Characterization of Stress-Responsive *CIPK* Genes in Rice for Stress Tolerance Improvement[1][W]

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Plants respond to adverse environments by initiating a series of signaling processes that often involves diverse protein kinases, including calcineurin B-like protein-interacting protein kinases (CIPKs). In this study, putative *CIPK* genes (*OsCIPK01–OsCIPK30*) in the rice (*Oryza sativa*) genome were surveyed for their transcriptional responses to various abiotic stresses. The results showed that 20 *OsCIPK* genes were differentially induced by at least one of the stresses, including drought, salinity, cold, polyethylene glycol, and abscisic acid treatment. Most of the genes induced by drought or salt stress were also induced by abscisic acid treatment but not by cold. A few *CIPK* genes containing none of the reported stress-responsive cis-elements in their promoter regions were also induced by multiple stresses. To prove that some of these stress-responsive *OsCIPK* genes are potentially useful for stress-tolerance improvement, three *CIPK* genes (*OsCIPK03*, *OsCIPK12*, and *OsCIPK15*) were overexpressed in *japonica* rice 'Zhonghua 11'. Transgenic plants overexpressing the transgenes *OsCIPK03*, *OsCIPK12*, and *OsCIPK15* showed significantly improved tolerance to cold, drought, and salt stress, respectively. Under cold and drought stresses, *OsCIPK03*- and *OsCIPK12*-overexpressing transgenic plants accumulated significantly higher contents of proline and soluble sugars than the wild type. Putative proline synthetase and transporter genes had significantly higher expression level in the transgenic plants than in the wild type. The differentially induced expression of *OsCIPK* genes by different stresses and the examples of improved stress tolerance of the *OsCIPK* transgenic rice suggest that rice *CIPK* genes have diverse roles in different stress responses and some of them may possess potential usefulness in stress-tolerance improvement of rice.

Plants often confront abiotic stresses such as drought, high salinity, and low temperature during their life cycles. These stresses can lead to dramatic changes in plant growth, development, and productivity. Severe stresses can even threaten the survival of plants. The mechanisms of plant responses to abiotic stresses have been extensively investigated in some model species, such as Arabidopsis (*Arabidopsis thaliana*), and resurrection plants (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, 2002). Plants can initiate a number of molecular, cellular, and physiological changes to respond and adapt to these stresses, thus enabling them to survive (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). During response and adaptation to the stresses, many stress-related genes are induced (Albrecht et al., 2003; Xiong and Yang, 2003; Jayasekaran et al., 2006) and the levels of a variety of stress resistance-related functional proteins are accumulated (Hansen et al., 1997; Xia et al., 1997; Xu et al., 1997; Tanaka et al., 2001; Chen et al., 2003).

Most abiotic stresses can elicit an increase of cytosolic free Ca^{2+} concentration in almost all eukaryotic cells, and the change of Ca^{2+} concentration has been generally accepted as a secondary messenger to transduce the cellular responses to extracellular stimuli (Sanders et al., 1999; Berridge et al., 2000, 2003; Hofer and Brown, 2003; Kolukisaoglu et al., 2004). In plants, calcium acts as a universal messenger in various signal transduction pathways, including responses to a diverse array of biotic (Chung et al., 2004; Ludwig et al., 2005) and abiotic stresses (Frohnmeyer et al., 1999; Knight and Knight, 2001; Kim et al., 2003a) and regulation of various cellular and developmental processes (Trewavas and Malho, 1998; Tran et al., 1999).

In plants, many Ca^{2+}-sensing protein kinases have been reported for their involvement in the stress responses. These protein kinases include calcium-dependent protein kinases (Sheen, 1996; Saijo et al., 2000; Romeis et al., 2001; Pagnussat et al., 2002; Asano et al., 2005; Zhang et al., 2005; Chandran et al., 2006; Mori et al., 2006) and Suc non-fermentation-related kinases (SnRKs; Nozawa et al., 2001; Guo et al., 2002). In Arabidopsis, there are 38 SnRKs (Hrabak et al., 2003), including the SnRK3 subfamily that has been called CIPK (calcineurin B-like [CBL] protein interaction protein kinase; Shi et al., 1999; Albrecht et al., 2001; Batistic and Kudla, 2004; Mahajan et al., 2006) or SOS2 (SALT OVERLY SENSITIVE2)-like protein kinase (PKS) associated with the AtCBL/SOS3-like

[1] This work was supported by grants from the National Program on the Development of Basic Research (2006CB100103), the National Program on High Technology Development, the National Natural Science Foundation of China, and the Rockefeller Foundation.

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[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.107.101295

calcium-binding proteins (SCaBPs; Kolukisaoglu et al., 2004). The plant CIPK contains a specific Ser/Thr protein kinase domain that is activated through interaction with CBL containing four EF hands for Ca^{2+} binding (Ishitani et al., 2000; Nagae et al., 2003). Activated CIPKs can subsequently transduce calcium signals by phosphorylating downstream signaling components (Liu et al., 2000). Sequence analysis of Arabidopsis genome revealed 10 *AtCBL/SCaBP* and 25 *CIPK/PKS* genes (Luan et al., 2002; Kolukisaoglu et al., 2004). Expression patterns of these *CBL/SCaBP* and *CIPK/PKS* genes suggest diverse functions in different signaling processes, such as light, hormone, sugar, and stress responses (Kudla et al., 1999; Guo et al., 2002; Gong et al., 2004; Pandey et al., 2004). In Arabidopsis, a SOS pathway containing SOS3 (a Ca^{2+} sensor of the CBL family; Ishitani et al., 2000; Sanchez-Barrena et al., 2005), SOS2 (a CIPK protein; Ishitani et al., 2000; Gong et al., 2004; Sanchez-Barrena et al., 2005), and SOS1 (a Na^+/H^+ antiporter activated by SOS2; Shi et al., 2000; Qiu et al., 2002, 2004) has been well elucidated for its role in transducing Ca^{2+} signal, thereby maintaining ion homeostasis during salt stress (Liu et al., 2000; Shi et al., 2000; Xiong et al., 2002; Zhu, 2002; Sanchez-Barrena et al., 2005). *SOS2* encodes a Ser/Thr protein kinase with an N-terminal catalytic domain (sharing sequence homology with SNF), C-terminal regulatory domain that is unique to CIPK family kinases, and a FISL/NAF motif containing 21 amino acids. Both the N-terminal and C-terminal domains are essential for salt tolerance (Albrecht et al., 2001), and the FISL motif acts as an autoinhibitory domain by interacting with the kinase domain to keep the kinase activity inactive under normal conditions. SOS3 can interact with SOS2 via the FISL motif thus to release SOS2 from an autoinhibition state to a kinase-active state in a calcium-dependent manner (Halfter et al., 2000). Through the SOS pathway, excess Na^+ ions can be transported out of the cell via the plasma membrane Na^+/H^+ antiporter SOS1 that is phosphorylated and activated by the SOS3-SOS2 complex, thus reinstating cellular ion homeostasis (Qiu et al., 2002). Constitutively overexpressing *SOS2* under the control of cauliflower mosaic virus 35S promoter can rescue the salt-hypersensitive phenotypes of the *sos3* and *sos2* mutants (Zhu, 2002; Cheng et al., 2004; Guo et al., 2004). Coexpression of all three functional components of the *SOS* pathway in a salt-sensitive yeast mutant led to a great increase in Na^+ tolerance (Quintero et al., 2002).

The CBL/CIPK or SCaBP/PKS signaling pathway has also been evidenced in rice (*Oryza sativa*; Ohba et al., 2000; Kim et al., 2003b). Sequence analysis suggested 30 putative *CIPK* genes in the rice genome (Kolukisaoglu et al., 2004), and the predicted rice CIPK proteins have the same domain compositions as Arabidopsis CIPK proteins. However, the functions of almost all rice CIPK proteins remain to be revealed except very few reports on the expression of rice *CIPK* genes, including *OsCK1*, which is induced by diverse stimuli such as cold, light, salt, sugar, calcium, and cytokinin (Kim et al., 2003b). Toward uncovering the roles of rice *CIPK* genes in responses to abiotic stresses, a complete survey of the expression levels of all the putative rice *CIPK* genes in response to various abiotic stress and abscisic acid (ABA) treatments was conducted in this study. Based on the gene expression patterns, three stress-responsive *CIPK* genes were selected and overexpressed in rice to evaluate the potential usefulness of the stress-responsive *CIPK* genes in genetic improvement of stress tolerance.

RESULTS

Identification and Sequence Analysis of *OsCIPK* Genes

To identify all putative genes of the CIPK family in the rice genome, the reported CIPK protein sequences from Arabidopsis (Kolukisaoglu et al., 2004) were used as queries to search against the rice genome annotation database release 5.0 (http://www.tigr.org/tdb/e2k1/osa1/) and the full-length cDNA database (http://cdna01.dna.affrc.go.jp/cDNA). Through this approach, all the 30 putative CIPKs that had been designated as *OsCIPK01* to *OsCIPK30* by Kolukisaoglu et al. (2004) were identified. Although no additional putative CIPK was identified, some information of the *OsCIPK* genes was updated, including accession numbers of full-length cDNAs (locus ID of the predicted genes in the updated The Institute for Genomic Research [TIGR] database if cDNA not available), accession numbers of corresponding BAC/PAC clones, and physical locations of *OsCIPK* genes on chromosomes (Table I).

