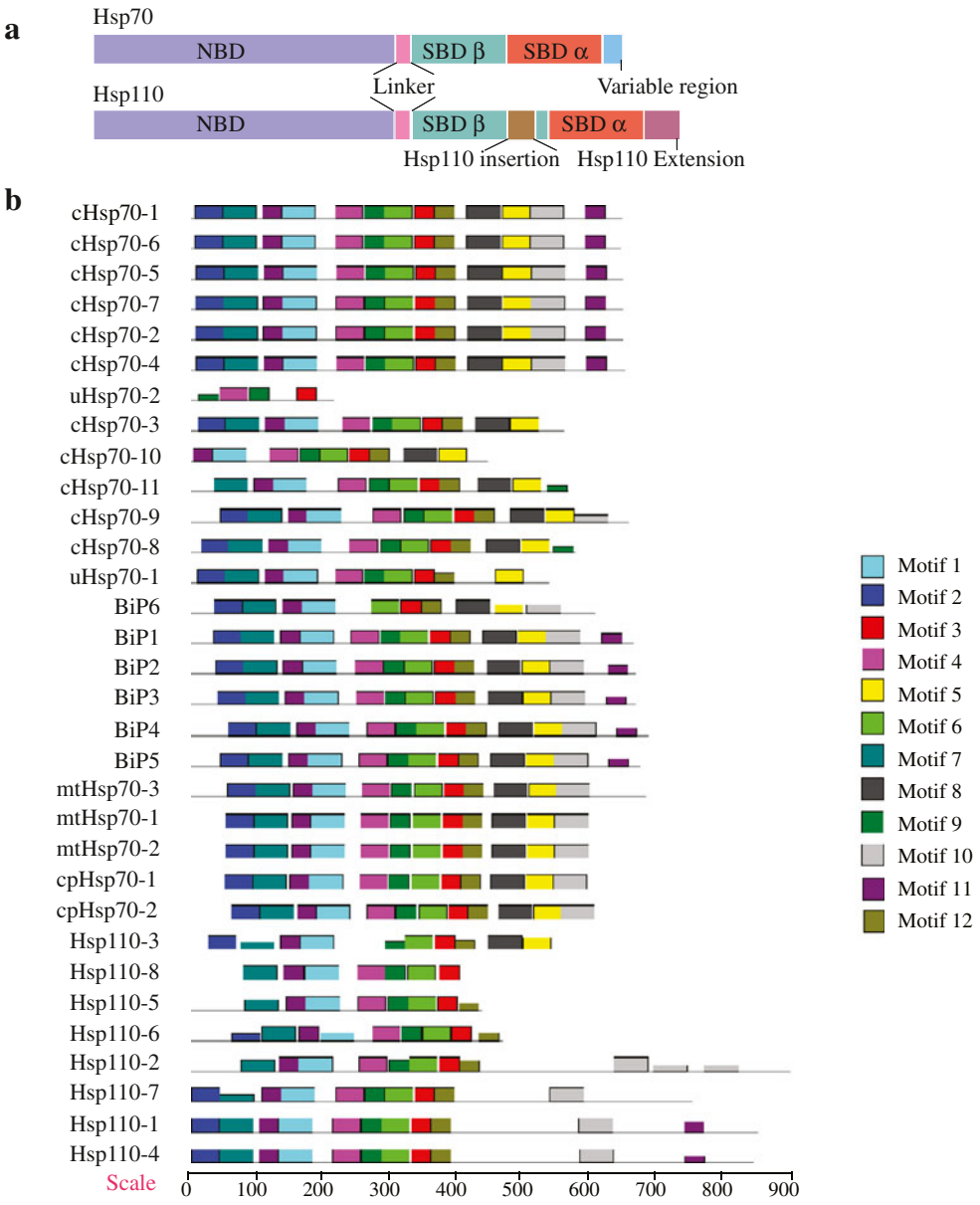
not possible to predict the functions of rice *Hsp70* genes that are different from canonical cytosolic *Hsp70* genes on the basis of this analysis only.

