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ORIGINAL ARTICLE

Application of MapMan and RiceNet Drives Systematic Analyses of the Early Heat Stress Transcriptome in Rice Seedlings

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Abstract High-throughput transcriptome analyses such as oligonucleotide microarray technology are powerful tools for identifying an entire set of transcripts under given experimental conditions. However, it is not a simple process to interpret which information is important from those large gene sets. Using oligonucleotide arrays, more than 3000 rice microarray data have been produced; all are available for public users from the NCBI gene expression omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/). In this study, we employed MapMan and RiceNet tools to drive systematic analyses of the early heat stress transcriptome in rice seedlings. We generated transcriptome data to identify 589 genes that respond to early during heat stress, and uploaded the list to various overviews installed in the MapMan tool. In the cellular-response overview, this investigation revealed that the heat stress MapMan term is the most dominant, fitting well to the purpose of transcriptome analysis for examining the early heat stress response. When we applied the regulation overview, we learned that genes associated with transcription factors, protein modification, and calcium regulation are more significantly coupled with early heat stress in rice seedlings. This suggests that essential components, comprising signaling pathways, are mediated by such stress. We also used RiceNet to determine the functional gene network mediated by this stress. This network development was based on genes with enriched MapMan terms, i.e., heat stress, transcription factors, protein modification, and calcium regulation. We expect that applications of MapMan and RiceNet to genome-wide transcriptome data will guide users to identify key elements for further analyses.

Keywords Heat stress response, MapMan analysis, Microarray, RiceNet, Rice seedlings

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Introduction

Heat stress can damage organisms when they are exposed to higher-than-optimal temperatures. It is a common problem in many cereal-growing regions, restricting both yield and quality. As a model crop, rice has been used for studying the molecular mechanism for the heat shock response. The knowledge gained can be further applied toward developing crops with enhanced tolerance to this stress. To investigate the roles of heat shock proteins and heat shock transcription factors (TFs) in rice, several research groups have explored differential expression patterns of genes and their developmental or anatomical expression patterns in response to various abiotic stresses (Hu et al. 2009; Mittal et al. 2009; Liu et al. 2010; Chauhan et al. 2011). A global view of heat stress in rice and a systematic analysis of genome-wide heat response genes might provide us with a better understanding of that mechanism (Jung et al. 2012). Results from our recent transcriptome investigation have suggested 589 candidate genes associated with the response to early heat stress in rice seedlings (Jung et al. 2012). We defined the early heat stress response as the stress response within an hour after high temperature treatment at more than 37°C in young seedlings. These genes may be valuable starting points for the further integrating of 'omics' analysis to elucidate the early heat stress response in that crop.

Genevestigator (https://www.genevestigator.com/gv/), the Rice Oligonucleotide Array Database (ROAD; http://www.ricearray.org/expression/meta_analysis.shtml), the Rice expression profile database (RiceXPro; http://ricexpro. dna.affrc.go.jp/data-set.html), the Plant expression database (PLEXdb; http://www.plexdb.org/), and Rice eFP browsers (http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi) are very useful on-line tools for evaluating the expression patterns of rice genes (Zimmermann et al. 2008; Jung et al. 2011; Sato et al. 2011). Coexpression analysis or coexpression network tools are methods for obtaining a comprehensive list of

significant candidates to guide further analyses. Following four web-databases retain above feature: the Gene co-expression network browser (http://www.clemson.edu/genenetwork/network.php), the OryzaExpress Gene Expression Network (GEN; http://bioinf.mind.meiji.ac.jp/Rice_network_public/script/index.html), PlantArrayNet (previously RiceArrayNet; http://arraynet.mju.ac.kr/arraynet/), and the ROAD coexpression network (http://www.ricearray.org/coexpression/network. shtml) (Lee et al. 2009b; Ficklin et al. 2010; Jung et al. 2011).

MapMan is a user-driven tool for displaying genomics data sets on diagrams of metabolic pathways and other biological processes (Peltier et al. 2004). This visualization and comparison of multiple omics data from plants can help determine the most biologically relevant data or provide a quick overview of diverse processes in large datasets. MapMan has been optimized to analyze *Arabidopsis* data, and a case study has been conducted with maize (*Zea mays*) (Xing et al. 1996; Peltier et al. 2004). MapMan has also been applied to other plants, i.e., *Brassica napus*, *Glycine max*, *Hordeum vulgare*, *Medicago truncatula*, *Nicotiana tabacum*, *Oryza sativa* ssp. *japonica*, *Populus trichocarpa,* and *Triticum aestivum (http://MapMan.gabipd.org/web/guest/MapManstore).*

Network analysis is useful for developing probable gene-to-gene networks based on wide-ranging evidence. Four good web-based tools are available for rice: RiceNet (http://www.functionalnet.org/ricenet/), Rice interaction viewer (Jung et al. 2011), Rice Protein Interaction Network (RPIN; http://bis.zju.edu.cn/prin/), and STRING (string-db.org/; (Gu et al. 2011; Jung et al. 2011; Lee et al. 2011; Seo et al. 2011; Szklarczyk et al. 2011). For example, network development via RiceNet is based on 24 types of data, as described on the related website. Using this program to develop a stress interactome mediated by Xa21, NH1, and SUB1, and to dissect the biotic stress response, Lee et al. (2011) have discovered two positive regulators and one negative regulator of the XA21 signaling pathway. Protein–protein interaction (PPI) maps are valuable for investigating direct regulators of target genes. The Rice interaction viewer, RPIN, and STRING all support the development of hypothetical PPI networks based on interlogs from other species in which large-scale PPI maps are available. However, except for an interactome of 199 kinases, a large-scale PPI map has not yet been generated for rice (Ding et al. 2009; Rohila et al. 2006; Jung et al. 2010).

We integrated our data for the transcriptome early heat stress response (Jung et al. 2012) into metabolic pathways and cellular regulatory processes and found that genes encoding TFs and those involved in protein modification and calcium regulation are more significantly associated with early heat stress in rice. We then implemented RiceNet with genes having enriched MapMan terms to produce a functional gene network. Our integrated omics data, which combine early heat stress-responsive genes with MapMan tools and RiceNet, provide new insights into how rice seedlings respond to that stress.

Materials and Methods

Sample Preparation and Microarray Analysis

To identify early heat responsive genes in rice seedlings, we carried out microarray analysis using NSF45K microarray (Jung et al. 2012). To do this, we germinated *Dongjin* wild type and *Dongjin* background transgenic rice seeds by imbibition for two days in water and subsequently planted in pots with soil and maintained in a growth incubator (Younghwa Science, Korea) for 2 weeks. Then, we incubated the seedlings in the growth incubator for 0 h, 1/2 h and 1 h at 37ºC. We collected at least ten leaves from the seedlings for each biological replicate and prepared at least three biological replicates for each genetic background. After total RNA extraction, we prepared cDNA probes incorporated with Cy3 or Cy5 and performed hybridization as we introduced previously (Jung et al. 2008b). Through the microarray data processing under criteria with less than 0.05 t-test p-value and more than 2 fold up-regulation in heat stress condition, we identified 589 genes (608 probes) (Table S1).