The 30 *OsCIPK* genes are distributed in all chromosomes except for chromosomes 4, 10, and 12 (Table I). In the Knowledge-based *Oryza* Molecular biological Encyclopedia (KOME) database, full-length cDNAs are available for all *OsCIPK* genes except for *OsCIPK04*, *06*, *09*, *13*, *22*, *27*, and *28*. Almost all OsCIPK proteins contain the complete protein kinase domain (except OsCIPK21) and the NAF domain (except OsCIPK08). OsCIPK21 has an incomplete kinase domain (Supplemental Fig. S1A) but contains the complete regulation domain. OsCIPK08 has lost the region containing the FISL/NAF motif (Supplemental Fig. S1B), but it contains the full N-terminal catalytic domain (sharing sequence homology with SNF). OsCIPK14 and OsCIPK15 have almost identical protein sequences except that five amino acids are missing at the C-terminal end of OsCIPK15 (Supplemental Fig. S1C). Comparison of the full-length cDNAs or predicted coding sequence (CDS) with the genomic sequences of *OsCIPK* genes revealed a great variation of the number of exons (ranging from one to 16; Table I). Based on the number of exons, *OsCIPK* genes can be obviously classified into two subgroups, the exon-rich subgroup (including *OsCIPK01*, *03*, *08*, *09*, *17*, *21*, *23*, and *24*) with each gene containing more than 10 exons and the exon-poor subgroup (the other 22 genes with zero to two introns for most of them; Fig. 1). We also noticed that the CDS of the protein kinase domain is interrupted by multiple introns

Table I. *The CIPK gene family in rice genome and primers used for amplification of gene-specific probes*

Gene[a]	Accession No.[b]	Genomic Locus[c]		Exons[d]	Chr.[e]	Primers for Probes or RT-PCR[f]	
		Accession No.	Mb			Forward Primer (5′–3′)	Reverse Primer (5′–3′)
OsCIPK01	Ak065588	AP002482	10.6	13	1	CATGAAAAATGGCAGGGGTT	CAGACGGGCAAGAAGAGAGC
OsCIPK02	AK072868	AP003757	28.7	3	7	ATGGGCATTGATGCAGAGAT	GCAAAAACAGTAACATCCAGAAAC
OsCIPK03	AK111929	AC119748	11.5	15	3	CAGGCACTGAATCTGGACAA	GCTACTCTACGGCGAACACC
OsCIPK04	Os12g41090	AL928781	25.4	3	12	CGTTCGACATCATCTCCATGTC	TGTCGTGTTCGCCGAACA
OsCIPK05	AK065589	AP003052	5.8	1	1	AAGAAGGGAGGAAAGGGCAG	GAACAGGAGATCCATGTAGACAAA
OsCIPK06	Os08g34240	AP004703	21.3	1	8	GATGCGGGTGACCAAGAG	ACCACCCAAACAGCGATACT
OsCIPK07	AK111510	AC145379	24.1	1	3	ATGGAGATGTCGGAGGTGTC	CATTCCTACCAACAATTTAG
OsCIPK08	AK120431	AP003449	19.8	16	1	TGAGGCATCCGAATGTGGTT	CGCCAGTGATGAACTCCAAGAT
*OsCIPK09**	OJ1015F07.8	AC104427	1.49	16	3	CTCGACCGCAACCATGTG	TCATTGTGGAAATCTCCCGTTT
OsCIPK10	AK066541	AC131175	12.6	2	3	TGCTAGCGACGAGGAACACTCT	GCGAGTGGCTGTAACACACAGA
OsCIPK11	AK103032	AP003230	35.5	2	1	GTGGTGGTGTTTGCTCATTTTG	TGTCCTAATTGCTTCCCAACCT
OsCIPK12[g]	AK101442	AP003409	32.2	3	1	TTTACCGGACCAAACCTGAC	AATGGCATCAGGAATGGAAG
*OsCIPK13**	OSJNBa0016I09.1	AP003052	5.8	1	1		
OsCIPK14	Os12g02200	BX000511	0.7	1	12		
OsCIPK15	AK121773	BX000512	0.6	2	11	AGGAGGGAAGGAATGGTGTT	TTGAAAGGCTCAAAGCCAAT
OsCIPK16	AK061220	AP005429	15.0	1	9	GAAAGGGTGGAAGCTGAGGT	CATCTCTCGATTTGGCCTGT
OsCIPK17	AK100498	AC093490	2.09	12	5	GCTTGGCCCTTCTGTAAATG	CGGTGGCTAGTTTTTGAACC
OsCIPK18	AK101355	AC105320	15.5	2	5	TGGTGTGCTGAAAATGGAAG	TTGTGCGAATTTCATTGAGC
OsCIPK19	AK069486	AC132485	25.4	2	5	CATAGAAGGCACGAGGGAAG	GCACCAAAACATGGACACTG
OsCIPK20	AK107068	AC104280	6.68	2	5	GATGTGACAGATTATCTAGGTG	GAGATTTCTAAAACATTCATA
OsCIPK21	AK107137	AP003749	26.5	15	7	ACATGTGGCAGCCCTAACTACA	TATGTCGGACAAAGAGCCATCA
OsCIPK22	Os05g26870	AC117264	15.5	4	5	TGGCCTTCTACACACAACTTGTG	CATACCCTTTTCGGCTGATCA
OsCIPK23	AK069726	AP003703	2.6	15	7	CAGGATTACCATCGCAGAGCTT	TGGAGGCTGATATCCCTTCTTG
OsCIPK24	AK102270	AP003629	24.0	15	6	TGTGCAACAAGATGGAAAGC	GAGCATTTCGACGTTCACAA
OsCIPK25	AK065374	AP004810	20.1	2	6	TCATGGTCCAGGTGTGCAAGA	CTTGAGCTCGTTGTCGCAGAAC
OsCIPK26	AK111660	AP004843	3.3	2	2	GCTGAGGTTTTTGAGCTTGC	TGTGACTCTGGCCTCTGATG
OsCIPK27	Os09g25100	AP005429	15.0	2	9		
OsCIPK28	Os05g39870	AC144743	23.4	7	5	CCCTTAAAGTCTCTGACTTTG	CACATGCGGTGTGGAGAAGA
OsCIPK29	AK111746	AP003757	28.7	1	7	AGGCCAAATTCAAGACCGAGTT	GGAGCATGCGAGTATGTCGAA
OsCIPK30	AK069231	AP003409	32.3	1	1	GAAGGAGAAAGGGAGGGTCG	AGATCAGCTGGACGGTGGTT

[a]Systematic nomenclature of rice *CIPK* genes followed Kolukisaoglu et al. (2004). [b]Accession numbers of full-length cDNA sequences from KOME (http://cdna.01.dna.affrc.go.jp/cDNA/) or TIGR locus ID (except *OsCIPK09* and *OsCIPK13* marked with asterisks). [c]Accession number of the BAC/PAC clones and approximate physical distance (Mb) of the chromosome in which the *OsCIPK* gene is located. [d]The number of exons for putative *OsCIPK* genes. [e]Chromosome in which the *OsCIPK* gene is located. [f]Primers are not listed for three *OsCIPK* genes that could not be amplified by RT-PCR. [g]Alternative name, *OsPK7* (Ohba et al., 2000).

at similar positions for the intron-rich subgroup but is seldom interrupted by introns for the intron-poor subgroup.

Stress-Induced Expression Profiles of *OsCIPK* Genes

To reveal the responses of *OsCIPK* genes to different stresses, RNA gel-blot analyses were performed using total RNA from the leaves of upland rice IRAT109 treated by drought, salt, cold, polyethylene glycol (PEG), and ABA. Reverse transcription (RT)-PCR and real-time PCR were also used because the hybridization signals of 11 *OsCIPK* genes in RNA gel-blot analyses were too weak to distinguish the difference between samples. Three genes (*OsCIPK13*, *14*, and *27*) could not be amplified in the leaves by RT-PCR (*OsCIPK13* and *OsCIPK27* had no cDNA support); the transcripts of all the other 27 *OsCIPK* genes could be detected by RNA gel-blot (Fig. 2) and /or PCR analyses (Fig. 3; Supplemental Fig. S2). Among them, 20 genes were induced by at least one of the stresses applied in this study. Among these 20 genes, 15 genes (*OsCIPK01*, *02*, *05*, *09*, *11*, *12*, *15*, *17*, *20*, *21*, *22*, *23*, *24*, *29*, and *30*) were induced by drought, 12 genes (*OsCIPK07*, *08*, *09*, *10*, *11*, *15*, *16*, *17*, *21*, *22*, *29*, and *30*) were induced by salinity stress, 12 genes (*OsCIPK01*, *03*, *09*, *12*, *15*, *16*, *17*, *21*, *22*, *23*, *24*, and *29*) were induced by PEG treatment, 16 genes (*OsCIPK01*, *02*, *03*, *05*, *07*, *09*, *11*, *12*, *15*, *16*, *17*, *20*, *22*, *24*, *29*, and *30*) genes were induced by ABA treatment, and three genes (*OsCIPK01*, *03*, and *09*) were induced by cold (Figs. 2 and 3).