Analysis of regulatory *cis*-elements in the promoters of Hsp70 superfamily genes

2 kB promoter upstream of ATG of *Hsp70* genes was analysed to find the *cis*-regulatory elements that may possibly govern their expression. Six *Hsp70* genes (cHsp70-11, BiP6, uHsp70-1, BiP2, BiP3, and BiP5) were not found in the database at Osiris (Morris et al. 2008). Promoters of five genes (cHsp70-1, cHsp70-2, cHsp70-4, cHsp70-7, and mtHsp70-1) were enriched in heat shock elements (HSEs) of perfect type (nTTCnnGAAnnTTCn, *P* value of $<10^{-4}$). Gap-type HSEs were detected in two genes (cHsp70-6 and cHsp70-10) and step-type HSEs were detected in cHsp70-2 (Table 2). Promoters of eight *Hsp70* genes were manually found to have a variant form of perfect HSEs in which a mismatch in the basic module is permitted (Table 2). Other *cis*-elements with higher frequency of their occurrence in Hsp70 promoters were motifI, SiteII, DRE, and ABRE. SiteII motifs have been implicated in the expression of genes in meristematic tissue and/or proliferating cells (Welchen and Gonzalez 2006), dehydration-responsive element/C-repeat (DRE/CRT) in drought, high light and cold stress responsive genes (Dubouzet et al. 2003), and ABRE and motifI in abiotic stress/ABA response genes (Mundy et al. 1990).

Hsp70 genes show constitutive as well as stress induced expression

Transcript expression profiles of *Hsp70* genes were assessed by semi-quantitative RT-PCR. In addition, heat stress



c

Motifs	width (aa)	Sequences
Motif 1	50	YLG[KE][TPK][VI][TK][NDK]AV[VI]TVP[AV]YFND[AS]QRQATKDA[GA][VAR]IAGLNV[ML]RIINEPTAAA[LI]AY
Motif 2	41	P[VA][IV]GIDLGTT[YN]SCV[AG]V[WYM][RQE][HGN][GD]R[VP]E[IV][IV][AT]ND[QE]GNR[TI]TPS[YWV]VAFT
Motif 3	29	[VI][DH][ED]V[VL]LVGGSTRIPKVQQ[LV][LV][QSK]D[FY]F[DGN][GK]K[EP]L
Motif 4	41	I[DES][NE]G[VI]FEV[KL]AT[AN]GDTHLGGEDFDNR[LM]V[DNKE]H[FL][VA]QE[FI]KRKH[GK][KIR]D
Motif 5	41	GIPPAPRGVPQIEV[TC]F[DE][IV]DANG[IV]L[NS]VSAEDK[TGA]TG[KQR][KS][NQ]KITI
Motif 6	41	SLx[DGE]G[VKI]DF[SY]ET[LI][TS]R[AS][RK]FEELNMDLF[ER][KR][CT]MEPV[EK]K[CA][LM][AR]DA[KG][LM]
Motif 7	50	[DG][GT]ERL[IV]G[DE]AAK[NR]Q[AV][AV]MNP[ET][NR]T[VI]FDAKRLIGR[RK]FSD[PA][SE]VQ[RS][DE][MI]K
		[LM][WLV]P[FY]KV[VI]
Motif 8	50	[MF][TS][KV][LV]IPRNTT[IV]PTK[KR][ST]QV[FY][TS]T[YA]ADNQTTVSI[KN]V[FL][EQ]GE[RS][SEA]M[TA][KR]
		DN[KRN]LLG[EKR]F
Motif 9	29	[ND][AP][RK]AL[RG][RK]LRT[AE][CA]E[RK]AKR[TA]LSS[TQ]AQTT[IV][EN][ILV]
Motif 10	50	[NG]DKGRLS[KQ]EEI[ED][KR][MA]V[QER]EAE[KE][FY][AK][AQ][EK]D[ERK][ER][HR][KR][EK][KT][VI][DE]A
		[KR]N[AS][LA][ED][NSAT]Y[VAI]Y[NS]M[REK][NK][TK][LIV][KG]
Motif 11	29	[VE][QV]Y[EK]G[ED]E[KR]QFS[PA]EE[IV]S[AS]MV[LI][AS]K[ML][KR]ETAEA
Motif 12	29	[KR][SGT][VI]NPDEAVAYGAA[VI]Q[AG][ASG]ILSG[DE][GV][DNK][ED]K[VT][LQ]