The Affymetrix raw data for various abiotic stress responses were downloaded from NCBI GEO (platform Accession Number is GPL2025): drought (GSE16108, GSE24048, and GSE26280), salt (GSE16108), cold (GSE16108), heat (GSE14275), anoxia (GSE6908), and submergence (GSE18930). We used the MAS 5.0 method provided by the R package affy for Affymerix array to convert probe level data to expression values (Affymetrix 2001).

MapMan Analysis

A total of 36 MapMan BINs were used for the Rice MapMan classification; these were extended in a hierarchical manner into subBINs (Usadel et al. 2005; Urbanczyk-Wochniak et al. 2006) (Table S2). For example, BinCode 29 indicates protein class; BinCode 29.5 indicates protein. degradation class; and BinCode 29.5.3 indicates protein. degradation. cysteine protease class. To integrate significant gene expression data from our transcriptome analysis into diverse MapMan tools, we generated a dataset using locus IDs in RGAP version 7 annotation, and the average fold-change data for heat vs untreated control. The information mapping RGAP version 7 annotation for MapMan is available at http://MapMan.gabipd.org/web/guest/MapManstore. To compile the statistics presented in Fig. 1, we used a Cellular_response_overview (Fig. 1A), Regulation_overview

(Fig. 1B), transcription (Fig. 1C), and Proteasome Detail And Autophagy (Fig. 1D), all installed in the MapMan toolkit. Detailed procedures for this analysis are depicted in Fig. S1.

Network Analysis

To perform our network analysis, we used RiceNet. In all, 240 genes were queried for protein, RNA, stress, signaling, and redox MapMan classes that had shown high abundance in their response to early heat stress in rice seedlings; 937 interactions were identified among 147 genes. We then downloaded the sif file retaining this interaction information. We also prepared detailed information of all components in the network (Table S4). Cytoscape, an open-source platform for complex network analysis and visualization, was used to integrate diverse data into nodes (circles) and to manipulate the network (Shannon et al. 2003). We imported the sif file from RiceNet into Cytoscape and developed a network through an edge-weighted spring-imbedded layout. The functional categories found by MapMan (Table S4) were integrated into the node colors of the network: brown indicating protein; green, signaling; yellow, stress: blue, RNA; and red, redox. Nodes were labeled as annotations (Fig. 2) and locus identifiers (locus_IDs) (Fig. S2) by the Michigan State University Rice Genome Annotation Project (MSU RGAP.) The network was mediated by OsSTI1 (Os04g45480), as highlighted in Fig. 2. Network components belonging to the same functional category were clustered together in same-colored rectangular boxes with nodes in each of those categories.

Results and Discussion

Overview from the MapMan Tool for Metabolic Pathways or Biological Processes Associated with Early Heat Stress in Rice

We obtained an overview of the metabolic pathways and regulatory processes related to heat stress in rice. Fold-change data for 589 genes that exhibited a >2-fold-induction

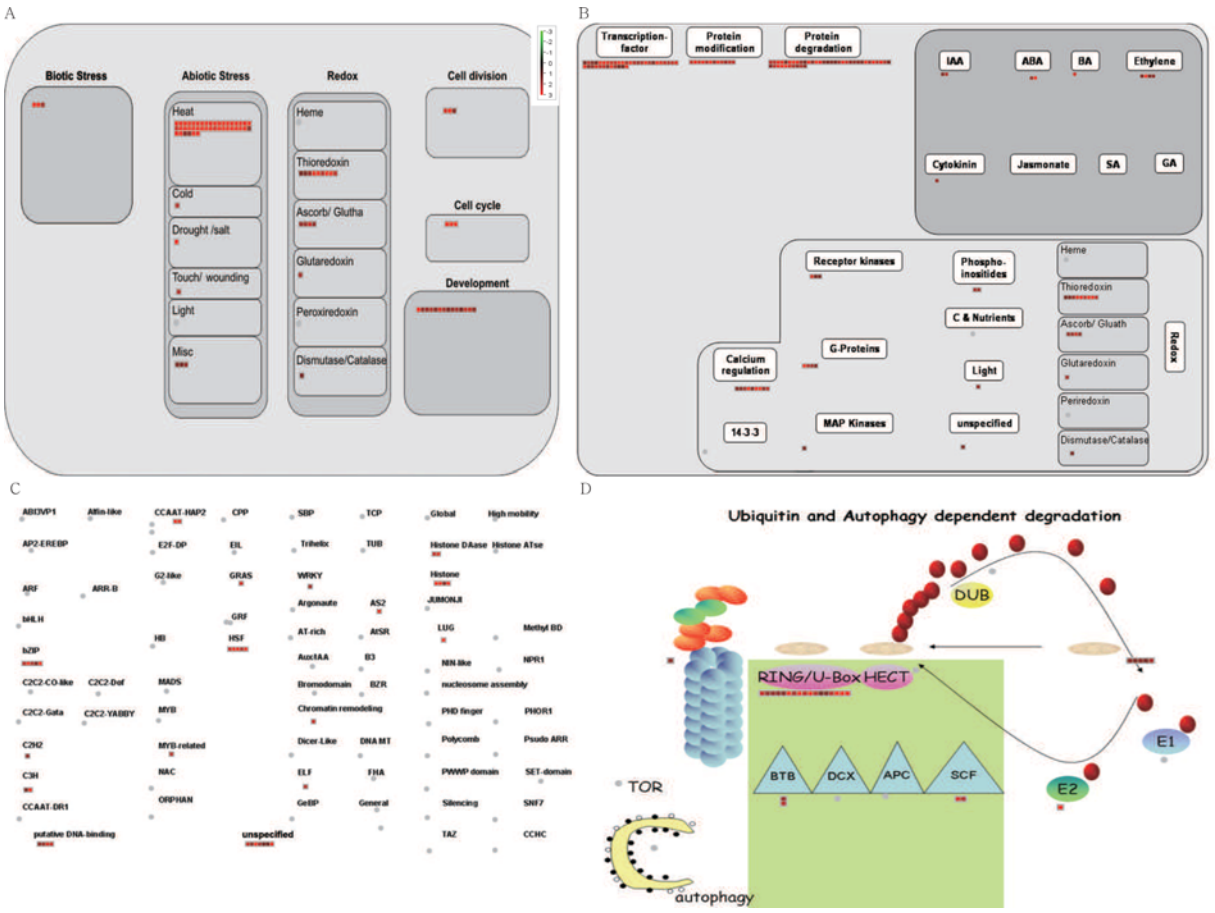

Fig. 1. MapMan analysis of early heat stress-responsive genes in rice.
(A) Cellular response overview in MapMan, (B) Regulation overview in MapMan, (C) Transcription overview in MapMan., (D) Ubiquitin and autophagy-dependent degradation overview in MapMan. Red indicates upregulation; green, downregulation. Detailed information about gene integration into the cellular response overview is shown in Table S2.

after plants were treated for 0.5 or 1 h (Materials and Methods) were uploaded to various tools installed in MapMan (Table S1; Fig. S1). The results are summarized in Table S2. This overview grouped the genes into 36 classes (bins). In all, we found 83 elements in the protein class, 67 related to RNA, 52 for stress, 21 for signaling, 18 for

Table 1. Gene list showing early heat stress response with heat stress MapMan term