Many *OsCIPK* genes were responsive to more than one stress (Fig. 4). Among the 15 drought-inducible genes, 13, 10, and eight genes were induced by ABA, PEG, and salt stress, respectively. Among the 12 salt-inducible genes, nine, eight, and seven genes were induced by ABA, drought, and PEG, respectively. Interestingly, some genes, such as *OsCIPK01* and *OsCIPK09*, were induced by multiple stresses, including cold, drought, PEG and ABA treatments. We also noticed that a few genes were induced mainly by one stress. For example, *OsCIPK03* was induced strongly by cold

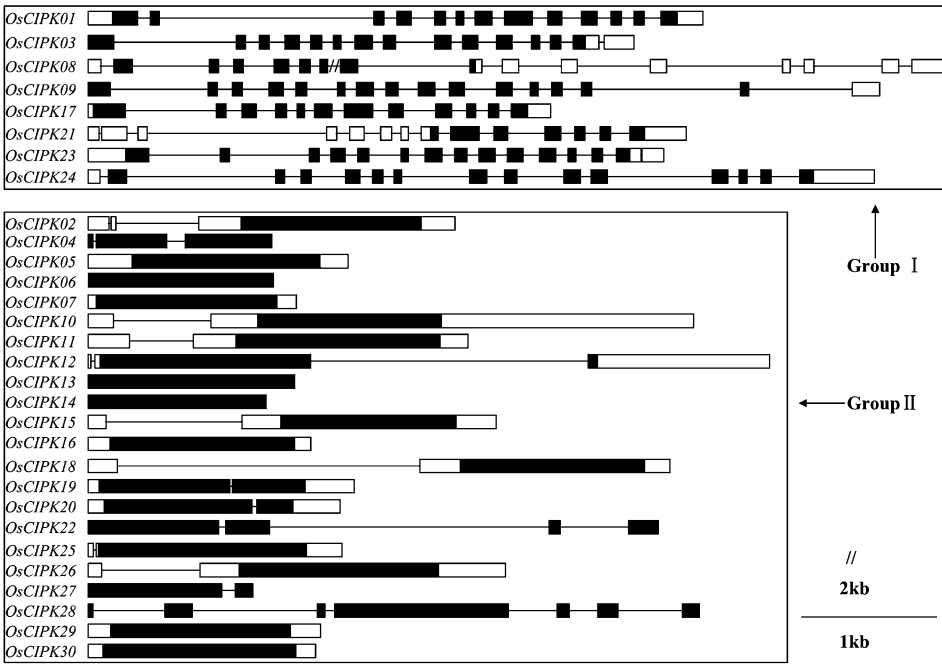

Figure 1. Intron-exon organization of the 30 *OsCIPK* genes. Rectangles and lines represent exons and introns, respectively. White rectangles represent UTR regions. The // and line bar represent 2 kb and 1 kb, respectively. Genes in Groups I and II have many (>10) and few (<5) exons, respectively.

stress, slightly by ABA and PEG treatments, but not by drought and salt stresses. These data indicate that some genes in the CIPK family of rice are involved in the responses to multiple abiotic stresses, whereas some genes of the family are responsive to specific stresses.

Generally, the *OsCIPK* genes induced by a specific stress treatment could be found in different subgroups based on protein sequence analysis (Kolukisaoglu et al., 2004) and in both exon-rich and exon-poor subgroups. This suggests that the specificity of stress responsiveness of *OsCIPK* genes has no obvious relationship with the CDSs or intron-exon structures of these genes. We picked up 1,000-bp sequences upstream the 5′ end of the full-length cDNAs for the stress-inducible *OsCIPK* genes to identify putative stress-responsive cis-elements that have been well characterized, such as ABA-responsive element (ABRE; Pla et al., 1993; Narusaka et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005), dehydration-responsive element (DRE)/C-repeat, and low-temperature-responsive element (LTRE; Yamaguchi-Shinozaki and Shinozaki, 1994; Shinwari et al., 1998; Narusaka et al., 2003). Sequence analysis suggested that 16 stress-responsive *OsCIPK* genes contain putative ABRE, DRE, or LTRE in their promoter regions (Fig. 4). Four genes (*OsCIPK02*, *21*, *24*, and *29*) containing DRE in their promoter regions were actually induced by drought. Among the 16 ABA-inducible genes, nine contain ABRE in their promoter regions. However, none of the three types of cis-elements was identified in the promoter regions of four genes (*OsCIPK07*, *08*, *12*, and *20*) that were induced by drought, salt, or ABA treatment.

To further confirm that some of these stress-responsive genes are potentially useful in improving stress resistance of crop plants, three genes (*OsCIPK03*, *OsCIPK12*, and *OsCIPK15* strongly induced by cold, drought, and salt stress, respectively) were overexpressed in rice 'Zhonghua 11' and the transgenic plants were tested for stress tolerance.

Overexpression of *OsCIPK03* in Rice Results in Enhanced Cold Tolerance

A total of 23 independent transgenic plants were generated for the *OsCIPK03* construct under the control of the cauliflower mosaic virus 35S promoter. RNA gel-blot analysis suggested that the gene was overexpressed in 15 independent plants (Fig. 5A). Transgenic seeds of four *OsCIPK03*-overexpressed T_1 families (T10, T24, T29, and T30) were germinated on hygromycin-containing Murashige and Skoog (MS) medium and at least 20 positive (hygromycin-resistant) seedlings for each family were selected for cold tolerance at the five-leaf stage. Overexpression plants and wild type with similar vigor under normal growth conditions (Fig. 5B) were used for treatment. After cold treatment (4°C for 5 d) and recovery (25°C for 5 d), almost all wild-type plants were completely dead, whereas most of the transgenic plants remained alive (Fig. 5C). In another cold treatment (4°C for 4 d and 25°C for 7 d for recovery) experiment, the survival rates of all the four transgenic families (68.2%–90.4%) were significantly (t test, $P < 0.01$) higher than the survival rate of the wild type (18.5%; Fig. 5D). These results suggested that overexpression of *OsCIPK03* could enhance the tolerance of transgenic plants to cold shock. In addition, no obvious difference in growth or development was observed between transgenic and wild-type plants under normal growth conditions (Supplemental Fig. S3). Drought and salt tolerance of *OsCIPK12* transgenic

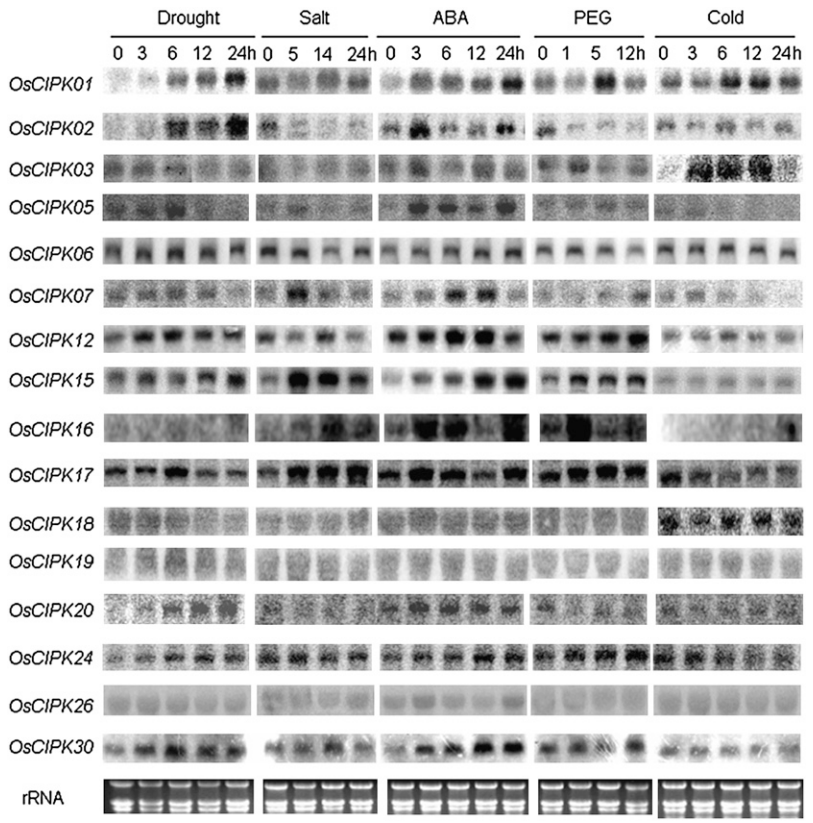

Figure 2. RNA gel-blot analysis of transcript levels for *OsCIPK* genes under drought, salt, cold, PEG, and ABA treatments. Multiple RNA blots were prepared from the same set of RNA samples (RNA loading of one set was shown as an example) and hybridized with gene-specific probes for different genes. Each stress treatment was repeated twice, and result from one repeat is shown.

plants were also tested, but no significant effect was detected for these two stresses (data not shown).