Fig. 2 Analysis of conserved motifs present in Hsp70 family proteins. **a** Domain architecture of Hsp70 and Hsp110 proteins. **b** Analysis of conserved motifs present in Hsp70 family proteins. Motifs were identified by MEME software using complete amino acid sequences of Hsp70 proteins. The protein sequences are arranged in the order as shown in tree in Fig. 1. Color code of the motifs is depicted on right side. **c** Multilevel consensus sequences of MEME-derived motifs. Single letters in the motif match that letter; groups of letters in square brackets match any of the letters in the group

Table 2 Enriched regulatory *cis*-elements in the promoters of Hsp70 superfamily genes

Regulatory *cis*-elements	Number of promoters	Predicted sites in promoters	*p* value	Genes
HSE-perfect type	5	5	10^{-4}	cHsp70-1, cHsp70-2, cHsp70-4, cHsp70-7, and mtHsp70-1
Nonenriched TFBS				
ABREmotif	12	18	0.0136	cHsp70-1, cHsp70-8, cHsp70-10, BiP-1, BiP-4, mtHsp70-1, cpHsp70-1, cpHsp70-2, Hsp110-1, Hsp110-2, Hsp110-5, and Hsp110-8
DRECRTCOREAT	14	19	0.034	cHsp70-1, cHsp70-2, cHsp70-3, cHsp70-4, cHsp70-5, cHsp70-6, cHsp70-7, cHsp70-8, cHsp70-10, BiP-4, mtHsp70-3, cpHsp70-2, uHsp70-2, and Hsp110-5
HSE gaptype	2	2	0.0205	cHsp70-6 and cHsp70-10
HSE steptype	1	1	0.485	cHsp70-2
SITEIIATCYTC	21	64	0.0069	cHsp70-1 cHsp70-2 cHsp70-3, cHsp70-4, cHsp70-5, cHsp70-6, cHsp70-7, cHsp70-8, cHsp70-10, BiP-1, BiP-4, mtHsp70-1, mtHsp70-2, mtHsp70-3, cpHsp70-2, uHsp70-1, Hsp110-1, Hsp110-3, Hsp110-4, Hsp110-5, and Hsp110-8
MotifI	3	3	0.0175	cHsp70-5, Hsp110-6, and Hsp110-7
Imperfect HSE				cHsp70-3, cHsp70-5, cHsp70-6, cHsp70-10, BiP-2, BiP-3, BiP-5, mtHsp70-3, and cpHsp70-1

p values represent statistically over-represented TF binding sites in selected set of promoters

HSE-perfect type nTTCnnGAAnnTTCn, *HSE-gaptype* nTTCnnGAAnnnnnnnTTCn, *HSE steptype* nTTCnnnnnnnTTCnnnnnnnTTCn, *ABRE-motif* TGACGT, *DRE/CRT motif* RCCGAC, *SiteII element* TGGGCY, *MotifI* RTACGTGGR

microarray data of rice (Sarkar et al. 2009) and public domain microarray data (genevestigator: www.genevestigator.com/ and rice oligonucleotide array database: www.ricearray.org/) were analyzed for stress induced expression and reproductive stage expression. From RT-PCR, it appears that ten *Hsp70* and four *Hsp110* genes (Hsp110-2, Hsp110-7, Hsp110-1, and Os05g8840) are expressed constitutively (Fig. 3). These genes can therefore be considered as Hsc70. The transcript expression of 12 genes was upregulated upon HS and among these, expression of two *Hsp70* and one *Hsp110* genes (Hsp110-3) was strictly induced by HS. Expression of two mitochondrial *Hsp70* genes (mtHsp70-2 and mtHsp70-3) was upregulated by ABA. Upregulation of both these genes was higher in heat stress in comparison to the *mtHsp70-1* gene. In response to anoxia stress, up-regulated expression of four *Hsp70* genes was detected. *cHsp70-4* and *cHsp70-6* genes were responsive to most of the stresses analyzed. Upregulation of two genes (cHsp70-6 and cHsp70-4) was noticed in CS in microarray profiles. Expression of ten *Hsp70* genes was not affected under stress and development (Fig. 3; Supplementary Fig. 3). Among the Hsp110 members, *Hsp110-5*, *Hsp110-6*, and *Hsp110-8* genes showed negligible expression in the microarray. However, proof of their transcript expression is supported by FL-cDNA for *Hsp110-6* (in flower and shoot library) and *Hsp110-5* genes (unknown library).