Functional category[a]	Gene model[b]	Putative Function	Fold change[c]
Stress	LOC_Os01g04340.1	HSP20/alpha crystallin family protein	1.82
Stress	LOC_Os01g04360.1	HSP20/alpha crystallin family protein	7.44
Stress	LOC_Os01g04380.1	HSP20/alpha crystallin family protein	8.82
Stress	LOC_Os01g08860.1	HSP20/alpha crystallin family protein	8.18
Stress	LOC_Os02g54140.1	HSP20/alpha crystallin family protein	7.72
Stress	LOC_Os03g14180.1	HSP20/alpha crystallin family protein	5.26
Stress	LOC_Os03g15960.1	HSP20/alpha crystallin family protein	7.47
Stress	LOC_Os03g16020.1	HSP20/alpha crystallin family protein	8.16
Stress	LOC_Os03g16030.1	HSP20/alpha crystallin family protein	8.67
Stress	LOC_Os03g16040.1	HSP20/alpha crystallin family protein	8.80
Stress	LOC_Os04g36750.1	HSP20/alpha crystallin family protein	9.16
Stress	LOC_Os06g14240.1	HSP20/alpha crystallin family protein	7.15
Stress	LOC_Os02g52150.1	HSP20/alpha crystallin family protein	7.03
Stress	LOC_Os06g11610.1	HSP20/alpha crystallin family protein	4.67
Stress	LOC_Os04g01740.1	heat shock protein (HSP90)	7.17
Stress	LOC_Os06g50300.1	heat shock protein (HSP90)	4.19
Stress	LOC_Os08g39140.1	heat shock protein (HSP90)	4.01
Stress	LOC_Os09g29840.1	heat shock protein (HSP90)	4.85
Stress	LOC_Os09g30418.1	heat shock protein (HSP90)	4.33
Stress	LOC_Os02g04650.1	activator of HSP90	3.65
Stress	LOC_Os06g49030.1	activator of HSP90	3.98
Stress	LOC_Os08g36150.1	activator of HSP90	4.86
Stress	LOC_Os05g06440.1	heat shock protein DnaJ (HSP40)	4.46
Stress	LOC_Os01g13760.1	heat shock protein DnaJ (HSP40)	2.95
Stress	LOC_Os05g48810.1	heat shock protein DnaJ (HSP40)	6.41
Stress	LOC_Os02g52270.1	heat shock protein DnaJ (HSP40)	3.36
Stress	LOC_Os02g54130.1	heat shock protein DnaJ (HSP40)	1.79
Stress	LOC_Os03g18870.1	heat shock protein DnaJ (HSP40)	2.54
Stress	LOC_Os06g09560.1	heat shock protein DnaJ (HSP40)	6.13
Stress	LOC_Os03g57340.1	heat shock protein DnaJ (HSP40)	4.18
Stress	LOC_Os06g02620.1	heat shock protein DnaJ (HSP40)	3.37
Stress	LOC_Os01g08560.1	DnaK family protein (HSP70)	4.58
Stress	LOC_Os02g02410.1	DnaK family protein (HSP70)	4.51
Stress	LOC_Os03g02260.1	DnaK family protein (HSP70)	5.52
Stress	LOC_Os03g11910.1	DnaK family protein (HSP70)	7.42
Stress	LOC_Os03g16920.1	DnaK family protein (HSP70)	4.89
Stress	LOC_Os05g23740.1	DnaK family protein (HSP70)	2.70
Stress	LOC_Os05g38530.1	DnaK family protein (HSP70)	7.82
Stress	LOC_Os11g47760.1	DnaK family protein (HSP70)	1.77
Stress	LOC_Os02g08490.1	chaperone protein clpB 1	7.62
Stress	LOC_Os03g31300.1	chaperone protein clpB 1	5.17
Stress	LOC_Os05g44340.1	heat shock protein 101	8.28
Stress	LOC_Os06g46900.1	phosphosulfolactate synthase-related protein	7.14
Stress	LOC_Os02g43020.1	heat shock protein STI (OsSTI2)	4.75
Stress	LOC_Os04g45480.1	heat shock protein STI (OsSTI1)	7.53

Functional category indicates the highest level of MapMan terms. [b]Gene model indicates the gene model annotated by rice genome annotation project team in Michigan State University, USA. [c]Fold change is $\log_2$ ratio of leaf samples exposed to heat stress for 0.5 h over untreated leaf samples.

transport, 17 for lipid metabolism, and 15 for redox (Fig. 1). These classes retained significant abundance associated with MapMan terms.

Cellular Processes Associated with Early Heat Stress

After integrating the expression patterns of the 589 genes showing an early heat stress response (Jung et al. 2012), we used the cellular response overview function of MapMan to determine that, within the 52-element class, the heat stress response (42 elements) was the most abundant (Fig. 1A; Table 1). They included 14 genes encoding HSP20/alpha crystallin family proteins (HSP20), nine for DnaJ domain-containing proteins, eight for DnaK family proteins (HSP70), and one each for heat shock protein 90 (HSP90), chaperone protein clpB 1, HSP101, a tetratricopeptide repeat (TPR) domain-carrying stress-inducible protein (STI), and a phosphosulfolactate synthase-related protein (Table 1). We had expected this type of identification because we had focused on early heat stress-responsive genes in our transcriptome analysis. Thus, we were able to evaluate the effectiveness of these microarray data. By comparing these results with additional microarray data gathered under heat stress (10 h at 42°C) in rice seedlings (GSE GSE14275), we confirmed that expression was up-regulated for eight *HSP20* genes (*Os01g04380*, *Os01g08860*, *Os02g54140*, *Os03g15960*, *Os03g16020*, *Os03g16030*, *Os03g16040*, and *Os04g36750*) (Hu et al. 2009). Our NSF 45K array showed that each fold-change was >100 except *Os01g04340*, *Os03g14180* and *Os06g11610*, indicating that these gene families are strongly associated with the early heat stress response in rice (Table 1). Expression patterns of two *HSP70* genes (*Os03g11910* and *Os03g16920*) and one *HSP90* gene (*Os04g01740*) were also confirmed by previous report (Hu et al. 2009).

In the redox class (15 elements), thioredoxin (9 elements) and ascorbate/glutathione (4 elements) were more significantly associated with early heat stress than any other components in that class. Overexpression of *Arabidopsis Thioredoxin* (*AtTDX*) results in thermo-tolerance (Lee et al. 2009a) and an *Arabidopsis* double mutant lacking *thylakoid ascorbate peroxidase* (*tylapx*) and cytosolic *ascorbate peroxidase1* (*apx1*) has enhanced tolerance to heat stress (Miller et al. 2007). These findings further support roles for those functional groups in the early heat stress response in rice. Of the genes not assigned to MapMan terms, we identified three -- *Os02g04650*, *Os06g49030*, and *Os08g36150* – that encode activators of HSP90. This suggests the presence of upstream regulators for early heat-responsive HSP90s.