Overexpression of *OsCIPK12* in Rice Resulted in Enhanced Drought Tolerance

A total of 27 independent transgenic plants were generated for the *OsCIPK12* overexpression construct. Among them, 15 plants showed overexpression of the gene, as determined by RNA gel-blot analysis (Fig. 6A). At least 20 hygromycin-resistant transgenic seedlings with similar plant height and vigor as the wild type (Fig. 6B) for each of six *OsCIPK12*-overexpressed T_1 families were selected for drought-resistance testing at the vegetative stage. With water withheld for 1 week, only a few plants of the transgenic families showed slight leaf-rolling, while almost all leaves of the wild type became rolled or withered. After recovery (rewatering when all leaves of the wild type were completely rolled) for 3 d, only about 30% of wild-type plants survived, whereas most of the transgenic plants (62.7% to 87.4%) survived quite well (Fig. 6C). The survival rates of the six transgenic families were significantly (*t* test, $P < 0.01$) higher than the survival rate of the wild type (Fig. 6D). This result suggested that overexpression of *OsCIPK12* could increase the drought tolerance of rice at the vegetative stage. We also tested the salt and cold tolerance of *OsCIPK12* transgenic plants, but no significant effect on improving tolerance to these two stresses was detected (data not shown).

Overexpression of *OsCIPK15* in Rice Resulted in Enhanced Salt Tolerance

A total of 27 independent transgenic plants were also generated for the *OsCIPK15* overexpression construct. RNA gel-blot analysis suggested that the gene was overexpressed in 17 independent plants (Fig. 7A). Transgenic seeds of four *OsCIPK15*-overexpressed T_1 families (T4, T14, T19, and T22) were germinated on MS medium containing hygromycin and then at least 20 seedlings for each family were transferred to MS medium containing 100 mM NaCl for salt-tolerance testing. Transgenic shoots with similar length and vigor as the wild type were used as control. At 12 d after transplanting, all the transgenic families on salt-containing medium had significantly (*t* test, $P < 0.01$) longer shoot and root length (Fig. 7, B and C) and higher fresh weight per plant (Fig. 7D) than the wild type. No significant difference in shoot and root length (data not shown) or fresh weight (white bars in Fig. 7D) was observed between transgenic families and the wild type growing in the normal MS medium. These results suggested that overexpression of *OsCIPK15* could enhance salt tolerance of rice. We also tested the drought and cold tolerance of *OsCIPK15* transgenic plants, but

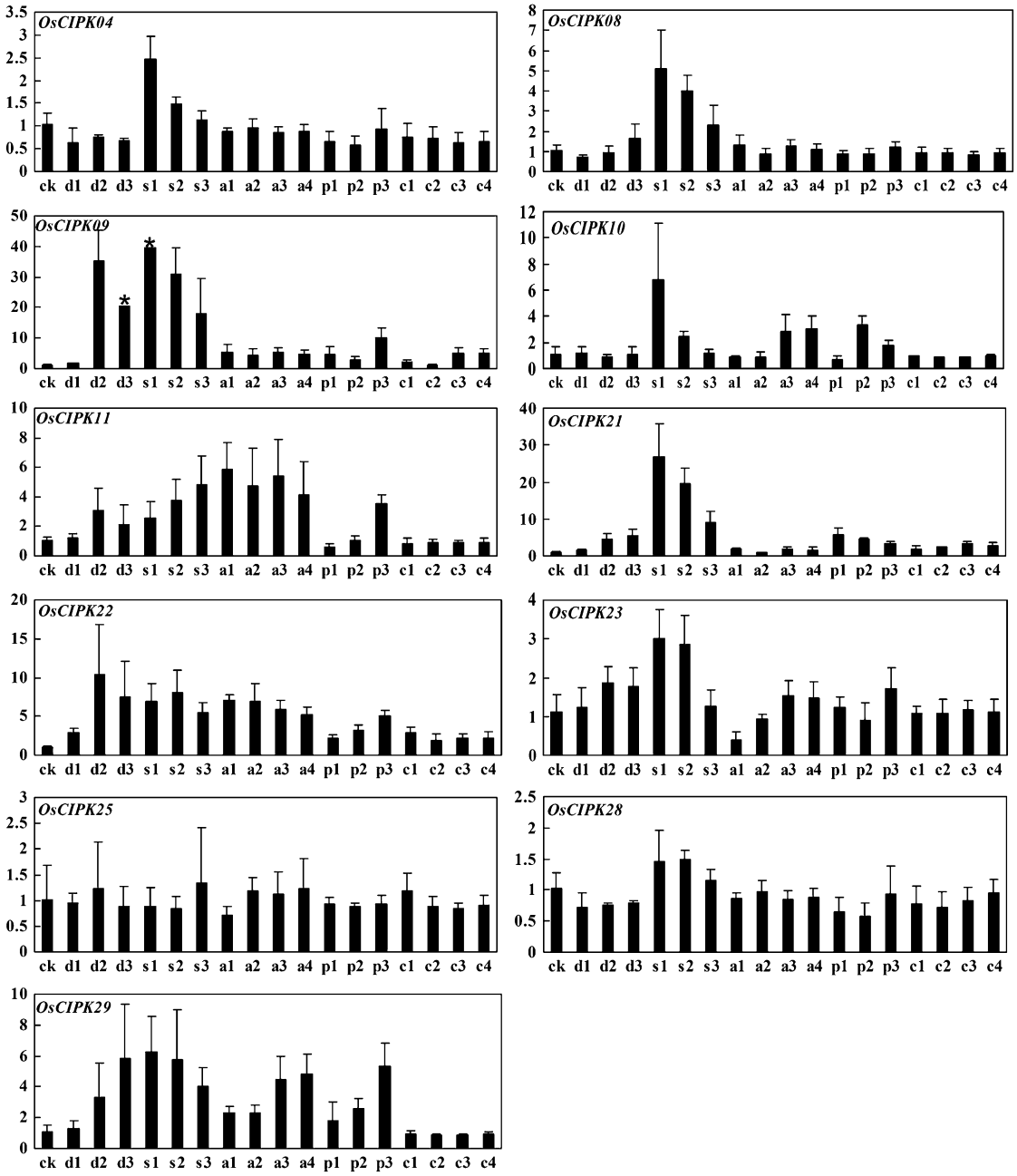

Figure 3. Real-time PCR analysis of expression levels of the *OsCIPK* genes that were undetectable by RNA gel blot. RNA samples were the same as those used in the RNA gel-blot analysis. The *x* axes are time courses of stress treatments and the *y* axes are scales of relative expression level. d, drought; s, salt; a, ABA; p, PEG; c, cold. For the gene *OsCIPK09*, the bars for d3 (>50-fold) and s1 (>70-fold) indicated by asterisks were intentionally reduced (fold of actual induction minus 30) so that the induction folds for other stresses could be seen clearly. A threshold of 2-fold was used to determine the stress responsiveness of genes.

no significant effect on improving tolerance to these two stresses was detected (data not shown).

Increased Pro and Soluble Sugar Contents in *OsCIPK03* and *OsCIPK12* Transgenic Plants under Stress Conditions

Plant adaptation to environmental stresses is often associated with metabolic adjustment, such as accumulation of Pro and soluble sugars (Abraham et al., 2003). To inquire the physiological basis for the improved stress tolerance of transgenic rice, we measured the Pro and soluble sugar contents in the overexpression plants of *OsCIPK* genes and the wild type under normal growth and stress conditions. No significant difference of Pro and soluble sugar contents in the leaves was detected between the overexpression plants of the three genes (*OsCIPK 03*, *12*, and *15*) and the wild type

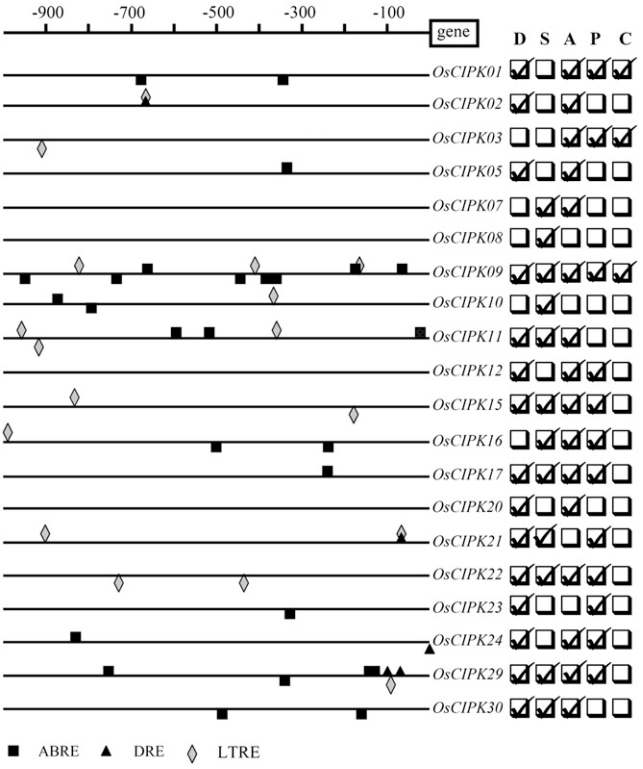

Figure 4. Putative ABRE, DRE, and LTRE core sequences in the 1-kb promoter regions of the stress-inducible genes identified by RNA gel-blot and RT-PCR analyses. The lines represent promoter sequences. The elements located in the forward strand (sense strand of the gene) and the reverse strand are indicated above and below the lines, respectively. Induction of the genes by stress treatments is indicated to the right of each gene with checks (D, drought; S, salt; A, ABA; P, PEG; C, cold).