During developmental stages, *cHsp70-7* gene was highly expressed throughout the life cycle of rice plant with varying expression levels (Supplementary Fig. 3). *cHsp70-4* gene was exclusively expressed during seed development stages with maximum expression in mature seed (Supplementary Fig. 3). The expression level of this gene was reduced sharply in germinating seeds. In case of chloroplast localized *Hsp70* genes, both the genes were expressed in seed development stages. However, expression level of *cpHsp70-2* gene was higher than cpHsp70-1. Expression of mitochondrial *Hsp70* genes was also differential during development: transcript expression signals for mtHsp70-2 and mtHsp70-1 were higher in seed development stages as compared with mtHsp70-3. On the other hand, expression of *mtHsp70-1* gene was higher than mtHsp70-2 and mtHsp70-3 during panicle development stages. *Hsp110-1*, *Hsp110-2*, *Hsp110-4*, and *Hsp110-7* genes showed varying intensity of expression in all stages of development. In contrast, expression of *Hsp110-3* gene was restricted to seed development stages.

Rice Hsp70: J-protein bichaperone machine complement corresponding yeast mutants

To assess functionality of *Hsp70* genes of rice, yeast mutant ssc1–3 defective in *mtHsp70* gene was employed. Mutation in *mtHsp70* gene renders yeast cells heat sensitive and cells exhibit growth defect at 37 °C (Gambill et al. 1993). Three putative mitochondrial *Hsp70* genes of rice namely mtHsp70-1, mtHsp70-2, and mtHsp70-3, were expressed in ssc1–3 yeast cells under the control of GPD promoter and growth phenotype was scored at 37 °C. ssc1-3 cells expressing these mitochondrial genes showed partial protection against HS as against ssc1–3 mutant cells (Fig. 4). Expression of mtHsp70-3

