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ORIGINAL ARTICLE

Deep Transcriptome Sequencing Reveals the Expression of Key Functional and Regulatory Genes Involved in the Abiotic Stress Signaling Pathways in Rice

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Abstract Drought, salt and cold are the major abiotic stresses that limit the rice production. Identification of the key functional and regulatory genes in the abiotic stress signaling pathways is important for understanding the molecular basis of abiotic stress tolerance. In this study, we investigated the transcriptomes of rice leaves and roots under cold, drought, and salt stresses using the massively parallel signature sequencing (MPSS) and sequencing by synthesis (SBS) technologies. About 1.8 to 2.6 million individual signatures were obtained from the seven abiotic-stressed and control libraries of the *japonica* cultivar Nipponbare. A total of 102,630 and 1,414,788 distinct signatures were obtained from the MPSS and SBS libraries, respectively. Clustering analysis identified many up- and down-regulated genes specifically and commonly expressed in the cold, drought and salt-treated plant leaves and roots. Data mining revealed the expression patterns of key functional and regulatory genes that were involved in different abiotic stress signaling pathways. Highly conserved *cis*-regulatory elements in the promoter of the up-regulated genes were identified. Our comprehensive and deep survey of abiotic stress transcriptome of rice has provided candidate genes for further understanding the molecular basis of abiotic stress tolerance in rice.

Key words: Abiotic stress, Expression profiling, Next generation sequencing, MPSS and SBS

Introduction

Cold, drought, and salt are the major abiotic factors limiting crop production worldwide. Crop losses due to abiotic stresses often reduce yields of major crops including rice by more than 50%, and yield losses in recent years have increased in many countries because of unpredictable weather and extremes associated with climate change (Boyer 1982; Bray et al. 2000; Dolferus et al. 2011; Lobell et al. 2011). Transcriptome analysis offers a powerful platform for determining the expression profiles of genes involved in abiotic stresses of plants at the molecular level. The most widespread and damaging abiotic stress experienced by rice is drought, which is especially severe in Asia where approximately one-third of the total rice area is subject to occasional or frequent drought stress (Huke and Huke 1997; Lafitte et al. 2004). Another important abiotic stress, low temperature, can affect about 15 million hectares of rice fields in 24 different countries (Lou et al. 2007). In Asia, cold weather in April often causes early season rice seedlings to rot (Xiong et al. 1984). Salt is another important stress, and millions of hectares of land suitable for rice production in Asia and Africa are currently not used because of high salt content in the soil associated with rising sea levels near coasts and excessive irrigation without proper drainage in

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inland. As a result of global warming, an increasing number of hectares in rice growing areas are predicted to be affected by drought, cold, and salt in the future (Lobell et al. 2011).

Plants adapt to abiotic stresses by altering thousands of genes and thereby changing cellular, physiological, and biochemical processes (Cushman and Bohnert 2000; Yamaguchi-Shinozaki and Shinozaki 2005; Umezawa et al. 2006). Microarrays and serial analysis of gene expression (SAGE) have been used to profile abiotic-stressed plants in order to identify transcripts and pathways related to stress tolerance mechanisms in model species such as *Arabidopsis* and rice (Desikan et al. 2001; Chen et al. 2002; Kreps et al. 2002; Seki et al. 2002a and 2002b; Oh et al. 2005; Mizuno et al. 2010; Yun et al. 2010; Mumomeni et al. 2011; Todaka et al. 2012). From these studies, thousands of genes with known or unknown functions relative to abiotic stresses and conserved or unique genes associated with responses to different abiotic stresses have been identified (Sreenivasulu et al. 2007). However, the relationships between the different signaling pathways involved in responses to diverse abiotic stresses are still not fully understood.

Abiotic stress pathways share common elements that are potential connection points for cross-talk. Cross-talk can also occur between abiotic stress pathways in different organs of the same plant when a systemic signal such as hydrogen peroxide moves from a stimulated cell into another tissue to elicit a response (Foyer et al. 1997; Knight and Knight 2001). Transcription factors (TFs) are known to play important roles in abiotic stress signal transduction (Chen and Zhu 2004). The expression of these genes is highly complex and is controlled at the transcriptional level by an intricate regulatory network. Abiotic stresses (including those generated by high salinity, osmotic change, cold, and jasmonic acid) induce the expression of TF genes like those encoding dehydration-responsive element/C-repeat (DRE/CRT) binding factors, CCA1 and Athb-8 proteins, Myb proteins, as well as bZIP/HD-ZIPs and AP2/EREBP domain proteins (Baima et al. 2001; Kizis et al. 2001). Seki et al. (2002a) employed a full-length cDNA microarray containing 7,000 independent *Arabidopsis* cDNAs to identify cold-, drought-, and salinity-induced genes and members of stress-related TF families such as DREB, ERF, WRKY, MYB, bZIP, helix-loop-helix and NAC. Water deficit conditions induce the production of abscisic acid (ABA), which plays an important role in the tolerance of plants to drought and high salinity. Exogenous application of ABA induces a number of genes that respond to dehydration and cold stress (Knight and Knight 2001; Zhu et al. 2002; Shinozaki et al. 2003; Chinnusamy et al. 2004; Nakashima et al. 2009). These studies suggest that cross-talk is common among the abiotic-stress signaling pathways.

Calcium acts as a second messenger in abiotic signal transduction (Ludwig et al. 2004). Calcium-binding proteins such as calmodulins, calmodulin-like proteins, calcineurin B-like proteins (CBL), calcium-dependent protein kinases (CDPKs), and calcineurin B-like interacting protein kinases (CIPK) play an important role in calcium-dependent abiotic stress signaling (Luan et al. 2002; Sanders et al. 2002). The other mechanism that connects extracellular stimuli to cellular response is the mitogen-activated protein kinase (MAPK) system, which is highly conserved in yeast, animals, and plants. The MAPK pathway is a three-component system that contains MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. Many MAPKs have been reported in rice for various abiotic stresses (Agrawal et al. 2003). The MAPK gene *OsMSRMK2* is highly induced by a variety of stresses including ABA, JA, SA, drought, and salt but not by cold (Agrawal et al. 2002).

In this study, we used both massively parallel signature sequencing (MPSS) and sequencing-by-synthesis (SBS) technologies to identify genes with altered expression patterns in the *Japonica* cultivar Nipponbare subjected to cold, drought, and salt stresses. Bioinformatic analyses revealed the expression of key functional and regulatory genes including those encoding MAPKs, SnRK2s, TFs, ion transporters, detoxifying proteins, late embryogenesis abundant (LEA) proteins, chaperons, heat shock proteins, and proteins in the calmodulin, ABA signaling, and ABA biosynthesis pathways. Promoter analysis of the highly up-regulated genes in stressed libraries identified conserved patterns of many *cis*-regulatory elements. Our results provide new insights into the regulatory networks of abiotic defenses in rice.

Results and Discussion

Characteristics of the MPSS Libraries

Seven MPSS libraries were constructed using the RNA samples extracted from leaf and root tissues of 2-week-old rice seedlings after subjecting to cold, drought, and salt treatments; these included three MPSS libraries from leaf tissue (subjected to cold, drought, and salt stresses), two MPSS libraries from root tissues (subjected to drought and salt stresses), and two control MPSS libraries (from untreated leaf and root tissues). About 1.8 to 2.6 million individual 17-base signatures were obtained in the seven MPSS libraries (Table 1). To compare the expression levels across the libraries, we normalized the frequency of signatures in individual libraries to one million (transcripts per million or TPM). We identified many genes that were commonly induced or suppressed by all three stresses and even more genes that were specifically induced or suppressed by only one of the three stresses. A total of