grown under normal growth conditions. After cold stress for 3 d, *OsCIPK03*-overexpressing plants (three independent lines were tested) accumulated approximately 8-fold higher content of Pro (Fig. 8A) and 5-fold higher content of soluble sugars (Fig. 8B) than the plants did before cold stress. The cold stress-induced increases of Pro and soluble sugar contents in the transgenic plants were significantly higher (*t* test, $P < 0.01$) than those in wild-type plants, in which only about 2- to 3-fold increases of Pro and the soluble sugars after cold stress were detected. Under drought stress, *OsCIPK12*-overexpressing plants (three independent lines were tested) also accumulated Pro and soluble sugars much quicker than wild-type plants. After water withholding for 6 d, transgenic plants accumulated approximately 7-fold higher content of Pro (Fig. 8C) and 5-fold higher content of soluble sugars (Fig. 8D) compared to the contents of Pro and sugars before drought stress. Such increases were significantly higher than that in wild-type plants, in which Pro and soluble sugars were increased less than 3-fold after drought stress. In *OsCIPK15*-overexpressing plants, however, the Pro and soluble sugar contents had no significant difference compared to the wild type under both normal and salt-stressed conditions (data not shown).

We further measured the expression levels of two putative Δ^1-pyrroline-5-carboxylate synthetase genes (*J033099M14* and *J033031H21*) and two putative Pro transporter genes (*03g44230* and *07g01090*) in the *OsCIPK03* and *OsCIPK12* transgenic plants stressed by cold and drought, respectively. As show in Figure 8E, all the four genes had significantly higher expression level (4- to 19-fold) in the *OsCIPK03* overexpression lines than in the wild type. In the *OsCIPK12* overexpression lines, one Pro synthetase gene (*J033031H21*) and the two Pro transporter genes also showed 1.5- to 4-fold higher expression than in the wild type (Fig. 8F).

For probing the mechanism of enhanced salt tolerance of *OsCIPK15*-overexpressing plants, concentrations of Na^+ and K^+ in the root, stem, and shoot were measured for transgenic and wild-type plants growing in the Hoagland solution containing 100 mM NaCl. Interestingly, no significant difference in the concentration of either ion was detected between transgenic plants and the wild type in the examined tissues, including root, leaf, and stem (Supplemental Table S1), despite the fact that the transgenic plants still showed improved salt tolerance similar to the result shown in Figure 7B.

DISCUSSION

Differential Responses of *OsCIPK* Genes to Abiotic Stresses

Rice is one of the most important crops and the model plant for monocot species. In this study, as a first step toward systematic functional characterization of CIPKs in rice, all the putative rice *CIPK* genes

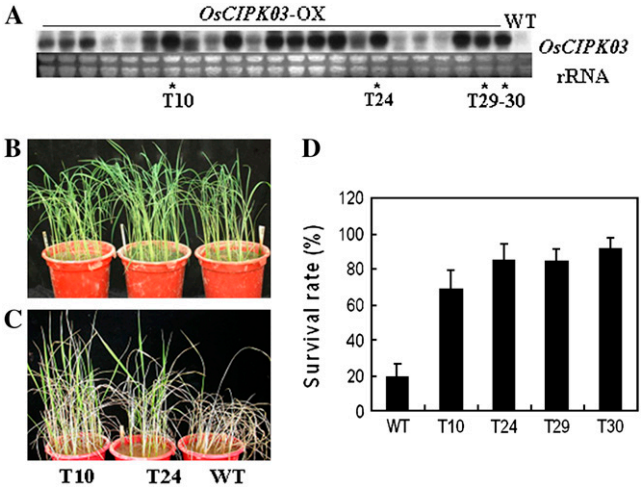

Figure 5. Identification and cold-tolerance testing of *OsCIPK03*-overexpressing rice. A, RNA gel-blot analysis of part of the transgenic plants and the wild type. B, Transgenic and wild-type plants right before cold treatment. C, Performance of cold tolerance of two transgenic families and wild-type plants after cold treatment (4°C for 5 d) and recovery (25°C for 5 d). D, Survival rate of four transgenic lines and the wild type after cold treatment (4°C for 4 d and 25°C for 7 d for recovery). Error bars are based on three replicates.

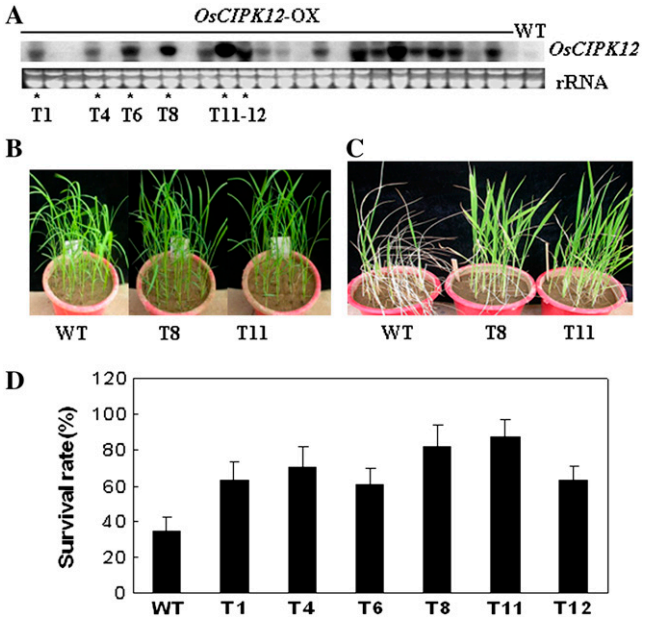

Figure 6. Identification and drought-tolerance testing of *OsCIPK12*-overexpressing rice. A, RNA gel-blot analysis of part of the transgenic plants and the wild type. B, Transgenic (two independent families are shown as examples) and wild-type plants right before treatment. C, Phenotypes of transgenic and wild-type plants after water withholding for 1 week and recovery for 3 d. D, Survival rate of transgenic lines and the wild type. Error bars are based on three replicates.

were checked for their expression changes upon various stress treatments. Our results clearly suggest that different *OsCIPK* genes have different responses to stresses. Among the 27 genes detected with transcripts by the techniques used in this study, 20 genes were found to be induced by single or multiple stresses. The *OsCIPK* genes induced by different stresses may provide new starting points to uncover the unique molecular basis of rice response to different stresses considering the nature of CIPKs as putative signaling components. Although the transcript levels of most *OsCIPK* genes were up-regulated by at least one stress, a few *OsCIPK* genes did not exhibit obvious change of transcript level upon the stress treatments. Since the CIPK family has been largely diversified and different members may be involved in responses to different stresses, it would be necessary to include more stress treatments in further studies to check the expression of *OsCIPK* genes, especially for those genes showing no response to the stresses in this study.

Besides the differential responses of *OsCIPK* genes to various stresses, responses of specific *OsCIPK* genes to multiple stresses were also detected. Noticeably, most ABA- or PEG-inducible genes were also induced by drought or salt stress, and most salt-inducible genes were also induced by drought (Fig. 4). These results indicate that *OsCIPK* genes may be involved in the substantial common regulatory systems or cross talks triggered by different stresses. As many abiotic stresses ultimately result in dehydration and osmotic imbalance of plant cells, there is a large overlap of genes induced by drought and salt stresses (Xiong et al., 2002; Ludwig et al., 2005; Nambara and Marion-Poll, 2005). Our results based on the stress-induced expression patterns of *OsCIPK* genes also support the notion of cross talks between drought and salt stresses (Seki et al., 2002a, 2002b). Stress-induced changes of gene expression in turn may affect the generation of hormones such as ABA, which has been considered an important "stress hormone" and plays a critical role in response to various stresses. The application of ABA to plant partially mimics the effect of stress conditions (Nambara and Marion-Poll, 2005). In this study, most ABA-inducible genes were also induced by drought or salt stress, suggesting that these *OsCIPK* genes may participate in the cross talks of signaling pathways for drought and salt stresses and ABA treatment. Studies have suggested that osmotic stress imposed by high salt or drought can be transmitted through ABA-dependent and ABA-independent signaling pathways (Uno et al., 2000; Chinnusamy et al., 2004). We also found that quite a few drought- or salt-inducible *OsCIPK* genes were