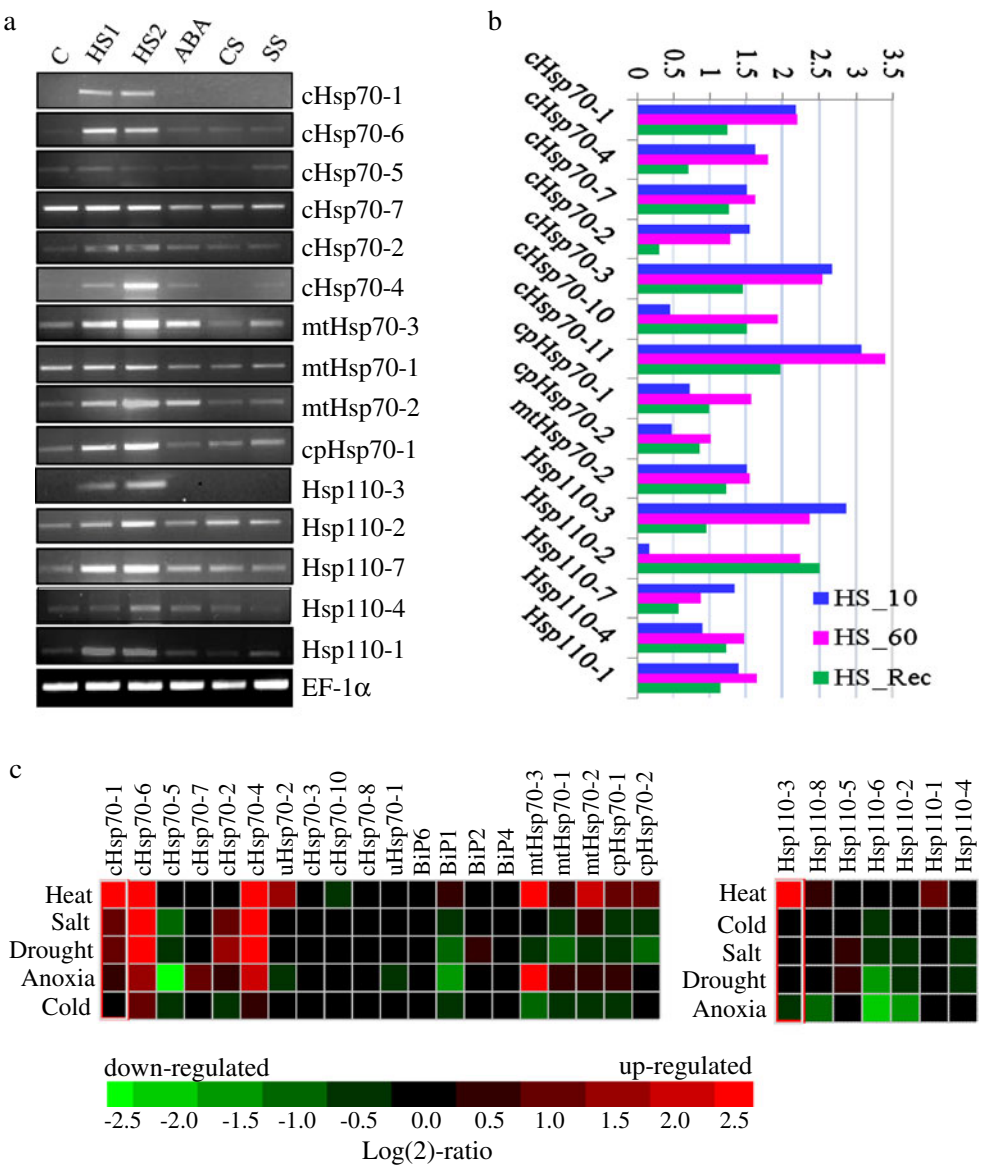


Fig. 3 Expression analysis of *Hsp70* genes of rice. **a** Semi-quantitative RT-PCR of selective *Hsp70* genes under stress conditions. Rice seedlings were given stress under different conditions as mentioned: *HS1* heat shock at 42 °C for 10 min, *HS2* heat shock at 42 °C for 1 h, *ABA* 100 μM abscisic acid for 3 h, *CS* cold stress at 6 °C for 6 h, *SS* salt stress in 150 mM NaCl for 6 h, *C* control maintained at 26 °C. **b** Microarray based expression profiles during heat stress and recovery conditions as described (Sarkar et al. 2009). *HS_10* heat stress at 42 °C for 10 min, *HS_60* heat stress at 42 °C for 1 h, *HS_Rec* 30 min recovery after heat stress at 42 °C for 1 h. **c** Heat map showing in silico expression data retrieved from Genevestigator (www.genevestigator.com/)

was least effective in complementing the of growth defect of ssc1–3 mutant cells (Fig. 4). From this analysis, it emerges that selective rice mitochondrial *Hsp70* genes can function in yeast mitochondrial chaperone system. The Ssc1 protein of yeast is the core component of mitochondrial import motor that facilitates translocation of polypeptides across mitochondrial membrane in ATP-dependent reaction in partnership with 2 J-domain co-chaperones. It is proposed that rice Hsp70 can work in conjunction with yeast Hsp40 to protect yeast cells under heat stress.

Discussion

The objective of this study was identification and functional characterization of Hsp70 genes in rice genome. This was considered important because Hsp70 proteins are associated with numerous cellular roles under unstressed as well as stressed conditions. Hsp70 proteins occupy central position in the cellular chaperone network interacting with chaperones of other families. We identified that 32 Hsp70 domain containing genes in rice genome constitute Hsp70 superfamily. This includes 24 *Hsp70* and 8 *Hsp110* genes. As against this, only 14 *Hsp70* and 4 *Hsp110* genes are reported in *Arabidopsis* (Lin et al. 2001). While there are 5 cytosolic *Hsp70* genes in *Arabidopsis*, the number of cytosolic genes is 11 in rice. Rice has six canonical (having EEVD at C terminus) and five nonclassical cytosolic *Hsp70* genes. The nonclassical *Hsp70* genes are not reported in *Arabidopsis*, indicating that this expansion in Hsp70 family may have occurred after the divergence of monocot–dicot lineage. It is known that segmental and tandem duplication has played a role in the evolution and expansion of gene families in plants (Cannon et al. 2004). The nonclassical rice genes