Transcriptional Regulation Associated with Early Heat Stress

Regulatory processes associated with early heat stress in crops could be interesting targets for controlling and improving plant tolerance. In our regulation overview, transcriptional regulation, protein modification, and protein degradation were the most frequently revealed within early heat stress-responsive genes (Fig. 1B; Table S2). We identified 67 elements in the RNA class, with 48 being specific to transcriptional regulation. These included nine heat shock transcription factors (HSFs), six bZIP transcription factors (bZIPs), four C2H2 zinc finger proteins, two C3H zinc finger family proteins, two C3HC4 type domain-containing proteins, three remorin family proteins, two for histone deacelylase (HDA), two nuclear transcription factor Y subunits (HAP2), two WD-40 repeat family proteins and other putative transcription factors (TFs) (Fig. 1C; Table 2 and Table S2). Of these, HSFs are well-known, essential components for heat perception and signaling in plants (Saidi et al. 2011). Expression patterns for *Os01g54550*, *Os03g06630*, *Os03g12370*, *Os03g53340*, *Os03g58160*, *Os04g48030*, *Os08g43334*, *Os09g35790*, and *Os10g28340* have previously been confirmed by real-time PCR analysis in a genome-wide examination of the rice HSF family (Hu et al. 2009; Wang et al. 2009; Liu et al. 2010; Chauhan et al. 2011). Overexpression of *HSFA* genes in transgenic *Arabidopsis* and tomato plants confers enhanced thermo-tolerance (Mishra et al. 2002; Nishizawa et al. 2006). Plant HSFs play other key roles in responses to oxidative, high-salinity, and chilling stresses (Li et al. 2005a; Li et al. 2005b; Nishizawa et al. 2006; Yokotani et al. 2008; Liu et al. 2010). *Arabidopsis* plants overexpressing the rice HSF gene *OsHsfA2e (Os03g58160)* exhibit salinity tolerance as well as thermo-tolerance (Yokotani et al. 2008). Therefore, rice *HSF* genes associated with early heat stress in rice seedlings can be good targets for studying the thermo-tolerance response.

Using affymetrix array data, we analyzed the differential expression patterns of these nine *HSF* genes in response to other abiotic stresses. Here, *Os04g48030*, *Os08g43334*, *Os09g35790*, and *Os10g28340* were significantly up-regulated by drought, salt, and anoxia, whereas *Os03g58160* showed significant up-regulation by drought, *Os03g12370* by cold, and *Os03g53340* by drought and anoxia, implying that they have roles in multiple stress responses (Table S3).

Rice and *Arabidopsis* bZIP TFs are generally involved in abscisic acid (ABA) signaling in response to abiotic stresses (Nakashima et al. 2009; Fujita et al. 2012). Based on the analysis of rice affymetrix array data (Table S3), we found that two of the six bZIP TFs (*Os01g64730/OsABF1* and *Os05g49420*) showed differential expression patterns in response to heat, cold, salt, and drought. Of the others, *Os05g34050* was significantly up-regulated by cold and submergence, *Os07g44950* by drought, *Os12g37410* by submergence, and Os06g41770 by cold. This suggested that they have regulatory functions in multiple abiotic stress

Table 2. Gene list showing heat early stress response in RNA category

Functional category	Gene model	Sub-functional class[a] (putative function)	Fold change
RNA	LOC_Os01g54550.1	HSF	1.32
RNA	LOC_Os03g06630.1	HSF	1.27
RNA	LOC_Os03g12370.2	HSF	2.48
RNA	LOC_Os03g53340.1	HSF	8.32
RNA	LOC_Os03g58160.1	HSF	1.53
RNA	LOC_Os04g48030.1	HSF	2.18
RNA	LOC_Os08g43334.1	HSF	2.98
RNA	LOC_Os09g35790.1	HSF	2.22
RNA	LOC_Os10g28340.1	HSF	5.80
RNA	LOC_Os01g64730.1	bZIP	3.07
RNA	LOC_Os05g34050.1	bZIP	2.23
RNA	LOC_Os05g49420.1	bZIP	1.69
RNA	LOC_Os07g44950.1	bZIP	3.44
RNA	LOC_Os12g37410.1	bZIP	2.29
RNA	LOC_Os06g41770.1	bZIP	2.22
RNA	LOC_Os04g02510.1	C2H2 zinc finger	1.81
RNA	LOC_Os01g09620.1	C2H2 zinc finger	3.07
RNA	LOC_Os05g10670.1	C2H2 zinc finger	1.57
RNA	LOC_Os03g07880.1	CCAAT box binding factor family, HAP2	2.71
RNA	LOC_Os08g09690.1	CCAAT box binding factor family, HAP2	4.51
RNA	LOC_Os02g12350.1	HDA	2.45
RNA	LOC_Os06g38470.1	HDA	2.68
RNA	LOC_Os03g08830.1	WD40	2.65
RNA	LOC_Os06g04040.1	WD40	2.87
RNA	LOC_Os08g24400.1	SWP	2.35
RNA	LOC_Os08g24420.1	SWP	1.61
RNA	LOC_Os07g08880.1	Chromatin Remodeling Factors	2.44
RNA	LOC_Os01g38530.1	ELF3	2.23
RNA	LOC_Os12g38490.1	GRAS	2.55
RNA	LOC_Os01g32770.1	AS2, Lateral Organ Boundaries Gene Family	2.73
RNA	LOC_Os02g56880.1	LUG	3.12
RNA	LOC_Os06g51260.1	MYB	2.29
RNA	LOC_Os11g09160.1	MYB	3.25
RNA	LOC_Os03g60800.1	putative transcription regulator (B3 DNA binding)	1.94
RNA	LOC_Os04g45070.1	putative transcription regulator	1.83
RNA	LOC_Os07g38170.1	putative transcription regulator (Remorin)	2.48
RNA	LOC_Os07g10780.1	putative transcription regulator (Remorin)	1.70
RNA	LOC_Os09g25440.1	putative transcription regulator (Remorin)	2.22
RNA	LOC_Os05g41530.1	putative transcription regulator (C2H2)	2.61
RNA	LOC_Os03g60560.1	putative transcription regulator (C2H2)	3.21
RNA	LOC_Os07g43740.1	putative transcription regulator (C2H2)	5.99
RNA	LOC_Os11g37230.1	putative transcription regulator (C3HC4)	2.87
RNA	LOC_Os01g61830.1	putative transcription regulator (C3HC4)	2.11
RNA	LOC_Os03g57900.1	putative transcription regulator	1.52
RNA	LOC_Os06g41930.2	putative transcription regulator	1.52
RNA	LOC_Os09g30414.1	putative transcription regulator	2.80
RNA	LOC_Os10g37640.1	putative transcription regulator	3.53
RNA	LOC_Os08g17400.1	WRKY	2.02

[a]Sub-functional class is bin code name provided by MapMan tool. For more detailed information of other column heads in this table, please refer those in Table 1.

responses, including heat. Of these, the homozygous T-DNA insertional mutants of *Os01g64730* encoding the rice ABA-responsive element binding factor 1 (*OsABF1*) are more sensitive than the wild-type (WT) plants to drought and salinity treatments, indicating their positive role in responding to those stresses (Hossain et al. 2010). In *Arabidopsis*, expression of the C2H2-type zinc finger protein is stimulated under heat stress; its overexpression is linked to enhanced salinity tolerance, which suggests that the rice homologs have a similar role (Ciftci-Yilmaz et al. 2007). Therefore, even though we have lacked experimental data about how the bZIP TFs identified in our study function under heat stress, we would also expect them to regulate multiple abiotic stress responses. Furthermore, we surmise that the other TF genes mentioned here have significant roles in that reaction, based on their expression patterns for early responsiveness under heat stress condition.