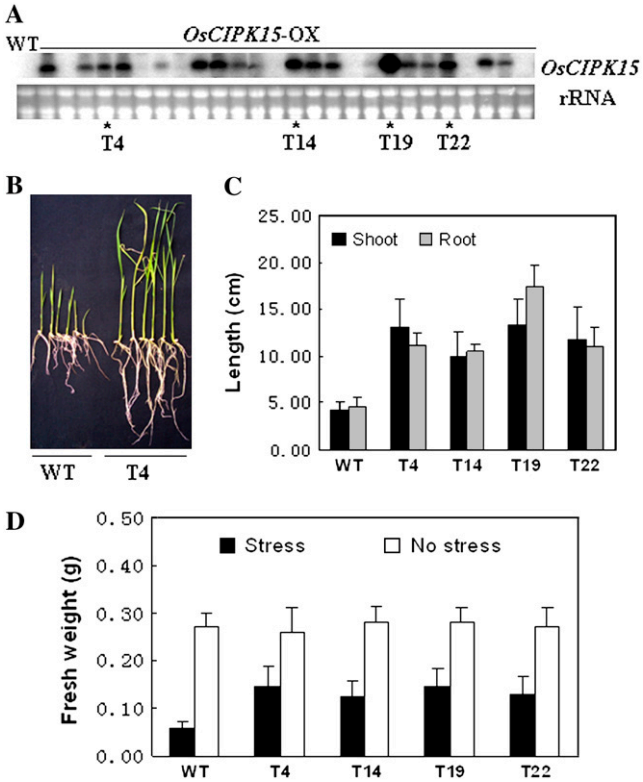

Figure 7. Identification and salt-tolerance testing of *OsCIPK15*-overexpressing rice. A, RNA gel-blot analysis of part of the transgenic plants and the wild type. B, Growth performance of transgenic (one family is shown as an example) and the wild type in medium containing 100 mM NaCl for 12 d. C and D, Shoot and root length (C) and fresh weight (D) of transgenic lines and wild type growing in the NaCl-containing medium for 12 d. The average values were calculated from measures of at least 20 plants.

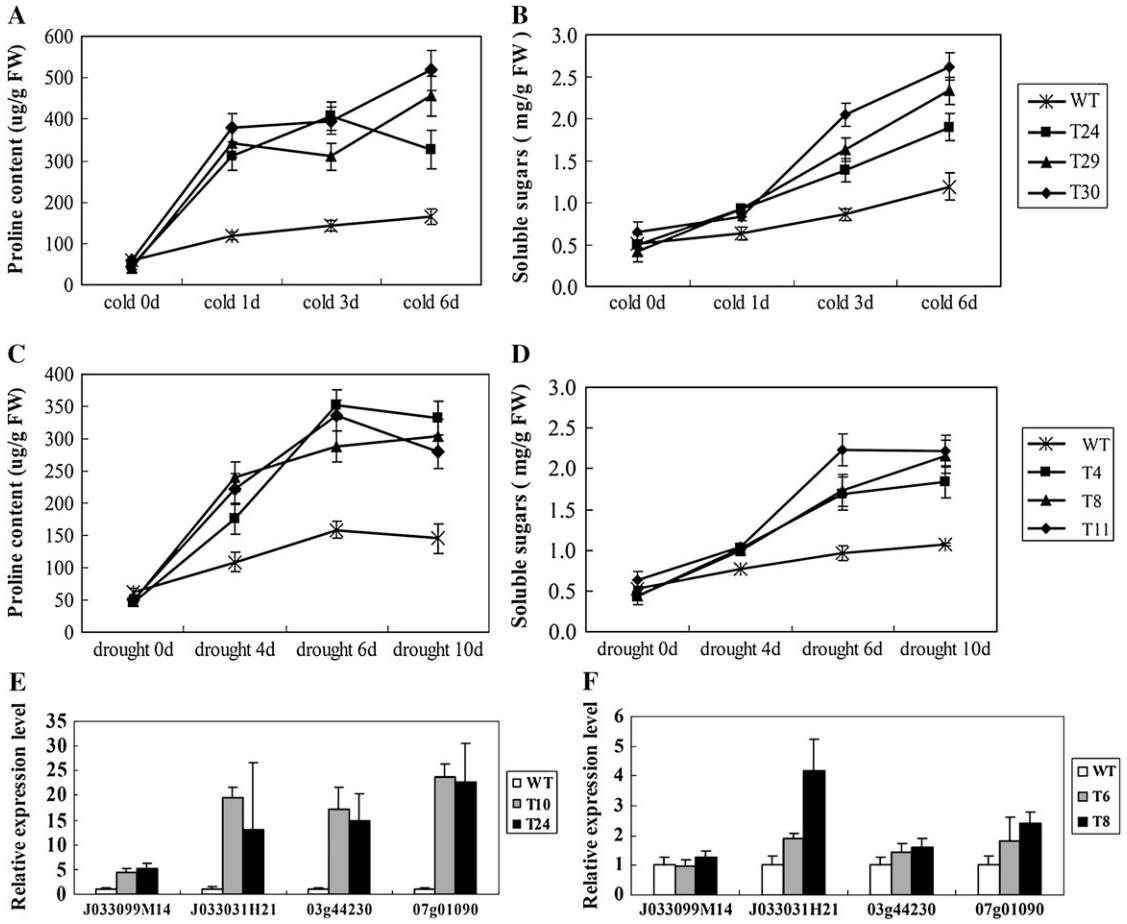

Figure 8. Contents of soluble sugars and free Pro in the transgenic rice plants overexpressing *OsCIPK03* and *OsCIPK12* genes compared to the wild-type plants. A and B, Free Pro (A) and soluble sugar (B) contents in the *OsCIPK03*-overexpressing plants under cold stress (4°C) for 0, 1, 3, and 6 d. C and D, Free Pro (C) and soluble sugar (D) contents in the *OsCIPK12*-overexpressing plants under drought stress for 0, 4, 6, and 10 d. Values are means ± SD ($n = 4$). FW, Fresh weight. E and F, Expression levels of putative Pro synthetase genes (*J033099M14* and *J033031H21*) and Pro transporter genes (*03g44230* and *07g01090*) in cold-stressed (4°C for 1 d) *OsCIPK03* (E) and drought-stressed (drought for 6 d) *OsCIPK12* (F) transgenic plants by real-time PCR. The bars represent three repeats. Accession numbers of the genes are as follows: *J033099M14*, AK102633; *J033031H21*, AK101230; *03g44230*, AK067118; and *07g01090*, AK0666298.

not induced by ABA treatment. However, further experiments are required to clarify which *OsCIPK* genes function in ABA-dependent or ABA-independent signaling pathways. In this study, most genes induced by drought, salt, ABA, or PEG were not induced by cold stress, which may imply that some *OsCIPK* genes might be specifically involved in the signaling pathways triggered by cold stress.

Complex Relationships of Predicted Stress-Related cis-Elements and Actual Stress Induction Patterns of *OsCIPK* Genes

cis-Elements and trans-acting factors are two basic types of molecular modulators for transcriptional regulation of genes controlling various biological processes, including abiotic stress responses. Several types of cis-elements have been identified and analyzed for their activation by different stresses. Among these stress-responsive cis-elements, ABRE, DRE, and LTRE have been extensively characterized for their important roles in activation of gene expression under stress conditions (Yamaguchi-Shinozaki and Shinozaki, 1994; Shinwari et al., 1998; Narusaka et al., 2003). In this study, 20 genes were induced by different stresses, including drought, salt, cold, PEG, and ABA treatments, which prompted us to investigate the relationship of induction pattern and distribution of putative stress-responsive elements of these genes. Most of the stress-inducible genes (16 of 20) contain at least one of the three cis-elements (DRE, ABRE, or LTRE) within the 1,000-bp promoter regions of these genes (Fig. 4). Generally, the genes containing putative stress-responsive cis-elements are actually induced by the corresponding stress, which is especially true for ABRE (all ABRE-containing genes are induced by ABA, drought, or salt stress). However, the predicted

cis-elements do not support the actual expression patterns for some genes. For example, there are four *OsCIPK* genes (*OsCIPK06*, *19*, *26*, and *28*) that are not induced by any of the stresses applied in this study but actually contain at least one of the three cis-elements in their promoter regions. On the other hand, there are four genes (*OsCIPK07*, *08*, *12*, and *20*) that contain none of the three cis-elements mentioned above in their promoter regions, but these four genes are actually induced by at least one stress. These discrepancies could be due to the sequence variation in the promoter regions since we used variety IRA109 in this experiment while the promoter sequences analyzed were from 'Nipponbare'. The discrepancies may also suggest that the activation of the cis-elements largely depends on the cis-background (sequences flanking the cis-element) and the trans-background (activity of the corresponding transcription factors). The discrepancies are not very surprising since only three major cis-elements were included in this analysis and increasing number of stress-responsive cis-elements have been identified and deposited in the PLACE database. It cannot be excluded that novel stress-responsive cis-elements yet to be identified may exist in the promoters of these stress-inducible genes containing none of the reported cis-elements.