Table 1. Characteristics of the MPSS and SBS libraries

Signature category	Control leaves		Cold-stressed leaves		Drought-stressed leaves		Salt-stressed leaves		Control roots		Drought-stressed roots		Salt-stressed roots	
	NYL	SNYL	NCL	SNCL	NDL	SNDL	NSL	SNSL	NYR	SNYR	NDR	SNDR	NSR	SNSR
Total reads sequenced	2,249,147	5,495,682	2,322,924	5,546,106	2,613,140	6,729,976	2,531,362	6,592,886	1,944,785	5,070,977	2,190,870	5,102,633	1,842,226	3,824,471
Distinct	28,685	338,129	30,446	322,697	31,763	354,816	30,462	343,017	27,246	274,105	33,794	349,676	32,278	286,938
Reliable*	25,225	215,647	26,282	198,365	25,647	233,898	26,587	232,378	21,974	179,736	27,867	191,550	25,408	185,114
Unreliable*	3,460	122,482	4,146	124,332	6,116	120,918	3,875	110,639	5,272	94,369	5,927	158,126	6,870	101,824
Significant *	25,317	77,390	26,400	72,338	25,739	82,218	26,492	83,343	22,775	80,015	27,918	83,715	26,667	79,438
Non-significant*	3,368	260,739	4,046	250,359	6,024	272,598	3,970	259,674	4,471	194,090	5,876	265,961	5,611	207,500
Reliable significant*	24,474	77,381	25,094	72,277	24,536	82,212	25,543	83,342	21,048	79,731	26,434	82,808	24,292	79,311
Reliable non-significant*	751	138,266	1,188	126,088	1,111	151,686	1,044	149,036	926	100,005	1,433	108,742	1,116	105,803
Reliable and significant ≥4TPM	19,648	50,029	19,592	45,752	18,989	45,225	20,483	44,044	17,130	35,419	21,333	38,904	19,722	40,828
1-100 TPM	25,853	334,370	28,009	319,300	29,131	350,985	27,605	339,132	24,693	270,317	31,163	345,709	29,969	282,972
101-1,000 TPM	2,678	3,554	2,262	3,129	2,506	3,647	2,721	3,679	2,405	3,580	2,505	3,782	2,143	3,777
1,001-10,000 TPM	150	200	172	263	123	180	133	201	143	205	124	183	160	187
>10,000 TPM	4	5	3	5	3	4	3	5	5	3	2	2	6	2
Distinct genes (≥4 TPM)	10,322	16,589	8,895	14,137	9,907	17,919	10,458	17,021	8,524	17,776	10,232	18,635	9,434	18,325
Signatures match Nipponbare genomic sequence (≥4 TPM)	17,203 (84%)	30,047 (60%)	17,492 (84%)	27,928 (61%)	16,471 (82%)	32,821 (73%)	17,522 (82%)	32,217 (73%)	13,805 (73%)	31,152 (88%)	17,484 (77%)	33,584 (83%)	16,312 (74%)	33,421 (82%)
Significant signatures identified by both MPSS and SBS	13,020 (66%)		11,914 (61%)		11,803 (62%)		13,561 (66%)		9,408 (55%)		12,095 (57%)		10,701 (54%)	

*Number of signatures passed through reliability and significance filters as described by Meyers *et al* (2004a and 2004b).
The code for the libraries: MPSS leaf library-NCL, SBS leaf library-SNCL, MPSS leaf library-NDL, MPSS root library-NDR, SBS leaf library-SNDL, SBS root library-SNDR, MPSS leaf library-NSL, MPSS root library-NSR, SBS leaf library-SNSL, SBS root library-SNSR, MPSS leaf library-NYL, MPSS root library-NYR, SBS leaf library-SNYL and SBS root library-SNYR

102,630 distinct 17-base signatures were obtained from the seven libraries (Fig. S1). These signatures were processed according to three filters as described in Meyers et al. (Meyers et al. 2004a and 2004b): significance, reliability, and genomic matches. When all the unique, reliable, and significant signatures (4 TPM) from the seven libraries were clustered, a total of 78,017 unique significant signatures were obtained. Clustering of reliable significant signatures identified 47,886 unique signatures that have only one hit in the rice genome (hits = 1) (Fig. S1; Table S2). When the seven libraries were compared, the number of distinct signatures ranged from 17,604 to 33,794, and the number of reliable signatures ranged from 21,974 to 27,867.

Validation of the MPSS Data

To validate the MPSS data, we selected six genes showing differential expression in the cold-, drought-, and salt-stressed leaf libraries for strand-specific RT-PCR. RNA isolated at different time intervals after the stress treatment was used. Similar to the expression levels in the respective MPSS libraries, expression levels in the leaves of cold-treated plants were relatively high for *AK100623* encoding a hypothetical protein and *AK070197* encoding a probable lipase. *AK109389* encoding a remorin-like protein and *AK058896* encoding a nonspecific lipid transfer protein 2 precursor (*Ltp2*) showed a much stronger expression in the leaves of drought-treated plants than in non-stressed plants similarly, Wong et al. 2006 reported abundance of transcripts to be significantly changed under drought, cold, high-salinity, corresponding to a gene encoding a member of the protease/lipid transfer protein (LTP) family (*At2g10940*). Further they reported that number of transcripts regulated by salinity was less than one-fifth and one-third of that of drought and cold-regulated genes, respectively. *AK072902*, encoding a putative RNA-recognition motif protein and *AK068486* encoding a putative U-box domain protein also showed a strong expression only in the leaves of salt-treated plants (Fig. 1; Table S1). It is noteworthy that the *Ltp2* gene was highly up-regulated 24 h after the drought treatment whereas its expression did not change after cold and salt treatments. Also, the expression of *AK072902* was moderate at 12 and 24h, but was significantly reduced at 48h, 5D, 6D, and 7D in the drought stressed leaves. In general, the expression patterns revealed by the MPSS and RT-PCR data were similar for about 70-80% of the genes.

Characteristics of the SBS Libraries

Seven SBS libraries were constructed using the same RNA samples that were used to construct the MPSS library described earlier. About 3.8 to 6.7 million individual 20-base signatures were obtained from the seven SBS libraries (Table 1), of which 1,414,788 were distinct signatures (Fig. S1). Like the MPSS data, the SBS signatures were processed for their significance, reliability, and genomic match as described in Meyers et al. (2004a and 2004b). A total of 589,645 reliable signatures and 825,142 unreliable signatures were identified in the seven libraries. Of the 75,296 reliable significant signatures, 67,550 (~89.7%) had one hit in the rice genome (hits=1) and 7,746 (~10.3%) signatures matched to the Nipponbare rice genomic sequence more than once (hits >1) (Fig. S1; Table S2). When all the seven libraries were compared, the number of distinct signatures ranged from 274,105 (control roots) to 354,819 (drought-stressed leaves) (Table 1). Among the SBS libraries, the number of distinct genes identified using the significant signatures (≥4 TPM) was highest in the drought-stressed roots (18,635 genes) (Table 1).

Correlation of the Transcriptome Results Generated by MPSS and SBS

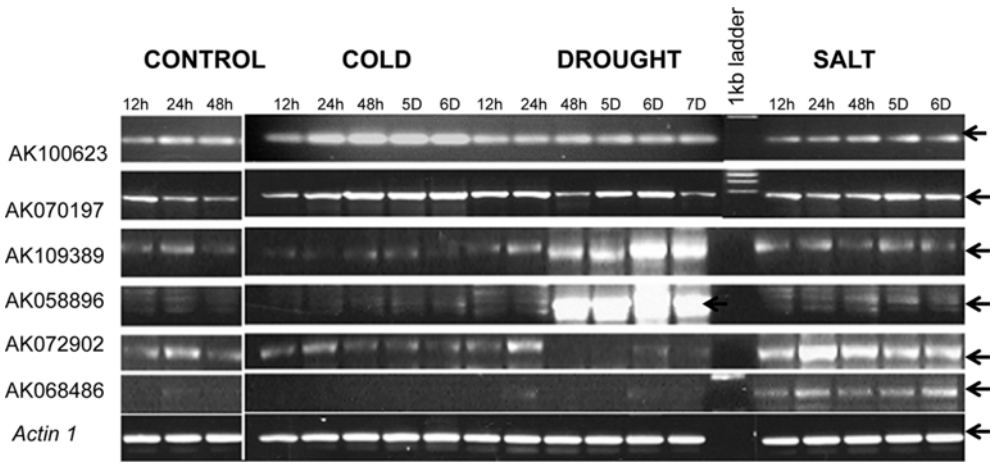

Fig. 1. RT-PCR validation of the differentially expressed genes in the abiotic-stressed and control leaves.