Protein Metabolism Associated with Early Heat Stress

The protein metabolism elements associated with early heat stress were those for degradation (41 elements), modification (12 elements), and folding (16 elements) (Fig. 1B; Table 3 and Table S2). Of these, degradation was related with five encoding ubiquitin domain-containing proteins, one for E2 ubiquitin-conjugating enzyme, and 21 for E3 ubiquitin ligases further classified to RING (17 elements), two SCF/F-box elements, and two BTB/POZ type elements as well as proteasomes, proteases, peptidases, and UBX domain-containing proteins (Fig. 1D; Table 3 and Table S2). Because protein degradation is severely affected by heat stress, the genes related to that process are significant in conferring tolerance. The E3 ubiquitin ligases have been identified as regulators of responses to disease, phosphate deficiencies, salinity, hot or cold temperature fluctuations, and drought

Table 3. Gene list showing heat early stress response in protein category

Functional category	Gene model	Sub-functional class (putative function)	Fold change
Protein	LOC_Os01g67590.1	degradation	2.28
Protein	LOC_Os02g50230.1	degradation	1.66
Protein	LOC_Os07g38590.1	degradation	2.40
Protein	LOC_Os08g43300.1	degradation	2.84
Protein	LOC_Os10g37630.1	degradation	2.82
Protein	LOC_Os04g01710.1	degradation.cysteine protease	3.28
Protein	LOC_Os08g39560.1	degradation.cysteine protease	3.47
Protein	LOC_Os02g13360.1	degradation.metalloprotease	3.12
Protein	LOC_Os03g51920.1	degradation.metalloprotease	6.09
Protein	LOC_Os06g12370.1	degradation.metalloprotease	1.93
Protein	LOC_Os08g39150.1	degradation.metalloprotease	3.58
Protein	LOC_Os05g45750.1	degradation.serine protease	2.03
Protein	LOC_Os05g49380.1	degradation.serine protease	1.61
Protein	LOC_Os06g30970.1	degradation.ubiquitin.E2	5.21
Protein	LOC_Os08g41150.1	degradation.ubiquitin.E3.BTB/POZ Cullin3.BTB/POZ	2.25
Protein	LOC_Os08g41180.1	degradation.ubiquitin.E3.BTB/POZ Cullin3.BTB/POZ	1.89
Protein	LOC_Os01g52110.1	degradation.ubiquitin.E3.RING	3.53
Protein	LOC_Os02g09060.1	degradation.ubiquitin.E3.RING	2.13
Protein	LOC_Os02g52210.1	degradation.ubiquitin.E3.RING	3.02
Protein	LOC_Os03g08000.1	degradation.ubiquitin.E3.RING	2.26
Protein	LOC_Os03g20870.1	degradation.ubiquitin.E3.RING	2.36
Protein	LOC_Os03g47500.1	degradation.ubiquitin.E3.RING	2.35
Protein	LOC_Os05g19970.1	degradation.ubiquitin.E3.RING	2.47
Protein	LOC_Os05g37900.1	degradation.ubiquitin.E3.RING	1.71
Protein	LOC_Os06g01200.1	degradation.ubiquitin.E3.RING	2.93
Protein	LOC_Os06g06490.1	degradation.ubiquitin.E3.RING	4.58
Protein	LOC_Os06g19680.1	degradation.ubiquitin.E3.RING	1.86
Protein	LOC_Os07g45350.2	degradation.ubiquitin.E3.RING	3.09
Protein	LOC_Os07g46700.4	degradation.ubiquitin.E3.RING	1.95
Protein	LOC_Os08g02850.1	degradation.ubiquitin.E3.RING	1.59
Protein	LOC_Os09g20900.1	degradation.ubiquitin.E3.RING	1.92

Table 3. Continued

Functional category	Gene model	Sub-functional class (putative function)	Fold change
Protein	LOC_Os10g35670.1	degradation.ubiquitin.E3.RING	2.95
Protein	LOC_Os12g43930.1	degradation.ubiquitin.E3.RING	1.77
Protein	LOC_Os01g65920.1	degradation.ubiquitin.E3.SCF.FBOX	2.20
Protein	LOC_Os11g14140.1	degradation.ubiquitin.E3.SCF.FBOX	2.62
Protein	LOC_Os07g36420.1	degradation.ubiquitin.proteasom	1.85
Protein	LOC_Os01g68950.1	degradation.ubiquitin.ubiquitin	1.59
Protein	LOC_Os02g06640.1	degradation.ubiquitin.ubiquitin	2.13
Protein	LOC_Os03g03920.1	degradation.ubiquitin.ubiquitin	1.77
Protein	LOC_Os09g31019.1	degradation.ubiquitin.ubiquitin	1.77
Protein	LOC_Os11g04880.1	degradation.ubiquitin.ubiquitin	1.61
Protein	LOC_Os02g01280.1	Folding (T-complex protein)	2.82
Protein	LOC_Os03g64210.1	Folding (T-complex protein)	1.94
Protein	LOC_Os05g46290.1	Folding (T-complex protein)	1.89
Protein	LOC_Os06g02380.1	Folding (T-complex protein)	3.45
Protein	LOC_Os10g32550.1	Folding (T-complex protein)	6.19
Protein	LOC_Os12g17910.1	Folding (T-complex protein)	2.03
Protein	LOC_Os03g25050.1	Folding (chaperonin)	4.40
Protein	LOC_Os06g09679.1	Folding (chaperonin)	2.88
Protein	LOC_Os07g44740.1	Folding (chaperonin)	5.06
Protein	LOC_Os09g26730.1	Folding (chaperonin)	3.35
Protein	LOC_Os10g41710.1	Folding (chaperonin)	2.56
Protein	LOC_Os02g39870.1	Folding (co-chaperone GrpE protein)	2.29
Protein	LOC_Os08g25090.1	Folding (co-chaperone GrpE protein)	1.68
Protein	LOC_Os09g11250.1	Folding (co-chaperone GrpE protein)	2.45
Protein	LOC_Os07g26940.1	Folding (ORM1)	3.31
Protein	LOC_Os05g38370.1	Folding (peptidyl-prolyl cis-trans isomerase)	1.78
Protein	LOC_Os03g53910.1	postranslational modification (TPR)	4.45
Protein	LOC_Os01g43540.1	postranslational modification (SGT)	3.75
Protein	LOC_Os09g11230.1	postranslational modification (Ser/Thr PPA)	2.40
Protein	LOC_Os02g13640.1	postranslational modification (PPA1 subunit SDS22)	2.47
Protein	LOC_Os08g01270.1	postranslational modification (Protein Kinase)	2.55
Protein	LOC_Os04g41100.5	postranslational modification (CDK)	1.98
Protein	LOC_Os05g11140.1	postranslational modification (CK1)	4.33
Protein	LOC_Os05g04340.1	postranslational modification (CGMC kinase)	2.28
Protein	LOC_Os03g14840.1	postranslational modification (AGC kinase)	5.07
Protein	LOC_Os03g06330.1	postranslational modification.kinase.receptor like cytoplasmatic kinase VII (Tyr kinase)	2.04
Protein	LOC_Os07g47270.1	postranslational modification.kinase.receptor like cytoplasmatic kinase VII (APK1)	2.42
Protein	LOC_Os07g49470.1	postranslational modification.kinase.receptor like cytoplasmatic kinase VII (APK1)	2.37

For more detailed information of column heads in this table, please refer those in Table 1.