Enhanced Stress Tolerance Mediated by *OsCIPK* Genes

Response of a gene to a specific stress at the transcriptional level does not necessarily mean that the gene must have significant effect in conferring tolerance to the stress. However, a survey of stress-induced expression patterns can often provide clues for speculating the putative functions of the genes, which may be especially true for a functionally diversified gene family such as the CIPK family. To prove that some of these stress-responsive *OsCIPK* genes may be potentially useful for enhancing stress tolerance in rice, three *CIPK* genes (*OsCIPK03*, *OsCIPK12*, and *OsCIPK15*, responsive to cold, drought, and salt stress, respectively) were chosen as examples and overexpressed in *japonica* rice 'Zhonghua 11'. Our results indicate that transgenic rice plants overexpressing *OsCIPK03*, *OsCIPK12*, and *OsCIPK15* can indeed significantly improve rice tolerance to cold, drought, and salt stress, respectively.

Plants have evolved an array of mechanisms in response to abiotic stresses, such as high salt, drought, cold, heat, and excessive osmotic pressure. In response to these stresses, many plants can accumulate compatible osmolytes, such as free Pro (Liu and Zhu, 1997; Armengaud et al., 2004; Ito et al., 2006) and soluble sugars (Gilmour et al., 2000; Garg et al., 2002; Tabuchi et al., 2004; Gupta and Kaur, 2005; Chyzhykova and Palladina, 2006), to protect their subcellular structures from damage by adjusting the intracellular osmotic potentials. Overproduction of Pro in tobacco (*Nicotiana tabacum*) could lead to an increase in tolerance of osmotic stress (Kishor et al., 1995). To address the possible mechanisms of the enhanced stress tolerance of the *OsCIPK* transgenic rice, we first checked the contents of Pro and soluble sugars in the transgenic plants under normal growth and stress conditions. The data showed that the *OsCIPK03*- and *OsCIPK12*-overexpressing plants indeed accumulated significantly more Pro and soluble sugars than the wild type under cold (for *OsCIPK03* overexpressor) and drought (for *OsCIPK13* overexpressor) stress conditions. Real-time PCR results also showed that Pro synthetase and Pro transport genes had significantly higher expression level in the *OsCIPK03* and *OsCIPK12* overexpression lines than in the wild type. The significantly improved Pro and sugar contents of *OsCIPK03*- and *OsCIPK12*-overexpressing plants under the stress conditions may partially explain the improved tolerance of *OsCIPK03*- and *OsCIPK12*-overexpressing plants to cold and drought stresses, respectively. No significant difference in Pro and sugar contents was detected between the transgenic and wild-type plants under normal growth conditions, despite the fact that the constitutive promoter was used to drive the transgenes. This might suggest that these CIPKs are not active under normal growth conditions. As for the *OsCIPK15*-overexpressing plants, we did not detect significant difference either in Pro and sugar contents or Na^+ and K^+ concentrations in root, stem, and leaf between the *OsCIPK15*-overexpressing plants and the wild type under both normal and salt stress conditions, which contrasts with the result of the homologous *CIPK* gene *SOS2* in Arabidopsis (Zhu et al., 1998; Zhu, 2002). Nevertheless, this observation has provided a starting point to identify a possible new mechanism of salt tolerance in addition to the SOS2-mediated pathway.

Although the molecular basis of the improved stress tolerance of the transgenic rice has not been completely resolved in this report, our data clearly suggest a functional diversification of the CIPK family in response to different stresses. Further characterization of the *OsCIPK* genes involved in different stress responses will largely expand our understanding the functions of the CIPK family. Even though only three *OsCIPK* genes were tested for their effectiveness in improving stress tolerance in rice, some other stress-responsive *OsCIPK* genes (most of them are under transgenic testing) may also be potentially useful for stress-tolerance improvement in rice.

MATERIALS AND METHODS

Identification and Sequence Analysis of *CIPK* Genes in Rice

The protein sequences of Arabidopsis (*Arabidopsis thaliana*) CIPK proteins available from The Arabidopsis Information Resource (http://www.arabidopsis.org; Garcia-Hernandez et al., 2002) and a previous report (Kolukisaoglu et al., 2004) were used to search for their homologous genes in the databases of rice, including the genome annotation database at TIGR (http://www.tigr.org/tdb/e2k1/osa1/) and the KOME (http://cdna01.dna.affrc.go.jp/cDNA). The resulting homologous protein sequences (with E-value less than $1.0E10^{-5}$) were manually checked for existence of the feature motifs and domains of CIPK proteins. The exon-intron organizations of the rice *CIPK* genes were determined by comparing the full-length cDNA or predicted CDSs with their corresponding genomic sequences. The position of each gene in the rice genome

was determined by BLASTN search against the genomic sequences of rice chromosomes available at the GRAMENE database (http://www.gramene.org).

To analyze putative cis-acting regulatory DNA elements (cis-elements) in the promoters of the rice *CIPK* genes, 1,000-bp regions upstream the 5′ end of the full-length cDNAs or predicted CDS were extracted from genomic sequences and subjected to cis-element search in PLACE 26.0, a frequently updated database of core DNA sequences of cis-elements identified in plants (http://www.dna.affrc.go.jp/PLACE/index.html; Higo et al., 1999).

Constructs of *OsCIPK* Genes and Rice Transformation

The full-length cDNAs of *OsCIPK03*, *OsCIPK12*, and *OsCIPK15* were amplified from upland rice IRAT109 (*Oryza sativa* L. ssp. *japonica*) by RT-PCR with the following three pairs of primers, respectively: 5′-CACCATGTATAGGGCTAAGAGGGCT-3′ and 5′-CTTGAAACTCACAAACTGTCA-3′, 5′-CACCATGCTGATGGCGACCGTCTC-3′ and 5′-GGAAGCTCCTGTCTCTAGCTC-3′ (the four underlined nucleotides, CACC, were added in front of the gene-specific forward primer to facilitate the TOPO cloning of the PCR fragment into the TOPO-D entry vector), and 5′-TAAGGTACCGGCTAAAGAATTGCAGTCCA-3′ and 5′-TAAGGATCCTTGCGACTGCTGCTATTC-3′ (the underlined sequences are for restriction sites *Kpn*I and *Bam*HI, respectively). For *OsCIPK03* and *OsCIPK12*, the PCR products were cloned into TOPO-D entry vector (Invitrogen), then introduced into destination vector pCB2004H (modified based on pCB2004) by LR reaction by following the manufacturer's instructions (Invitrogen). The *Bar* gene in pCB2004 (provided by Dr. Chengbin Xiang) was replaced by hygromycin-resistance gene *Hpt* (hygromycin phosphotransferase), resulting in the pCB2004H. For *OsCIPK15*, the sequencing-confirmed PCR fragments were double digested by *Kpn*I and *Bam*HI and ligated into the binary expression vector pCAMBIA1301U digested with *Kpn*I and *Bam*HI, thus allowing the genes to be driven by a maize (*Zea mays*) ubiquitin promoter. The constructs were introduced into *japonica* 'Zhonghua 11' by *Agrobacterium*-mediated transformation (Hiei et al., 1994).

Plant Growth and Treatments

For transcript level measurement of *OsCIPK* genes, rice plants of IRAT109 were grown in the green house with a 14-h-light/10-h-dark cycle. Two-week-old seedlings were treated with chemical and abiotic stresses. Chemical treatments were conducted by spraying leaves with 0.1 mM ABA followed by sampling at 0, 3, 6, 12, and 24 h, or irrigating the plants with 20% PEG 6000 followed by sampling at 0, 1, 5, and 12 h. Abiotic treatments were conducted essentially according to Saijo et al. (2000). For cold stress, seedlings were transferred to a growth chamber at 4°C and sampled at 0, 3, 6, 12, and 24 h after treatment. Drought stress was applied by exposing intact plants in the air without water supply and plant leaves were sampled at 0, 3, 6, 12, and 24 h after treatment. The 2-week-old seedlings were submerged with 200 mM NaCl solution for salt stress and sampled at 0, 5, 14, and 24 h after treatment.

For drought-resistance testing of transgenic rice at the seedling stage, the plantlets that germinated on plates with 50 mg/L hygromycin and positive seedlings and wild-type control (both with shoot height of 4–5 mm) were transferred into barrels filled with a mixture of soil and sand (1:1). To minimize the experimental error, each barrel was filled with 2 kg of soil/sand mixture and supplied with the same volume of water. When the plants grew up to five-leaf stage, water supply was cut off to allow drought stress to develop. About 10 d later, the survival rates of plants and the dry weight per plant were investigated. For salt-tolerance testing, transgenic seeds were germinated on MS medium with 50 mg/L hygromycin and wild-type seeds were germinated on MS medium without hygromycin at 26°C. The plantlets (shoot length 4–5 mm) were transferred onto MS medium containing 100 mM NaCl and allowed to grow in a growth chamber at 26°C with a 14-h-light/10-h-dark cycle. Plants were measured for fresh weight, shoot length, root length, and number of roots at 12 d after transplanting. For cold-tolerance testing, positive transgenic and wild-type plantlets (with shoot height 2–3 cm) were transferred into pots filled with soil. When the plants grew up to the five-leaf stage, the plants were transferred to growth chamber at 4°C and subsequently kept under normal growth conditions for 5 d. The survival rate as well as the area of green leaves after cold stress were investigated.