Table 2A. Correlation between the transcriptomes revealed by the MPSS vs SBS technologies

Library	Correlation coefficient	
	Before removing outliers*	After removing outliers*
Untreated control leaves	0.4	0.6 (removal of 172 out of 9447 signatures)
Cold-stressed leaves	0.3	0.6 (removal of 83 out of 8722 signatures)
Drought-stressed leaves	0.3	0.5 (removal of 132 out of 6918 signatures)
Salt-stressed leaves	0.5	0.6 (removal of 55 out of 9830 signatures)
Untreated control roots	0.4	0.6 (removal of 197 out of 7580 signatures)
Drought-stressed roots	0.4	0.6 (removal of 118 out of 9495 signatures)
Salt-stressed roots	0.3	0.6 (removal of 186 out of 8226 signatures)

*Genome matched signatures used

Table 2B. Correlation between stress transcriptomes detected by MPSS and SBS technologies

	Correlation coefficient	
Library	MPSS	SBS
Cold vs drought	0.24	0.32
Cold vs salt	0.52	0.61
Drought vs salt	0.74	0.80

About 54 to 66% of the significant signatures overlapped between the MPSS and SBS libraries (Table 1). We subjected all the significant signatures in the MPSS and SBS libraries to Pearson correlation coefficient analysis. The correlation coefficients were low when the unfiltered MPSS and SBS data were used (Table 2). Removal of a few outliers (~0.6-2.5% of the signatures) substantially increased the correlation coefficients in all seven libraries (Table 2A). For example, the correlation coefficient between the two cold-stressed leaf libraries was increased from 0.3 to 0.6 after removal of only 83 of the 8722 signatures. To detect the similarity between different stress transcriptomes, we compared the frequencies of all the signatures obtained in the cold, drought, and salt libraries (cold vs. drought, cold vs. salt, and drought vs. salt) by Pearson correlation coefficient analysis. The correlation was very high between the drought- and salt-stressed transcriptomes in both MPSS (0.74) and SBS (0.80) libraries. The correlation was moderate to high between cold- and salt-stressed transcriptomes in the MPSS (0.52) and SBS (0.61) libraries. The correlation was weak, however, between the cold- and drought-stressed transcriptomes in both the MPSS (0.24) and SBS (0.32) libraries (Table 2B).

Matching to Genomic Databases to Identify Antisense and Alternative Transcripts

The reliable significant (≥4TPM) MPSS and SBS experimental signatures were matched to the rice Nipponbare genomic sequence and annotated genes in order to identify sense, antisense, and novel transcripts expressed in the stressed and control libraries. The analysis for the leaf libraries indicated that more significant MPSS signatures (82-84%) than significant SBS signatures (60-73%) matched the rice genome. For the root libraries, however, more significant SBS signatures (82-88%) than significant MPSS signatures (73-77%) matched the rice genome (Table 1). About 10 to 17% and 11.4 to 14% of the signatures represent antisense transcripts in the MPSS and SBS libraries, respectively (Table S2). A high number of antisense signatures (classes 3, 6) were found in both the drought-stressed (MPSS 2,276; SBS 3,926) and salt-stressed (MPSS 2,308; SBS 4,041) root libraries in both the MPSS and SBS data. The control root libraries had fewer antisense signatures (MPSS 1,433; SBS 3,543) than any of the other libraries (Table S2).

The reliable significant (≥4TPM) MPSS and SBS signatures were matched to the rice annotated genes (version 6) to determine how many different transcript tags were matched to a single annotated gene and to thereby identify alternative splicing forms. Cluster analysis revealed that many genes undergoing alternative splicing/termination in both the MPSS and SBS libraries (Table S3). In addition, many genes were commonly present in both the MPSS and SBS libraries and others were specifically expressed in one library. In the cold-stressed leaf library, among 579 genes, the gene family *Os04g33640* "Glycosyl hydrolases family 17" showed highest 11/8 (SBS/MPSS) alternate transcripts. Similarly, among the 455, 296 genes which showed formation of alternate transcripts in drought and salt leaf library, *Os10g05990* (pollen Ole e I allergen and extensin family protein) and *Os11g42550* (dirigent protein) displayed highest alternate transcripts. Flavonol synthase gene (*Os10g40934*) and OsWAK receptor like cytoplasmic kinase (*Os01g20900*) showed the formation of maximum alternate transcripts in drought-stressed and salt-stressed root libraries respectively (Table 3 and Table S3). Additionally, we identified several genes that are known to have potential roles in constitutive and alternate splicing mRNA like *Os12g38430* (SR repressor protein), *Os03g27840* (splicing factor, arginine/serine-rich), *Os07g38730* (tubulin/FtsZ domain containing

Table 3. List of important stress-regulated genes specifically expressed in each stress condition and undergoing alternative splicing

Tissue	Gene ID	Gene description	No of alternative transcripts (SBS)	No of alternative transcripts (MPSS)
Cold-stressed leaves	Os04g33640	Glycosyl hydrolases family 17	11	5
	Os03g26910	Glycosyl hydrolase Rv2006/MT2062	10	8
	Os09g39910	ABC transporter ATP-binding protein	7	4
	Os07g05940	9-cis-epoxycarotenoid dioxygenase 1-	6	3
	Os12g43720	Early-responsive to dehydration protein	5	7
	Os03g03370	Fatty acid hydroxylase	7	2
	Os02g52780	bZIP transcription factor	5	4
	Os05g46350	Calmodulin-binding motif domain containing protein	5	2
	Os03g09170	Ethylene-responsive transcription factor	4	4
Drought-stressed leaves	Os10g05990	Pollen Ole e I allergen and extensin family protein	7	2
	Os03g60340	Leaf senescence-related protein	4	5
	Os01g02870	Auxin-induced protein 5NG4	4	2
	Os05g48390	Ubiquitin conjugating enzyme protein	3	3
	Os09g30400	OsWRKY80 - Superfamily of TFs having WRKY and zinc finger domains	3	2
	Os09g36580	Thaumatin	3	2
Salt-stressed leaves	Os11g26790	Dehydrin	7	4
	Os01g59819	beta-glucosidase	6	2
	Os05g07940	Glyoxalase family protein	6	4
	Os06g48300	Protein phosphatase 2C	4	2
	Os06g14030	Potassium channel SKOR	4	2
Drought-stressed roots	Os10g40934	Flavonol synthase	5	2
	Os08g20130	Flavonol sulfotransferase	4	4
	Os01g10600	Aquaporin protein	3	2
	Os01g69090	CBS-domain containing protein	3	2
	Os09g33810	ankyrin repeat domain contain protein	3	2
	Os11g04460	Calcium-transporting ATPase	3	2
Salt-stressed roots	Os01g20900	OsWAK receptor-like cytoplasmic kinase	3	2
	Os02g11740	ATP/ADP-transporter	2	3
	Os04g36740	Potassium channel SKOR	2	3
	Os03g38740	Dicer, putative	3	2
	Os03g16960	Cysteine-rich repeat secretory protein 55 precursor	4	2

protein), *Os04g40310* (dehydrogenase) and *Os03g22380* (RNA recognition motif containing protein) (Anireddy 2004; Lida et al. 2006; Wang and Brendel, 2006) (Table S3). Thus, the alternate splicing events may play a role in response to abiotic stresses in rice.

Comparative Analysis of the Cold-, Drought-, and Salt-stressed Transcriptomes of Rice Leaves and Roots

We classified the up- or down-regulated genes in the stress libraries into two categories: 1) genes specifically expressed in the stressed plants and absent in the control plants; and 2) genes differentially (up- or down-regulated) expressed in the stressed vs. control plants.