(Ma et al. 2004; Zeng et al. 2004; Guo et al. 2006; Rees et al. 2009; Josine et al. 2011). Thus, our identification of 21 heat-inducible E3 ubiquitin ligases suggests that their modulation is an adaptive response, via protein ubiquitination, to early heat stress in rice.

From the protein modification elements, we identified eight kinases [two APK1Bs; a cyclin-dependent kinase G-2 (CDK); a CGMC kinase including CDA, MAPK, GSK3, and CLKC; a casein kinase 1; a AGC kinase including protein kinase A (PKA), PKG, and PKC; a tyrosine protein kinase; and a putative protein kinase], plus a phosphatase, a phosphatase regulatory subunit SDS22, a TPR domain-containing protein, and a suppressor of the G2 allele of SKP1 (SGT) (Table 3). Phosphorylation by kinases and de-phosphorylation by phosphatases are highly studied types of protein modification. For example, human PKA phosphorylates the serine 320 of human HSF1 as part of a heat-inducible response, and phosphorylated HSF1 transcriptionally regulates the expression of heat shock proteins (Murshid et al. 2010). The interaction of HSP90 with casein kinase II (CKII)

protects mature CKII (Miyata et al. 1992). Exposure of A431 human carcinoma cells to a rapid rise in temperature induces augmented expression and the tyrosine phosphorylation of protein kinase FA/glycogen synthase kinase-3 alpha (kinase FA/GSK-3 alpha) (Yang et al. 1997). Likewise, the binding of TPR domain-containing proteins to HSP90 is essential to the latter's chaperone function in vivo (Ramsey et al. 2000). The interaction between protein phosphatase 5 (PP5) and cytosolic HSP90 is part of a tomato R protein complex that is important to its folding and functioning (de la Fuente van Bentem et al. 2005). *Arabidopsis* SGT1 proteins act with cytosolic HSP70 to enable the operation of the cytosolic HSP70 chaperon (Noel et al. 2007). Therefore, we suspect that the rice homologs identified here would have similar roles in heat stress responses.

With respect to protein-folding, 16 elements were found: six T-complex proteins, five chaperonins, three co-chaperon GrpE proteins, one Orosomucoid 1 (ORM1), and a peptidyl-prolyl cis-trans isomerase (Table 3 and Table S2). Chaperonin, co-chaperon GrpE protein, and T-complex protein are members of the chaperonin complex, which is critical to the folding of proteins into stable and functional forms in response to diverse stresses (Fenton et al. 2003; Coghlin et al. 2006). Protein-folding is a major molecular activity by heat shock chaperon proteins, which comprise a major gene family that is responsive to heat stress. This regulation is critical in preventing the aggregation and mis-folding of proteins under stressful growing conditions (Chen et al. 2008). Therefore, these related elements are good targets for helping us understand the molecular mechanisms underlying

Table 4. Gene list showing heat early stress response in signaling and redox reaction categories.

Functional category	Gene model	Sub-functional class (putative function)	Fold change
Signaling	LOC_Os07g29794.1	Calcium (calmodulin binding heat shock protein)	2.31
Signaling	LOC_Os03g61670.1	Calcium (calreticulin precursor)	3.29
Signaling	LOC_Os04g32950.1	Calcium (calreticulin precursor)	4.42
Signaling	LOC_Os05g43170.1	Calcium (calreticulin precursor)	1.69
Signaling	LOC_Os07g14270.1	Calcium (calreticulin precursor)	3.24
Signaling	LOC_Os06g46950.1	Calcium (EF hand family protein)	1.70
Signaling	LOC_Os05g31620.1	Calcium (calcium sensor protein)	2.03
Signaling	LOC_Os05g13580.1	Calcium (calcium sensor protein)	1.68
Signaling	LOC_Os03g53200.1	Calcium (calcium sensor protein)	1.88
Signaling	LOC_Os07g43470.1	G-proteins (GTP-binding protein)	3.15
Signaling	LOC_Os05g28290.1	G-proteins (ranBP1)	2.07
Signaling	LOC_Os05g27530.1	G-proteins (ras-related protein)	1.64
Signaling	LOC_Os12g43550.1	G-proteins (ras-related protein)	2.28
Signaling	LOC_Os02g41550.1	Light (FAD binding domain)	1.89
Signaling	LOC_Os06g48590.2	MAP kinases (CGMC)	1.53
Signaling	LOC_Os06g09540.1	Phosphinositides (SAC)	2.06
Signaling	LOC_Os08g34950.1	phosphinositides.phosphatidylinositol-4-phosphate 5-kinase	1.95
Signaling	LOC_Os01g52000.1	receptor kinases.DUF 26	1.51
Signaling	LOC_Os09g04550.1	receptor kinases.DUF 26	1.57
Signaling	LOC_Os04g03180.1	receptor kinases.leucine rich repeat XI	2.59
Redox	LOC_Os04g44830.1	Thioredoxin (TPR)	2.79
Redox	LOC_Os05g07690.1	Thioredoxin (thioredoxin)	1.50
Redox	LOC_Os05g41120.1	Thioredoxin (thioredoxin)	1.93
Redox	LOC_Os07g08840.1	thioredoxin	3.79
Redox	LOC_Os08g15204.1	Thioredoxin (thioredoxin)	5.10
Redox	LOC_Os09g27830.1	Thioredoxin (thioredoxin)	4.13
Redox	LOC_Os10g35720.1	Thioredoxin (protein disulfide isomerase)	2.29
Redox	LOC_Os11g09280.1	Thioredoxin (glutaredoxin)	1.61
Redox	LOC_Os02g42890.1	ascorbate and glutathione (cytochrome b561)	1.89
Redox	LOC_Os03g17690.1	ascorbate and glutathione.ascorbate (Ascorbate Peroxidase)	1.85
Redox	LOC_Os05g02530.1	ascorbate and glutathione.ascorbate (glutathione S-transferase)	1.96
Redox	LOC_Os09g39380.1	ascorbate and glutathione.ascorbate (monodehydroascorbate reductase)	2.53
Redox	LOC_Os07g46990.1	dismutases and catalases (copper/zinc superoxide dismutase)	1.52
Redox	LOC_Os01g34620.4	Glutaredoxins (glutaredoxin)	2.06

For more detailed information of column heads in this table, please refer those in Table 1.

the early heat stress response in rice.