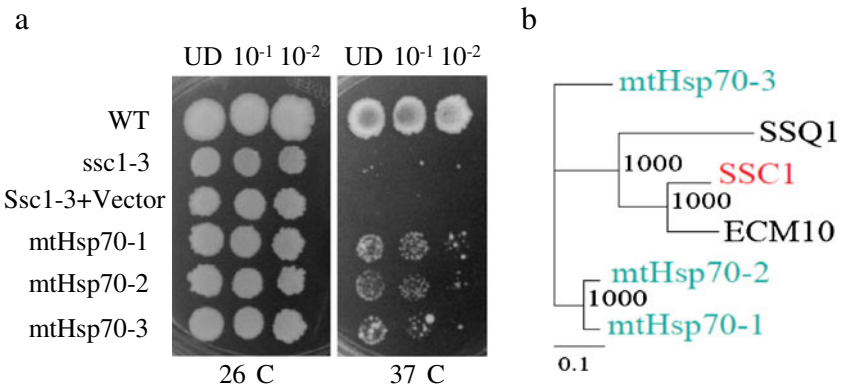


Fig. 4 Expression of rice mitochondrial Hsp70 proteins partially complement yeast Hsp70 mutant. **a** The mitochondrial *Hsp70* genes of rice were cloned under the GPD promoter in p426 vector and transformed in yeast mutant cells (ssc1-3/PK83: MATα ssc1-3 ade2 lys2 ura3 trp1) having mutation in mitochondrial *Hsp70* gene (*Ssc1*). The cells were grown to log phase, growth was normalized to 0.2 OD, and tenfold serial dilutions of wild type, yeast mutant cells transformed with vector and transformed with rice Hsp70 were spotted on selection medium and plates were incubated at 26 and 37 °C. UD is normalized OD_{600} 0.2. Tree depicts rice and yeast mitochondrial *Hsp70* genes. **b** Subset of the phylogenetic tree showing relationship amongst rice and yeast proteins analyzed in this assay

are tandem duplicates. These genes are found in sorghum genome as well, suggesting a monocot-specific clade. However, a definite interpretation in this regard can be only drawn after larger number of genomes are sequenced and analyzed. This is the first comprehensive study of rice Hsp70 superfamily, though isolated studies on mitochondrial Hsp70 and BiPs of Hsp70 family of rice are published (Qi et al. 2011; Oono et al. 2010). Multiple Hsp70 members were identified in various cellular compartments in rice in our analysis. Overall, 11 putative nucleo/cytosolic, 3 mitochondrial, 6 ER, and 2 chloroplastic Hsp70 proteins were identified. Localization of two proteins remained ambiguous due to multiple prediction sites in different localization tools used herein. Eleven Hsp70 proteins predicted to be localized to cytosol or nucleus by different tools were considered as nucleo/cytosolic considering the reports that cytosolic Hsp proteins relocate to nucleus in response to heat stress (Kose et al. 2012). Under heat stress, nuclear localized Hsp70 prevented fragmentation of DNA and provided thermotolarance (Cho and Choi 2009). Qi et al. (2011) reported two mtHsp70 proteins and Oono et al. (2010) reported ten BiPs proteins in rice. In this study, we found that these ten BiPs consist of six Hsp70 and four Hsp110 proteins.

Hsp110 family genes are considered essential for the functioning of *Hsp70* genes. This study indicates that rice Hsp110 family contains eight proteins. Four proteins reportedly form Hsp110 family in *Arabidopsis* (Lin et al. 2001) Yeast Hsp110 family consists of three proteins. Both these species contain one ER localized Hsp110 protein and remainder are cytosolic Hsp110 proteins. In *Arabidopsis*, Hsp110 members Hsp70-14 and Hsp70-15 represent highly homologous proteins localized predominantly to cytosol. AtHsp70-15 has been implicated in stomatal closure response in unstressed as well as heat stress conditions (Jungkunz et al. 2011). No study has been published on Hsp110 family of rice so far.