For the category 1 genes, we identified the significant signatures present in both the stress MPSS and SBS libraries and subtracted those that were present in the control libraries for cluster analysis. When all the genes specifically expressed in the stress libraries were clustered, a total of 294 genes in leaves (cold, drought, and salt) and 105 genes in roots (drought and salt) were commonly expressed under all the conditions (Fig. 2A and 2B). When genes specifically expressed in each stress condition were compared, more drought-stress specific genes (1,114 in leaves and 1,828 in roots) were expressed than salt-stress specific genes (866 in leaves and 1,030 in roots) (Fig. 2A and 2B).

For category 2 genes, a gene with ≥3-fold expression in the stressed library than in the control in both the MPSS and

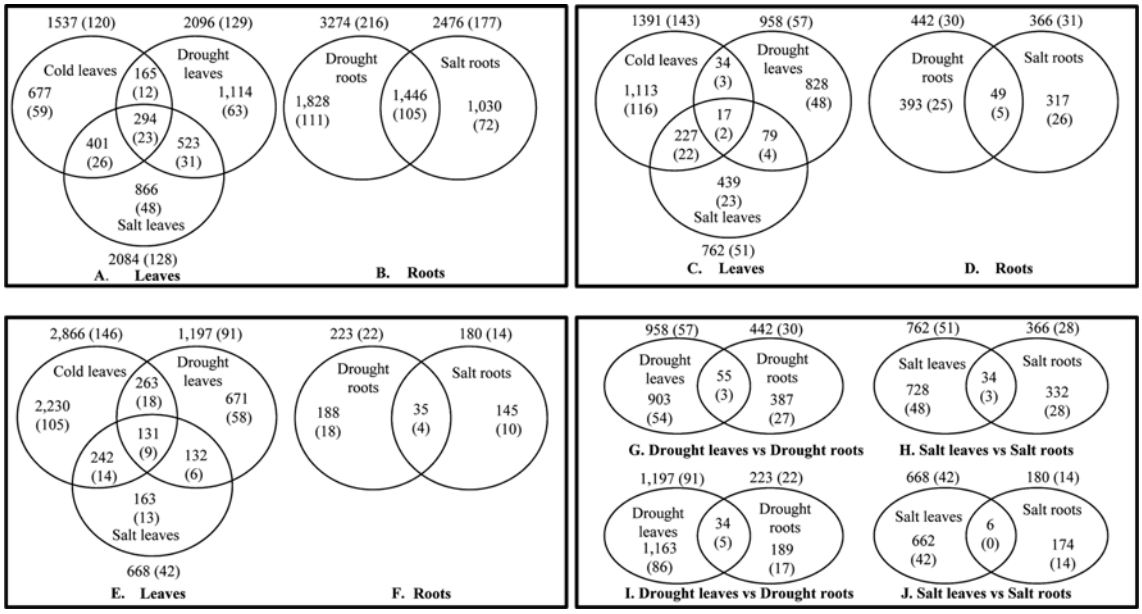

Fig. 2. Cluster analyses of genes up- and down-regulated in plants subjected to stress (relative to expression in non-stressed plants). The distribution of genes specifically and commonly expressed in the cold-, drought-, and salt-stressed Nipponbare leaves (A) and roots (B) compared to leaves and roots of nonstressed control plants (absent). Values in parentheses indicate the number of transcription factor genes expressed. Cluster analysis showing the distribution of 3-fold up-regulated genes (C and D) and down-regulated genes (E and F) genes specifically and commonly expressed in stressed leaves and roots. Values in parentheses indicate the number of transcription factor genes up-regulated (C and D) or down-regulated (E and F). Cluster analysis showing the root-specific and leaf-specific up- and down-regulated genes. Genes with 3-fold up-regulation (G and H) and down-regulation (I and J) in drought- and salt-stressed leaves and roots were compared. Values in parenthesis indicate the number of transcription factor genes up-regulated (G and H) or down-regulated (I and J). Only the genes commonly identified in both the MPSS and SBS libraries were used for the cluster analyses.

SBS libraries was considered to be stress-induced, and a gene with ≤3-fold expression in the stressed library than in the control in both the MPSS and SBS libraries was considered to be stress-repressed. Cluster analysis showed that 17 and 131 genes were commonly up- and down-regulated, respectively, in the stressed-leaf libraries relative to the control library (Fig. 2C and 2E; Table S4 and Table 4). Similarly, 49 and 35 genes were up- and down-regulated in both the drought- and salt-stressed roots, respectively (Fig. 2D and 2F; Table S4 and Table 5). More genes were commonly up- (244) and down-regulated (373) between the cold- and salt-stressed leaves than between cold- and drought-stressed leaves or between drought- and salt-stressed leaves. A group of genes (836 genes in SBS and 1365 genes in MPSS) with ≥3-fold up-regulation in the leaf transcriptomes was evident after drought and salt stresses, suggesting the existence of a common regulatory system or cross-talk between the two stress responses (Table S4). Several studies have shown that different abiotic stresses induce similar stress-inducible genes, indicating a cross-talk between the stresses (Nordin et al. 1993; Knight 2003; Mantyla et al. 1995). The cross-talk between any two stresses was established based on the number of similar up- or down-regulated genes and the number of stress-specific genes. From the analysis, a moderate to high degree of cross-talk was evident between drought- and salt-stress responses, and between cold- and salt-stress responses in leaves.

We identified induced and repressed genes in the cold-stressed leaves (1,391 induced, 2,866 repressed), drought-stressed leaves (958 induced, 1,197 repressed), salt-stressed leaves (762 induced, 668 repressed), drought-stressed roots (442 induced, 223 repressed), and salt-stressed roots (366 induced, 180 repressed) (Fig. 2C, 2D, 2E and 2F). More genes were specifically up-regulated than down-regulated in the drought-stressed leaves and roots (828 vs. 393) and in the salt-stressed leaves and roots (439 vs. 317). In the case of the cold-stressed leaves, more genes (2,230) were down-regulated than up-regulated (Fig. 2E). We also observed that most up-regulated genes were stress-specific in both the leaf and root libraries. Our observations are consistent with the findings in cold, salt and drought stress rice (Rabbani et al. 2003; Mizuno et al. 2010; Moumeni et al. 2011), cold- and high-salinity-stressed *Arabidopsis* (Kreps et al. 2002) and with those in drought- and high-salinity-stressed barley (Ozturk et al. 2002). These genes might be involved in the signaling perception of stress responses at early treatment stages. It is not known whether these genes are also involved in different stress responses or not. Therefore, further

Table 4. The abiotic stress-related genes commonly up- and down-regulated in the cold-, drought-, and salt-stressed leaves

Gene category	Gene ID	Gene name	MPSS signature	SBS signature	Cold (ratios)		Drought (ratios)		Salt (ratios)	
					MPSS	SBS	MPSS	SBS	MPSS	SBS
Commonly expressed genes in cold-, drought-, and salt-stressed leaves	Os07g44180	Hydrophobic protein LTI6A	GATCCGGAGCCTGTGAT	GATCCGGAGCCTGTGATTGG	10	4	7	4	29	15
	Os07g04700	MYB transcription factor	GATCTCAGTAGTCCCAG	GATCTCAGTAGTCCCAGGAT	17	4	10	9	5	6
	Os03g30570	RNA-binding protein	GATCTCGTCCGACTCGC	GATCTCGTCCGACTCGCCGC	44	10	34	4	14	4
	Os01g65670	Amino acid transporter	GATCGGGTGCGTCGGGT	GATCGGGTGCGTCGGGTACG	9	12	32	6	23	4
	Os09g34910	Nucleolar RNA helicase 2	GATCAATTTTGCCATCG GATCATCATAATCTCGA*	GATCAATTTTGCCATCGCAA GATCATCATAATCTCGAACA*	18	5	17	4	17*	3*
	Os01g08700	GIGANTEA protein	GATCATCTGTAACTCTC* GATCAGCGAGTAGGAAA	GATCATCTGTAACTCTCATT* GATCAGCGAGTAGGAAAGGA	10*	17*	13	6	5	4
Commonly suppressed genes in drought-, and salt-stressed roots	Os01g04370	169 kDa class I heat shock protein 1	GATCGGTGAGGGAAGAAGTC	GATCGGTGAGGGAAGAA	-44	-25	-44	-6	-44	-6
	Os01g04800	DNA-binding protein RAV1	GATCATTCAAGTATGTACAA	GATCATTCAAGTATGTA	-86	-9	-14	-4	-3	-5
	Os01g71090	Xylanase inhibitor	GATCCTCGGAGGGTTCCAGA	GATCCTCGGAGGGTTCC	-85	-4	-85	-7	-85	-4
	Os03g58980	Germin-like protein subfamily member 2 precursor	GATCATCAAACTGCTCACGT	GATCATCAAACTGCTCA	-90	-7	-4	-7	-10	-7
	Os04g41229	DNA-binding protein	GATCATTAGACATTATCCCG	GATCATTAGACATTATC	-6	-6	-6	-4	-6	-4