Signaling Pathway Associated with Early Heat Stress

We identified 21 elements for signaling pathways. These included nine genes related to calcium regulation, three for G-proteins, four for receptor kinase, two for phosphoinositide, one for MAP kinase, one for light signaling and an unspecified signaling gene (Fig. 1B; Table 4 and Table S2). Of these, the ones for calcium regulation and G-protein are more significantly enriched in the signaling pathway. The redox response element thioredoxin was dominant here in the cellular processes of early heat stress-responsive genes in rice. This suggests a hierarchy of signaling pathways in which G protein-mediating calcium-signaling cascades function upstream of heat stress-responsive TFs in order to regulate the redox reaction via thioredoxin activity, thus triggering tolerance to heat stress.

The capacity to sense cytoplasmic calcium during heat stress is important for the accumulation of GABA (4-Aminobutyric acid) in *Arabidopsis*. We believe that the three calcium sensor proteins found here (Os05g31620, Os05g13580, and Os03g53200) have a similar role in the heat stress response by rice (Table 4). When *calmodulin* genes 5 and 6 (*cam 5* and *cam6*) mutant *Arabidopsis* tissues are exposed to 42°C for 2 h, seed germination is reduced and seedling survival is lower than that of the WT (AL-Quraan et al. 2012). Another four rice genes (*Os03g61670*, *Os04g32950*, *Os05g43170*, and *Os07g14270*) encode calreticulin precursor proteins that bind Ca^{2+} ions similarly to calmodulin. However, their presence in the ER implies that calcium-signaling through the ER is important for the response to early heat stress by rice seedlings (Coppolino et al. 1998). For the G-proteins, we identified *Os07g43470*, encoding GTP-binding protein; *Os05g28290*, encoding the ras-related nuclear binding protein 1 (ranBP1) domain-containing protein; and *Os05g27530* and *Os12g43550*, both encoding ras-related proteins (Table 4). The involvement of GTP(-binding) proteins has been reported in a Ca^{2+}-stimulated exocytotic pathway within barley aleurone cells (Homann et al. 1997). Therefore, we might easily expand this relationship to include the early heat stress response in rice.

The role of G-proteins has also been analyzed with transgenic tobacco plants that constitutively over-express Gα and Gβ, thereby conferring heat tolerance (Mongrand et al. 2004). This study of PPIs by Mongrand et al. (2004) has revealed that the Gα subunit works with pea phospholipase C (PLCδ) in the calcium-binding domain. Activation of the cholesterol pathway and Ras maturation in humans are associated with enhanced tolerance to heat (Shack et al. 1999). Furthermore, two yeast two-hybrid studies have demonstrated a relationship between *Laccaria bicolor HSP* (*LbHSP*) and the *Laccaria bicolor Ras* gene (*Lbras*), suggesting that LbHSP has a supporting role in *ras*-mediated mycorrhizal-signaling pathways during various stages of ectomycorrhizal development. This is accomplished through activation of the cholesterol pathway and Ras maturation in response to stress. A high-affinity purification assay of *Xenopus* eggs, using Ran as bait, has revealed co-immunoprecipitation of cytosolic HSP70 and RanBP1 (Saitoh et al. 1996). All of these findings further suggest that the Ras-related protein and ranBP1 are functionally associated with the heat shock response in rice.

Mitogen-activated protein kinase (MAPK) cascades of *Nicotiana benthamiana* function downstream of HSP90 for *N* gene-dependent cell death (Takabatake et al. 2007). In *Arabidopsis*, inositol 1,4,5-trisphosphate (IP_3) is primary involved in transducing heat shock signals (Liu et al. 2006). Similarly, we would expect MAPKs and phosphoinositides to have significance in the early heat shock response by rice seedlings. We have explained above the relationship the thioredoxin family are associated with the redox response to early heat stress. Bigelow and Squier (2011) have previously reported that thioredoxin-dependent redox regulation, through the reversible oxidation of methionines, plays an important role in calmodulin mediation of the stress response in plants. All of these are evidence for the cooperative roles of G-proteins, calcium regulation, and thioredoxin in response to heat stress in rice.

Development of a Functional Gene Network Associated with MapMan Terms that are Enriched in the Early Heat Stress Response

To develop a functional gene network from our MapMan analysis, we used RiceNet to identify 240 genes from the functional classes that were enriched in MapMan terms. From this network, we discovered 937 interactions among 147 genes. The functional categories from the MapMan tool were then integrated into the network, where signaling, RNA, stress, protein, and redox nodes were represented by green, blue, yellow, brown, and red, respectively (Fig. 2; Table S4). These nodes were labeled based on annotations from the Michigan State University Rice Genome Annotation Project database (MSU RGAP; http://rice.plantbiology.msu.edu/) (Ouyang et al. 2007). This network is a useful platform from which to generate a hypothetical model mediated by components of interest.

Previous screening of our promoter trap lines had allowed us to identify rice Line 0-087-04 as having beta-glucuronidase (GUS) activity under the control of the *OsSTI1* (*Os04g45480*) promoter that encodes an STI-carrying TPR domain. Our current analysis suggested that the functional network is mediated by *OsSTI1*. The stress

category had 16 components associated with OsSTI1 signalling: eight for protein, three for RNA, and three (Table S4). Among its predicted interaction partners, we found five HSP70s (Os01g08560, Os02g02410, Os03g16920, Os05g38530, and Os11g47760) and three HSP90s (Os04g01740, Os08g39140, and Os09g30418), suggesting a role for OsSTI1 as a co-chaperone of HSP70 and HSP90. Indeed, one stress-inducible co-chaperone, *Caenorhabditis elegans* Sti1/HSP70/HSP90-organizing protein (Hop), functions as an adaptor protein that simultaneously binds to HSP70 and HSP90 to transfer client proteins between the two (Smith et al. 1993; Song et al. 2009). This further supports our prediction of a role for OsSTI1. Calreticulin is transcriptionally up-regulated by heat shock in humans (Nguyen et al. 1996). Similarly, two rice genes (Os03g61670 and Os07g14270) encoding calreticulin are stimulated in response to heat stress, implying that they operate in the signal pathway upstream of the OsSTI1–HSP70–HSP90 complex.

RiceNet predicted four DnaJ proteins (Os01g13760, Os03g57340, Os05g48810, and Os06g02620) as having a functional association with OsSTI1–HSP70–HSP90 complex. The DnaJ (Hsp40) co-chaperone regulates the DnaK (Hsp70) chaperone by accelerating ATP hydrolysis (Genevaux et al. 2002), supporting a functional association with HSP70 of the OsSTI1–HSP70–HSP90 complex and DnaJ in rice. A family of ubiquitin-like proteins binds the ATPase domain of Hsp70-like protein (Kaye et al. 2000), also suggesting a functional association of ubiquitin-like protein (Os03g03920) with that complex. Likewise, identification of an activator of HSP90 (Os08g36150) in the network suggests a possible interaction between HSP90 in the OsSTI1–HSP70–HSP90 complex and an activator of HSP90. Two zinc finger (C3HC4) proteins (Os06g19680 and Os07g43740), an HSF (Os10g28340), and a nuclear TF Y subunit (Os08g09690) might be transcriptional regulators modulating activity of OsSTI1–HSP70–HSP90 complexes, the biological functioning of which should be elucidated by further studies. Small heat shock proteins (sHSPs) of *Escherichia coli* cooperate with the chaperone protein ClpB/HSP100 and the DnaK (HSP70) protein in vitro and in vivo, forming a functional triad of chaperones (Mogk et al. 2003). This also implies a functional association of HSP20 (Os03g16020) and chaperone protein clpB/HSP100s (Os02g08490, Os03g31300, and Os05g44340) with HSP70 of the OsSTI1–HSP70–HSP90 complex. The predicted functional network with chaperonin (HSP60, Os03g25050 and Os07g44740), T-complex protein (Os10g32550), and the TPR domain protein (Os03g53910) of OsSTI1 might be mediated by HSP70 in OsSTI1–HSP70–HSP90 complexes. Indeed, the reported formation