Transcript of Hsp70 superfamily genes are expressed constitutively and during development and the expression is enhanced significantly under various stress conditions, suggesting that *Hsp70* genes have critical role(s) during growth and stress. The stress induced expression profile of Hsp70 superfamily genes showed diverse response. This diversity in their expression mosaic may be attributed to the regulatory *cis*-elements present in the promoter region of these genes. Maximum number of *Hsp70* genes were upregulated by heat stress. In accordance, HSE elements were enriched in the promoters of the Hsp70 family genes. Consequently, *Hsp70* genes have been implicated in development of thermotolerance. Over-expression of mtHsp70 of rice protected rice suspension culture cells from heat stress induced programmed cell death (Qi et al. 2011). *Cis*-elements like ABRE, DRE, and SiteII coincided with the expression of *Hsp70* genes in other stresses and with tissue specific expression.

This study indicates that multiple Hsp70 proteins are present in each of the cellular compartments. It appears that the Hsp70 proteins of the same cellular compartment may not be functionally redundant as the protein isoforms present in specific cellular compartments are noted to be differentially expressed. For instance, of the six classical cytosolic *Hsp70* genes, only cHsp70-7 transcript was expressed at all the developmental and anatomical stages. The transcript of this gene was constitutively high and not affected much by heat stress. On the other hand, it also is the case that functional redundancy has been observed in most gene families in spite of expressional diversification (Kafri et al. 2006). In *Arabidopsis* chloroplast localized two Hsp70 proteins show differential transcript expression. However, only

one of these (i.e., cpHsp70-1) is shown to be essential (Su and Li 2010). Hsp70 proteins of mitochondria, chloroplast and ER function as motors driving the translocation of proteins across membranes into the organelles (Su and Li 2010). Rice ER contains six Hsp70 proteins (BiP1-BiP6), and these are functionally diverse under normal growth conditions. Among the six BiP isoforms, BiP1 appears to be highly expressed constitutively in all tissues and is heat induced as well. In contrast, other BiPs are not expressed constitutively (Wakasa et al. 2012). BiPs are upregulated in response to accumulation of misfolded proteins in ER, in unfolded protein response (UPR) pathway. BiP4 and BiP5 were specifically upregulated upon DTT treatment, a UPR inducer. It is further noted that knockdown of BiP1 leads to induction of BiP2, BiP3, BiP4, and BiP5 (Wakasa et al. 2012), suggesting distinct functional diversity. We observed that BiP1 was highly expressed in seed development stages in agreement with earlier report (Wakasa et al. 2011), indicating its role in protein quality control in seed maturation. In concord, both over-expression and knockdown of BiP1 resulted in ER stress and compromised seed quality (Yasuda et al. 2009; Wakasa et al. 2011). Overall, it will be worth analyzing as to how many genes are dispensable in Hsp70 family in rice. The mutants of all the *Hsp70* genes are not available in genetic mutant resources of rice. Arabidopsis genetic resources are more extensively available but *Arabidopsis* mutants always cannot be used in such analysis especially for genes that have monocot-specific lineages.

The functionality of a subset of *Hsp70* genes of rice was analyzed in this study by expressing three Hsp70 mitochondrial proteins in ssc1–3 mutant cells of yeast. Of the three mitochondrial *Hsp70* genes in yeast, Ssc1 is the most abundant and essential protein of mitochondrial matrix involved in import and subsequent folding of the precursor proteins (Gambill et al. 1993). ssc1-3 mutants have mutation in the ATPase domain that renders the cells temperature sensitive and hinders the growth at nonpermissive temperature (Gambill et al. 1993). Partial functional complementation of growth defect of ssc1–3 yeast mutant cells by rice mitochondrial *Hsp70* genes suggests that rice Hsp70 proteins can possibly interact with yeast mitochondrial protein import machinery though not as effectively as the native yeast proteins. Functional distinction could be ascribed to the interaction of Hsp70 with its co-chaperones. Tutar et al. (2006) also showed that growth of a yeast mutant in cytosolic Hsp70s was faster when supported by its own Ssa1p than Arabidopsis or primate Hsc70. Partial complementation signifies that though Hsp70 are evolutionarily highly conserved, species specificity still plays a role. Taken together, the results demonstrate that rice Hsp70 machinery can interact with yeast Hsp70 chaperone machinery.

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