*Ratios refer to up-regulation (positive values) or down-regulation (negative values) relative to the non-stressed control

Table 5. The abiotic stress-related genes commonly up- and down-regulated in the drought- and salt-stressed roots (compared to the controls)

Gene category	Gene ID	Gene name	MPSS signature	SBS signature	Drought (ratios)		Salt (ratios)	
					MPSS	SBS	MPSS	SBS
Commonly 3-fold up-regulated genes in drought- and salt-stressed roots	Os09g16510	OsWRKY74 Superfamily of rice TFs having WRKY and zinc finger domains	GATCCTATCTGAATTTC	GATCCTATCTGAATTTCTGA	5	5	3	5
	Os08g41880	Nucleotide pyrophosphatase/phosphodiesterase	GATCGTGTTTCTCTTCC	GATCGTGTTTCTCTTCCTCA	56	14	6	3
	Os05g41210	Calmodulin	GATCATCTCGTCGACTT	GATCATCTCGTCGACTTCCT	13	4	22	3
	Os08g38020	bZIP protein gene	GATCGATTTTTCTGTTT	GATCGATTTTTCTGTTTCGA	17	6	9	10
	Os02g40730	Ammonium transporter 1, member 2	GATCATCTGTTTGGAAC	GATCATCTGTTTGGAACAAA	142	4	205	4
Commonly 3-fold down-regulated genes in drought- and salt-stressed roots	Os07g48090	CBL-interacting serine/threonine-protein kinase 11	GATCATCACCCACCCGT	GATCATCACCCACCCGTGGT	-22	-3	-22	-3
	Os10g31640	glycine-rich cell wall structural protein 2 precursor	GATCCCCAGTTACCCCC	GATCCCCAGTTACCCCCAGC	-73	-4	-48	-4
	Os06g13180	metalloendoproteinase 1 precursor	GATCACGGCGACGTCGC	GATCACGGCGACGTCGCAGA	5	5	3	5
	Os02g06930	serine/threonine-protein kinase receptor precursor	GATCTTAAGGTCAGGAG	GATCTTAAGGTCAGGAGCAT	56	14	6	3
	Os07g37400	F-box domain containing protein gene	GATCCCTTAACTCCTCC	GATCCCTTAACTCCTCCAAA	13	4	22	3

Table 6. Identification of abiotic stress-related *cis* elements in the promoter regions of 3-fold up-regulated genes in the cold-, drought-, and salt-stressed rice leaves and roots compared to the controls (values in parentheses indicate the % of genes)

Sl No	Motif Name	Sequence	Cold-stressed leaves	Drought-stressed leaves	Salt-stressed leaves	Drought-stressed roots	Salt-stressed roots	Stress	Reference
1	ACGTATERD1	ACGT	1,225(88.3)	802(83.9)	635(83.4)	380(86)	305(83.3)	Light/dark, Dehydration	(Simpson et al. 2003)
2	MYBCORE	CNGTTR	1,228(88.5)	872(91.2)	687(90.3)	391(88.5)	322(88)	Dehydration/water stress	(Solano et al. 1995)
3	GT1GMSCAM4	GAAAAA	1,180(85)	806(84.3)	650(85.4)	390(88.2)	307(83.9)	Pathogen, Salt	(Park et al. 2004)
4	ABRELATERD1	ACGTG	1017(73.3)	594(62.1)	519(68.2)	300(67.9)	247(67.5)	Dehydration/ABA/Light	(Simpson et al. 2003)
5	MYB2CONSENSUSAT	YAACKG	935(67.4)	672(70.3)	545(71.6)	294(66.5)	240(65.6)	Dehydration/ABA	(Abe et al. 2003)
6	LTRECOREATCOR15	CCGAC	911(65.6)	528(55.2)	458(60.2)	234(52.9)	216(59)	cold, drought, ABA	(Baker et al.1994)
7	CBFHV	RYCGAC	878(63.3)	538(56.3)	461(60.6)	252(57)	218(59.6)	Cold stress	(Xue 2002)
8	DRECRTCOREAT	RCCGAC	692(49.9)	393(41.1)	344(45.2)	172(38.9)	154(42.1)	drought, high salt, high light, cold/Heat	(Dubouzet et al. 2003)
9	MYCATERD1	CATGTG	626(45.1)	466(48.7)	340(44.7)	217(49.1)	167(45.6)	water stress	(Simpson et al. 2003)
10	MYCATRD22	CACATG	626(45.1)	466(48.7)	340(44.7)	217(49.1)	167(45.6)	water stress/ABA	(Abe et al. 1997)
11	ACGTABREMOTIFA2OSEM	ACGTGKC	420(30.3)	168(17.6)	225(29.6)	82(18.6)	73(19.9)	Salt and Dehydration	(Hattori et al. 2002)
12	DRE2COREZMRAB17	ACCGAC	385(27.7)	217(22.7)	175(23)	108(24.4)	87(23.8)	Drought, Cold, Salt, ABA	(Busk et al.1997)
13	ABREOSRAB21	ACGTSSSC	314(22.6)	120(12.6)	148(19.4)	72(16.3)	67(18.3)	Osmotic stress	(Marcotte et al.1989)
14	MYB2AT	TAACTG	323(23.3)	292(30.5)	212(27.9)	106(24)	99(27)	water stress	(Urao et al. 1993)
15	AGCBOXNPGLB	AGCCGCC	199(14.3)	123(12.9)	100(13.1)	52(11.8)	46(12.6)	salt/pathogen	(Hart et al. 1993)
16	CRTDREHVCBF2	GTCGAC	168(12.1)	99(10.4)	87(11.4)	39(8.8)	40(10.9)	cold stress	(Xue et al. 2003)
17	ABREATRD22	RYACGTGGYR	147(10.6)	111(11.6)	61(8)	56(12.7)	33(9)	water stress	(Iwasaki et al. 1995)
18	MYBATRD22	CTAACCA	107(7.7)	30(3.1)	52(6.8)	11(2.5)	11(3)	Dehydration/water stress/ ABA	(Abe et al 1997)
19	ABREZMRAB28	CCACGTGG	87(6.3)	27(2.8)	43(5.7)	17(3.8)	8(2.2)	Cold, ABA and Water stress	(Suzuki et al. 2005)
20	LTREATLTI78	ACCGACA	95(6.8)	84(8.8)	50(6.6)	33(7.5)	31(8.5)	cold stress	(Nordin et al. 1993)

detailed functional analysis of these genes may provide new insights into the abiotic stress signaling mechanism.

In addition, drought- and salt-stress transcriptomes were highly correlated in both MPSS (r = 0.74) and SBS (r = 0.8) data, and cold- and salt-stress transcriptomes were moderately to highly correlated in the MPSS (r = 0.52) and SBS (r = 0.61) data (Table 2B). In all the cases, the number of genes whose up-regulation or down-regulation was specific to one of the three stresses was quite high compared to those commonly expressed by any two stresses.