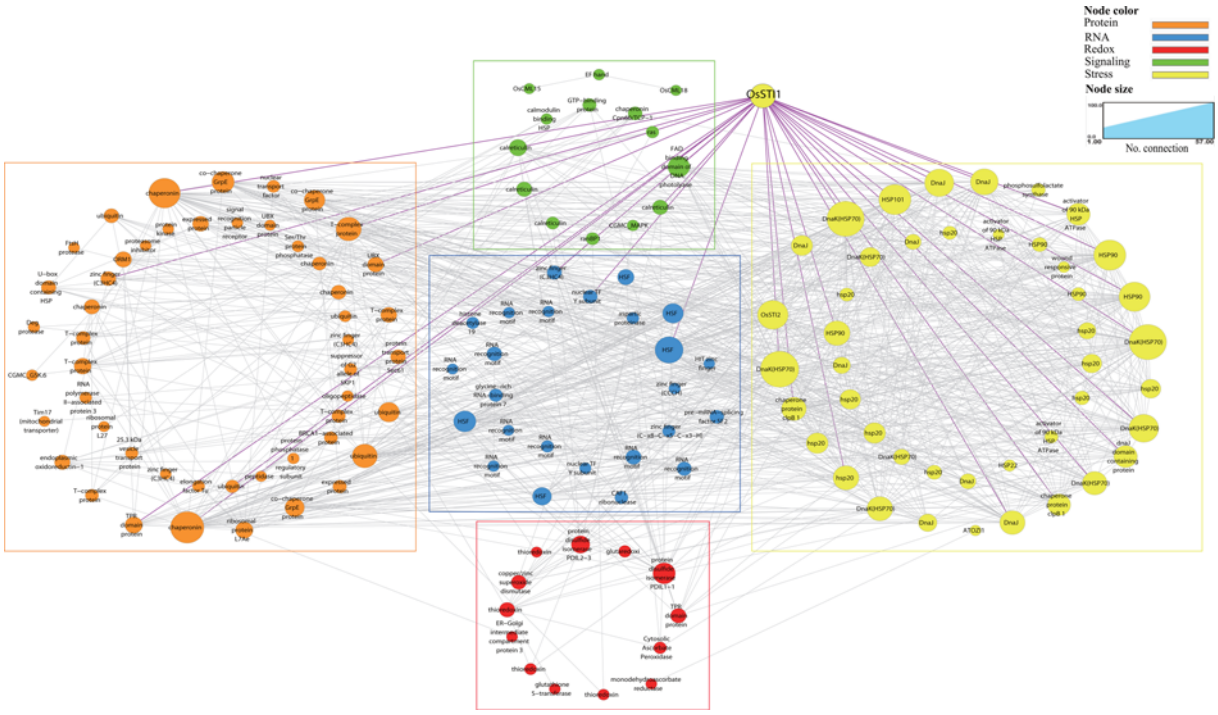

Fig. 2. Functional gene networks associated with early heat stress-responsive genes using RiceNet. The query covered 240 genes, revealing 937 interactions among 147 genes. Cystoscape (version 2.8.1) was used to develop network and to integrate functional categories per MapMan by node color: brown, protein; green, signaling; yellow, stress: blue, RNA; and red, redox. Nodes were labeled as annotations from Rice Genome Annotation Project database (Fig. 2) and locus identifiers (locus_IDs) (Fig. S2). The network mediated by OsSTI1 (Os04g45480) is highlighted in Fig. S2. Detailed information for all network components is shown in Table S4.

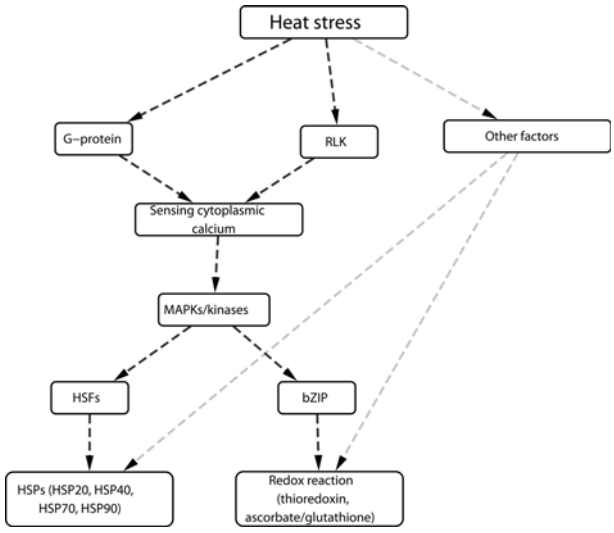

Fig. 3. Proposed model for signaling pathway mediated by heat stress in rice seedlings. Black dotted arrows indicate possible pathways identified from this analysis; and weak grey arrows show those not estimated.

of a stable complex between chaperonin-containing complex protein 1 and HSP70 in eukaryotes (Cuellar et al. 2008), the interaction of HSP60 and HSP70 in *Histoplasma capsulatum* (Guimaraes et al. 2011), and the participation of TPR domains in the assembly of HSP70–HSP90 multichaperone complexes (Scheufler et al. 2000) support the relationships predicted by RiceNet.

Conclusions and Future directions

Our MapMan analysis effectively suggested candidate genes in protein, RNA, stress, signaling, and redox categories that are highly accumulated for the early heat stress response in rice seedlings. Its effectiveness was demonstrated by our finding that the "heat stress" MapMan term was the most highly accumulated in response to heat stress. Based on this, we generated a model of signaling pathways to cope with heat stress in rice seedlings (Fig. 3). The elements found at each step of the model are good candidate genes for further functional analyses. Application of RiceNet after the MapMan analysis provides a molecular framework for understanding the early heat stress response in rice. In our network, the functional interaction partners of OsSTI1 were highlighted and sorted into different functional categories, suggesting diverse roles for *OsSTI1* and presenting avenues for future evaluation. Genome-wide gene-indexed mutant populations of rice have already been developed through international efforts (Jung et al. 2008a). Therefore, functional validation of these bioinformatics analysis results should be examined further. Systematic functional analysis of the components identified via this network might accelerate the discovery of the molecular mechanism underlying the early heat stress response in rice.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Guide for installing MapMan to analyze rice genes.

Fig. S2. Locus_ID version of Fig. 2.

Table S1. Early heat stress-responsive genes identified by NSF 45K array.

Table S2. Results of MapMan analysis for 589 early heat-inducible genes in rice.

Table S3. Fold-change data for nine HSF and six bZIP TF genes in response to drought, salt, cold, submergence, anoxia, or prolonged heat.

Table S4. Detailed information for the 147 components shown in Fig. 2.

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