Conserved *cis*-regulatory Elements in the Promoters of Up-regulated Genes

We analyzed the promoters of the up-regulated genes in all the libraries. The analysis revealed the existence of many abiotic stress-related conserved *cis* elements in the promoters of the genes that were ≥3-fold up-regulated in the cold-stressed leaves (783 genes), drought-stressed leaves (430 genes), salt-stressed leaves (252 genes), drought-stressed roots (121 genes), and salt-stressed roots (104 genes). The conserved motifs that were detected in the promoter regions of these up-regulated genes and that have previously been reported to be involved in abiotic stress signaling pathways are listed in Table 6. For example, the *cis* elements belonging to the following families were highly represented among the genes up-regulated in the stressed libraries: WRKY71OS, ACGTATERD1, MYBCORE, GT1GMSCAM4, WBOOXA-TNPR1, ABRELATERD1, MYB1AT, MYB2CONSEN-SISAT, ASF1MOTIFCAMV, CBFHV, LTRECOREATCO-R15, GCCCORE, DRECRTCOREAT, and MYCCONSEN-SUSAT (Table 6). Genes containing these motifs are induced in response to cold, salt, drought, ABA, wounding, or pathogens in rice and other plant species (Oh et al. 2005; Xue 2003; Eulgem et al. 1999; Kizis and Pages 2002; Svensson et al. 2006). The DRE motif with the core sequence

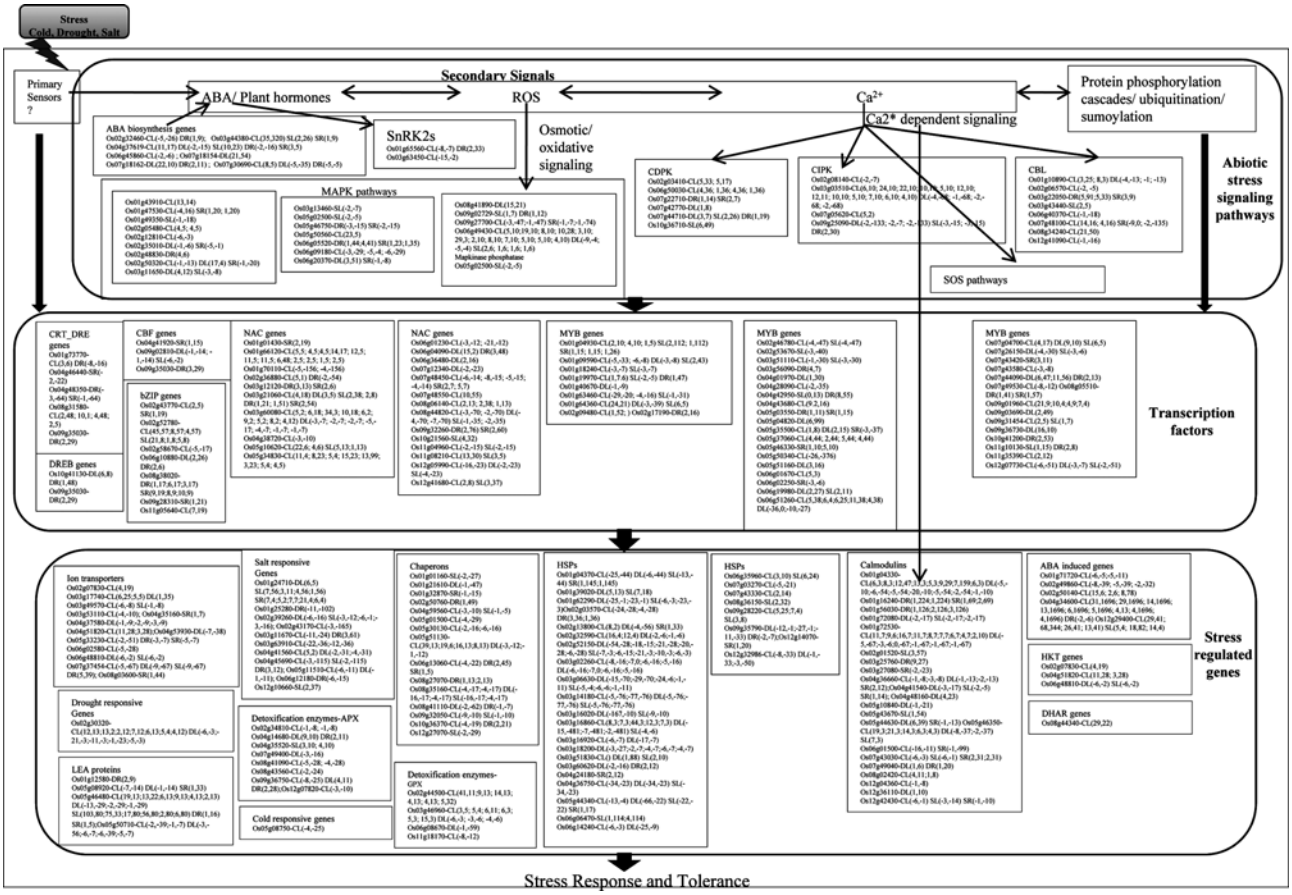

Fig. 3. The expression of the key genes in the networks of abiotic stress-related pathways. These genes belong to MAP kinase, Ca^{2+}-dependent signaling, and osmotic or oxidative signaling pathways in rice. The transcripts identified in both the MPSS and SBS libraries were used for the analysis. The positive numbers in parentheses indicate up-regulation, and the negative numbers in parentheses indicate down-regulation. The first and second values before the semicolon in the parentheses indicate the fold-change in gene expression in SBS and MPSS data, respectively, identified in abiotic-stress libraries (CL, cold stressed leaves; DL, drought stressed leaves; SL, salt stressed leaves; DR, drought stressed roots; SR, salt stressed roots). Many genes produced multiple transcripts because of alternative transcription, and the fold change of such alternative transcripts in both SBS and MPSS data are separated by semicolon within the parentheses for each gene.

A/GCCGAC was identified as a *cis*-acting promoter element in regulating gene expression in response to drought, high-salinity, and cold stresses in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki. 1994). Many stress-inducible genes were also induced by exogenous application of ABA. These genes contain potential ABA-responsive elements (ABREs) in their promoter regions (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 2000). Interestingly, many of these motifs were detected in the promoter region of the stress-responsive genes identified in this study. Thus, it is likely that these *cis* elements are evolutionarily conserved among the stress-responsive genes across plant species. Further evaluation of these *cis* elements will be useful for the development of synthetic promoters that are responsive to multiple stresses (Walley et al. 2007).

Abiotic Stress Signaling and Defense Genes

Abiotic stress signaling includes calcium-dependent pathways, ABA-dependent or -independent pathways, and MAP kinase pathways. Many genes involved in abiotic stress signaling and transcription regulation were up- or down-regulated in the stressed libraries (Fig. 3). Some of the important genes showing significant transcriptional changes in response to the three abiotic stresses are summarized in the following sections.

Genes in the calcium-dependent pathway

Calmodulins are Ca^{2+}-binding proteins that are highly conserved in eukaryotes; they can sense the changes in the intracellular concentration of Ca^{2+} resulting from any stress. Calmodulin protein genes such as those encoding calmodulin-related protein 2 (*Os01g04330*), calmodulin-related protein (*Os01g59530*), calmodulin-like protein 41 (*Os01g72530*), and IQ calmodulin-binding region protein (*Os05g46350*) were up-regulated in the cold-stressed leaves but were down-regulated in the drought-stressed leaves.

The calcineurin B-like (CBL) proteins contain calcium-binding motifs and interact specifically with a group of Ser/Thr protein kinases designated as CBL-interacting protein kinases (CIPKs). The CBL proteins form a complex network with their target kinases CIPKs and regulate target gene expression (Shi et al. 1999; Das and Pandey 2010; Kolukisaoglu et al. 2004). The activity of the CIPK protein kinase SnRk3 is dependent on CBL in plants (Hrabak et al. 2003). In the cold-stressed leaves, genes encoding CBL-interacting serine/threonine protein kinase 15 (*Os01g10890, Os07g48100*, and *Os08g34240*) were up-regulated in both the MPSS and SBS libraries. The gene encoding the CBL-interacting serine threonine protein kinase 24 (*Os06g40370*), however, was down-regulated in the cold-stressed leaves in both the MPSS and SBS libraries. During drought stress, the CBL-interacting serine threonine protein kinase 15 (*Os03g22050*) was up-regulated in both the leaves and roots, and was also induced in the salt-stressed roots, suggesting a cross-talk among these two stresses through this protein (Fig. 3 and Table S4). Many CIPK genes were differentially expressed in the stressed leaves and roots as revealed by both MPSS and SBS technologies. For example, the *CIPK* genes *Os01g10890, Os07g48100, Os08g34240, Os02g06570*, and *Os12g41090* and the CBL-interacting serine threonine protein kinase gene *Os06g40370* showed differential expression in cold-stressed rice leaves compared to controls. Similarly, the CIPK-like protein 1 genes, *Os03g20380, Os07g05620*, and *Os09g25090*, were up-regulated in the cold-stressed leaves and drought-stressed roots compared with that in the controls. Interestingly, two CIPK-like protein 1 genes, *Os03g03510* and *Os02g08140*, were up- and down-regulated, respectively, in the cold-stressed leaves (Fig. 3). Xiang et al. (2007) observed that 20 *CIPKs* are differentially induced by drought, salt, cold, and ABA in rice. The transgenic plants over-expressing *OsCIPK03* and *OsCIPK12* produce significant levels of proline and soluble sugars, which are responsible for the induced tolerance to cold and drought stresses in these plants. Ca^{2+}-dependent protein kinases (CDPKs) contain calmodulin-like Ca^{2+}-binding domains and a kinase domain. Genes encoding CDPKs such as *Os02g03410* and *Os06g50030* were highly up-regulated in the cold-stressed leaves (Fig. 3). The rice CDPK gene *CPK7* is induced in response to high salinity stress, and the transgenic plants expressing this gene showed different levels of tolerance to cold, salt, and drought stresses in rice (Saijo et al. 2000). Therefore, manipulating the expression of *Os02g03410* and *Os06g50030* in transgenic rice may lead to enhanced tolerance to abiotic stresses.

Genes in the ABA pathway

ABA is involved in the regulation of many aspects of plant growth and development and also is the major hormone that controls plant responses to abiotic stresses (Wasilewska et al. 2008). The genes encoding the SNF1-related protein kinase 2 (SnRK2, *Os01g65560*) and the molybdenum cofactor synthesis protein 3 (*Os02g32460*) were up-regulated in the drought-stressed roots but down-regulated in the cold-stressed leaves. The aldehyde oxidase gene *Os07g18162* was up-regulated in drought-stressed roots. However, the zeaxanthin epoxidases genes *Os04g37619* and *Os07g30690* were down-regulated in the drought-stressed leaves and roots. The 9-cis-epoxycarotenoid dioxygenase 2 gene *Os03g44380* was up-regulated in the cold- and salt-stressed leaves and salt-stressed roots (Fig. 3).

Genes in the MAP kinase pathway

MAP kinase genes that were highly up-regulated in the cold-

stressed leaves included *Os06g49430, Os01g43910, Os02g05480, Os02g54600, Os05g49140*, and *Os05g50560*. In contrast, some MAP kinase genes such as Os01g47530, *Os02g12810, Os02g50320, Os06g09180, Os06g48590,* and *Os09g27700* were down-regulated in the cold-stressed leaves. Several MAP kinase genes such as *Os03g11650, Os03g12390, Os06g20370*, and *Os08g41890* were up-regulated in the drought-stressed leaves. Similarly, in salt-stressed leaves and roots, the MAP kinase genes *Os02g35010, Os02g50320, Os03g11650, Os03g13460, Os05g02500, Os05g46750, Os06g20370*, and *Os09g27700* were down-regulated, but *Os01g47530, Os06g05520*, and *Os06g49430* were up-regulated (Fig. 3).

TF genes

TFs play an important role in activating stress related genes and regulating stress signal transduction (Agarwal et al. 2006; Shinozaki and Yamaguchi-Shinozaki, 2007; Lata and Prasad, 2011). Various kinds of TFs with RING finger, bZIP, zinc finger, MYB, and NAC motifs are involved in the activation of stress-responses. The TF genes belonging to AP2-EREBP and bHLH families were highly represented among the up- or down-regulated genes in the leaf libraries. Many TFs belonging to AP2-EREBP (15), bHLH (13), HB (7), NAC (12), MYB (12), and bZIP (6) families were up-regulated in the cold-stressed leaf library than that in other libraries. The TFs specifically or commonly expressed ≥3-fold in the leaf and root libraries are shown in Fig. 2C, 2D, 2E, 2F, 2G and 2H (Table S5; Table S6; Table S7). Two and nine TFs were commonly up- and down-regulated, respectively, in response to all three stresses in the leaf libraries (Fig. 2C and 2E). The number of TFs specifically up-regulated (116) or down-regulated (105) in the cold-stressed leaves was higher than that in the drought- or salt-stressed leaves (Fig. 2C and 2E).

When the leaves were subjected to the cold treatment, *Os01g73770* (DREB protein 1C) and *Os08g31580* (DREB factor1) were up-regulated, but the DREB-like gene *Os10g41130* was up-regulated only in the drought-stressed roots. In addition, the genes *Os04g46440* (DREB protein 1C) and *Os04g48350* (DREB protein 1D) were down-regulated in salt-stressed roots (Fig. 3). The CBF-coding genes *Os04g41920* and *Os09g35030* were up-regulated in salt- and drought-stressed roots (Fig. 3).

About 30 NAC domain-containing TF genes were differentially expressed in the stressed leaves and roots in the MPSS and SBS libraries compared to the controls. For example, the NAC domain TF gene *Os03g21060* was up-regulated under all three stresses in both roots and leaves. In contrast, the NAC genes *Os03g21030* (GRAB2 protein) and *Os03g60080* (NAC domain containing protein 67) were up-regulated in the cold-stressed leaves but down-regulated in the drought-stressed leaves. Among the 30 NAC domain-containing TF genes, *Os01g66120, Os05g34830*, and *Os08g06140* were substantially up-regulated in the cold-stressed leaves. The NAC domain-containing protein-48 gene (*Os01g66120*) produced 12 alternative transcripts. Interestingly, all of them were up-regulated in the cold-stressed leaves. In addition, the NAC domain transcription factor gene *Os03g21060* was up-regulated in roots and leaves in response to all three stresses (Fig. 3). NAC TFs bind to a particular motif that is involved in the induction of dehydration-tolerance genes in *Arabidopsis* (Tran et al. 2004). Hu et al. 2006 observed an enhanced tolerance to drought in rice transgenic plants over-expressing *STRESS-RESPONSIVE NAC1* TF. They showed that these plants lose water much slower than their controls by closing their stomata, suggesting that NAC TFs may play an important role in ABA-controlled processes.

The bZIP transcription factor genes *Os02g52780* and *Os02g58670* were highly up- and down-regulated, respectively, in the cold-stressed leaves. The bZIP TF genes *Os08g38020, Os09g28310*, and *Os06g10880* were also up-regulated in salt- and drought-stressed roots (Fig. 3). The MYB family was the largest group (>70) of differentially expressed TF genes (≥3.0-fold change in expression relative to the control) in the abiotic-stressed leaves and roots. One of them encoding a DNA-binding protein, *Os08g04840*, was up-regulated in the cold-stressed leaves. Of the 15 MYB genes identified in the drought-stressed roots, most (13) were up-regulated and only two, *Os02g46030* and *Os08g06110*, were down-regulated. Further characterization of the TF genes identified in our study will increase our understanding of their functions in response to abiotic stresses in rice.

Other stress-regulated genes encoding ion transporters, heat shock, and LEA proteins

We identified two up-regulated metal ion transporter genes, *Os04g35160* and *Os08g03600*, in the salt-stressed roots in both the MPSS and SBS libraries. A few other genes, including *Os03g49570* encoding an organic anion transporter, *Os06g48810* encoding the HKT1 protein, and Os07g37454 encoding the organic cation transporter 3, were down-regulated in the salt-stressed leaves. Four ion transporter genes, i.e., *Os02g07830* encoding the cation transporter HKT6, *Os03g06090* encoding a nickel ion transporter, *Os03g17740* encoding an organic anion transporter, and *Os04g51820* encoding the cation transporter HKT4, were up-regulated in the cold-stressed leaves. We also observed differential expression of more than 50 heat shock protein (HSP)-coding genes in the abiotic stress libraries, and most were down-regulated in response to all three abiotic stresses. More down-regulated HSP genes (>50) than up-regulated HSP genes (13) were found in the

stressed leaves. Some of the up-regulated HSP genes in cold-stressed leaves included *Os01g08560* (heat shock 70 kDa protein 4), *Os02g10710* (chloroplast small heat shock protein), *Os02g13800* (heat shock factor protein 1), *Os03g12370* (heat shock factor protein HSF8), and *Os03g16860* (heat shock cognate 70kDa protein 2). HSP genes down-regulated in the drought-stressed leaves included those encoding the bc1 complex from mitochondria (*Os01g21460*), mitochondral chaperone BCS1 (*Os05g51130*), chaperone protein dnaJ (*Os08g35160*), and chaperone protein dnaJ10 (*Os08g41110*). LEA proteins are mostly dehydration- or osmotic stress-responsive proteins. LEA proteins are implicated in the mechanism of desiccation tolerance (Goyal et al. 2005). Overexpression of *OsLEA3-1* in rice improved grain yield under drought conditions (Xiao et al. 2007). We found that the LEA protein gene *Os05g46480* (belonging to LEA protein group 3) was highly up-regulated in the cold- and salt-stressed leaves and in the drought- and salt-stressed roots, but was down-regulated in drought-stressed leaves.

Conclusions

Deep sequencing of rice transcriptomes in response to cold, drought and salt stresses revealed the expression patterns of many key functional and regulatory genes that are involved in the abiotic stress signaling pathways. We found that a lot of genes are commonly or specifically induced or suppressed in response to three abiotic stresses, suggesting that rice plants not only have a common mechanism to respond to multiple stresses but also have unique mechanisms to respond to each stress. Promoter analysis of highly up-regulated genes in the stressed libraries revealed conserved patterns of *cis*-regulatory elements. This study has provided new information on the regulatory and metabolic networks of abiotic defenses in rice. Detailed characterization of these genes may provide candidate genes for the development of molecular markers for rice breeding programs and for the engineering of new rice cultivars that are highly tolerant to abiotic stresses.

Materials and Methods

Plant material and growth conditions

Nipponbare plants used for cold-, drought-, and salt-stress treatments were grown in a growth chamber at 80% relative humidity with 12 h of light (500 μmol photons m^{-2} sec^{-1}) at 26ºC followed by 12 h of dark at 20ºC. Two-week-old seedlings were subjected to cold, drought, and salt treatments as described by Dubouzet et al. (2003) with a few modifications. For cold treatment, plants were kept at 4ºC for 24 h; for drought treatment, water was withheld from plants for 5 d (until they began to wilt); and for salt treatment, plants were immersed in 250 mM NaCl for 24 h (Dubouzet et al. 2003). Total RNA isolated from both leaves and roots of these plants was used for MPSS and SBS library construction. For time-course RT-PCR validation experiments, total RNA was isolated from leaf and root tissues at 12 h, 24 h, 48 h, 3 d, 5 d, 6 d, and 7 d after stress treatment.

RNA Isolation and RT-PCR

Total RNA was isolated from the stressed and control rice plants using Trizol reagent (INVITROGEN) according to the manufacturer's instructions. The RT-PCR experiment was conducted as described previously (Venu et al. 2007). PCR of the control gene *Actin1* was performed with the sense primer 5'-CGTCTGCGATAATGGAAC-TGG-3' and antisense primer 5'-CTGCTGGAATGTGCTGAGA-GAT-3'. Genes specifically expressed in each library were amplified with the gene-specific primers listed in Table S1.

Construction of the MPSS and SBS Libraries, Sequencing, and Bioinformatics

The following libraries were constructed: two MPSS and SBS cold libraries (MPSS leaf library-NCL; SBS leaf library-SNCL), four drought libraries (MPSS leaf library-NDL; MPSS root library-NDR; SBS leaf library-SNDL; SBS root library-SNDR), four salt libraries (MPSS leaf library-NSL; MPSS root library- NSR; SBS leaf library-SNSL; SBS root library- SNSR), and four control libraries (MPSS leaf library-NYL; MPSS root library-NYR; and SBS leaf library-SNYL; SBS root library-SNYR). The MPSS and SBS libraries were constructed and sequenced essentially as previously described (Meyers et al. 2004a and 2004b; Nobuta et al. 2007; Venu et al. 2010; Venu et al. 2011a and 2011b). Data were analyzed to identify the genes specifically and commonly expressed in cold-, drought-, and salt-stressed leaves and roots compared to the controls using our established procedures (Venu et al. 2011a and 2011b). TF genes expressed in stressed libraries were identified with a homology search of the rice TF database (http://ricetfdb.bio.uni-potsdam.de/v2.1/). For the identification of highly conserved *cis* motifs, the promoter sequences (1.0 kb before the ATG site) of the up-regulated genes (3-fold) in both the MPSS and SBS libraries were analyzed using the 'PLACE Signal Scan Search' software (http://www.dna.affrc.go.jp/htdocs/PLACE/) (Venu et al. 2010; Venu et al. 2011a and 2011b). Bioinformatic analyses including identification of antisense transcripts, alternative transcripts, and TFs were conducted as previously described (Nobuta et al. 2007; Venu et al. 2010; Venu et al. 2011a and 2011b). The entire MPSS data set (accession numbers GSM170905, GSM170912, GSM169568, GSM170914, GSM169564, GSM169567, and GSM170907) and SBS data set (accession numbers GSM629198, GSM629199, GSM629202, GSM629203, GSM629205, GSM629210, GSM629211, GSM629213, GSM629215, GSM629216, and GSM629218) are available at the NCBI's Gene Expression Omnibus (GEO) database. The raw and normalized MPSS can be accessed at http://mpss.udel.edu/rice.

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Authors' Contributions

RCV and MVS performed abiotic stress treatments, constructed SBS libraries, validation experiments, data mining and gene discovery,

bioinformatic analysis of MPSS and SBS data and wrote the manuscript; KN and SC analyzed the data; MS, YJ and KMM revised the manuscript; BCM and G-LW designed the experimental plan and revised the manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

Supporting Information

Fig. S1. Filter results for the seven MPSS and SBS libraries The signatures were processed according to three filters-significance, reliability, and genomic match-as described by Meyers et al 2004a, b.
Table S1. List of primers used for the validation of genes identified in the MPSS abiotic stress libraries using RT-PCR.
Table S2. Classification of MPSS and SBS signatures. The reliable significant (4TPM) MPSS and SBS signatures from the seven libraries were classified based on their location on the annotated gene (hits = 1) (See Meyers et al. 2004a, b for details).
Table S3. List of putative genes with alternative splicing/termination identified in both the MPSS and SBS libraries. These genes were specifically expressed in the abiotic-stressed leaves and roots but were absent in the respective controls.
Table S4. List of commonly and specifically expressed genes (3-fold up- and down-regulated genes) in the abiotic-stressed leaves and roots compared to their respective controls identified by cluster analysis (Fig. 2C, 2D, 2E and 2F). The transcripts commonly identified in both the MPSS and SBS libraries were used for cluster analysis.
Table S5. List of the TF genes specifically and commonly expressed in the abiotic-stressed leaves and roots (Fig. 2C, 2D, 2E and 2F) that were used for cluster analysis.
Table S6. List of commonly and specifically expressed genes (3-fold up- and down-regulated genes) in the drought- and salt-stressed leaves and roots. The drought-stressed leaf and root libraries were compared with the salt-stressed leaf and root libraries to identify commonly and specifically up-regulated genes (Fig. 2G and 2H) and down-regulated genes (Fig. 2I and 2J). The transcripts identified in both the MPSS and SBS libraries were used for cluster analysis.
Table S7. List of TF gene families identified in the abiotic-stress leaf and root libraries. The up-regulated (3 fold) TF genes in the abiotic stress libraries were used to identify the TF gene.

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