RNA Gel-Blot and Real-Time PCR Analyses

Total RNA was isolated from rice leaves using TRIzol reagent (Invitrogen). Total RNA (15 μg from each sample) was separated on a 1.2% agarose gel containing 2% formaldehyde and then transferred onto a nylon membrane. RNA gel blots were hybridized with α-^{32}P-dCTP-labeled gene-specific probes (primers for amplifying the *OsCIPK* gene probes are listed in Table I) overnight using Perfect-HYB Plus buffer (Sigma) at 65°C. Blots were washed three times (twice with 2× SSC/0.1% SDS for 10 min and once with 0.5× SSC/0.1% SDS for 5 min) at 65°C. The blots were briefly dried and then subjected to radiophotography. For real-time PCR analysis, first-strand cDNAs were synthesized from DNaseI-treated total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in an optical 96-well plate with an ABI PRISM 7500 real-time PCR system (Applied Biosystems). Each reaction contained 10 μL of 2× SYBR Green Master Mix reagent (Applied Biosystems), 1.0 μL of cDNA samples, and 200 nM gene-specific primer in a final volume of 20 μL. The thermal cycle used was as follows: 95°C for 3 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The primers for real-time PCR analysis of genes related to Pro synthesis and transport were as follows (in parentheses): *J033099M14* (5′-CTCAAATCAAGGCGTCAACTAAGA-3′ and 5′-TTTGTCAATATATACGTGGCATATACCA-3′), *J033031H21* (5′-CGCCCCTCCCCGTATCT-3′ and 5′-AGGAATGCGGCAACAAGTG-3′), *03g44230* (5′-AGGGACGATGGAGTTCTAAAGCT-3′ and 5′-GGGATTCCAAAGGCAAAAAGA-3′), *07g01090* (5′-GAGGAGGCTACCTGACTGTCAAC-3′ and 5′-GCTCATGAAGTCGCCAAGGA-3′). Rice *Actin1* gene (accession no. X16280) was used as internal control with primers 5′-TGGCATCTCTCAGCACATTCC-3 and 5′-TGCACAATGGATGGGTCAGA-3′. The relative expression levels were determined as described previously (Livak and Schmittgen, 2001).

Measurement of Pro and Sugar Contents

Transgenic plants at the four-leaf stage were used for biochemical analysis. Free Pro content in leaves was determined by essentially following the reported methods (Troll and Lindsley, 1955). About 50 mg of leaves were homogenized in 1 mL of sulfosalicylic acid (3%) and the homogeneous mixture was centrifuged at 13,000 rpm for 15 min at 4°C. The extract (200 μL) was transformed to a new microcentrifuge tube and mixed with 200 μL of acid ninhydrin (0.1 g ninhydrin dissolved in 2.4 mL of glacial acetic acid and 1.6 mL of 6-mortho-phosphoric acid) and 200 μL of acetic acid. The reaction mixture was boiled in a water bath at 100°C for 30 min and cooled down at 4°C for 30 min. Then 400 μL of toluene was added to the leaf extract and thoroughly mixed. Finally, 120 μL of the toluene phase was removed for absorbance measurement at 520 nm in a DU640 spectrophotometer (Beckman).

Total soluble sugars in leaves were determined by the modified phenol-sulfuric acid method (Dubois, 1956). About 0.1 g of leaves were homogenized in 8 mL of double-distilled water, then boiled twice in a water bath at 100°C for 30 min. The extract (about 500 μL) was transferred to a new microcentrifuge tube, added with 1.5 mL of double-distilled water, 1 mL of 9% (v/v) phenol, and 5 mL of sulfuric acid added, and kept at room temperature for 30 min. The absorbance was measured at 485 nm in a DU640 spectrophotometer (Beckman).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Table I.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Partial sequence alignment of part of *OsCIPK*s.

Supplemental Figure S2. RT-PCR analysis of nine *OsCIPK* genes that could not be detected with signals in northern analysis.

Supplemental Figure S3. Fresh weight of transgenic plants at four-leaf stage.

Supplemental Table S1. Content (mg/g) of Na^+/K^+ in root, stem, and leaf of *OsCIPK15*-overexpressing transgenic seedlings under normal and salt stress conditions.

ACKNOWLEDGMENTS

We thank Dr. Chengbin Xiang for providing the pCB2004 vector and Yinglong Cao for providing the modified pCAMBIA1301U vector with maize *ubiquitin* promoter.

Received April 19, 2007; accepted May 22, 2007; published May 25, 2007.

LITERATURE CITED

Abraham E, Rigo G, Szekely G, Nagy R, Koncz C, Szabados L (2003) Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in Arabidopsis. Plant Mol Biol **51:** 363–372

Albrecht V, Ritz O, Linder S, Harter K, Kudla J (2001) The NAF domain defines a novel protein-protein interaction module conserved in Ca^{2+}-regulated kinases. EMBO J **20:** 1051–1063

Albrecht V, Weinl S, Blazevic D, D'Angelo C, Batistic O, Kolukisaoglu U, Bock R, Schulz B, Harter K, Kudla J (2003) The calcium sensor CBL1 integrates plant responses to abiotic stresses. Plant J **36:** 457–470

Armengaud P, Thiery L, Buhot N, Grenier-De March G, Savoure A (2004) Transcriptional regulation of proline biosynthesis in Medicago truncatula reveals developmental and environmental specific features. Physiol Plant **120:** 442–450

Asano T, Tanaka N, Yang G, Hayashi N, Komatsu S (2005) Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: comprehensive analysis of the CDPKs gene family in rice. Plant Cell Physiol **46:** 356–366

Batistic O, Kudla J (2004) Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. Planta **219:** 915–924

Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol **4:** 517–529

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol **1:** 11–21

Chandran V, Stollar EJ, Lindorff-Larsen K, Harper JF, Chazin WJ, Dobson CM, Luisi BF, Christodoulou J (2006) Structure of the regulatory apparatus of a calcium-dependent protein kinase (CDPK): a novel mode of calmodulin-target recognition. J Mol Biol **357:** 400–410

Chen X, Goodwin SM, Boroff VL, Liu X, Jenks MA (2003) Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. Plant Cell **15:** 1170–1185

Cheng NH, Pittman JK, Zhu JK, Hirschi KD (2004) The protein kinase SOS2 activates the *Arabidopsis* H^+/Ca^{2+} antiporter CAX1 to integrate calcium transport and salt tolerance. J Biol Chem **279:** 2922–2926

Chinnusamy V, Schumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot **55:** 225–236

Chung E, Park JM, Oh SK, Joung YH, Lee S, Choi D (2004) Molecular and biochemical characterization of the *Capsicum annuum* calcium-dependent protein kinase 3 (CaCDPK3) gene induced by abiotic and biotic stresses. Planta **220:** 286–295

Chyzhykova OA, Palladina TO (2006) The role of amino acids and sugars in supporting of osmotic homeostasis in maize seedlings under salinization conditions and treatment with synthetic growth regulators. Ukr Biokhim Zh **78:** 124–129

Dubois M (1956) Colorimetric method for determination of sugars and related substances. Anal Chem **28:** 350–356

Frohnmeyer H, Loyall L, Blatt MR, Grabov A (1999) Millisecond UV-B irradiation evokes prolonged elevation of cytosolic-free Ca^{2+} and stimulates gene expression in transgenic parsley cell cultures. Plant J **20:** 109–117

Garcia-Hernandez M, Berardini TZ, Chen G, Crist D, Doyle A, Huala E, Knee E, Lambrecht M, Miller N, Mueller LA, et al (2002) TAIR: a resource for integrated *Arabidopsis* data. Funct Integr Genomics **2:** 239–253

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA **99:** 15898–15903

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis *CBF3* transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol **124:** 1854–1865

Gong D, Guo Y, Schumaker KS, Zhu JK (2004) The SOS3 family of calcium sensors and SOS2 family of protein kinases in Arabidopsis. Plant Physiol **134:** 919–926

Guo Y, Qiu QS, Quintero FJ, Pardo JM, Ohta M, Zhang C, Schumaker KS, Zhu JK (2004) Transgenic evaluation of activated mutant alleles of *SOS2* reveals a critical requirement for its kinase activity and C-terminal regulatory domain for salt tolerance in *Arabidopsis thaliana*. Plant Cell **16:** 435–449

Guo Y, Xiong L, Song CP, Gong D, Halfter U, Zhu JK (2002) A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in Arabidopsis. Dev Cell **3:** 233–244

Gupta AK, Kaur N (2005) Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. J Biosci **30:** 761–776

Halfter U, Ishitani M, Zhu JK (2000) The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc Natl Acad Sci USA **97:** 3735–3740

Hansen JD, Pyee J, Xia Y, Wen TJ, Robertson DS, Kolattukudy PE, Nikolau BJ, Schnable PS (1997) The *glossy1* locus of maize and an epidermis-specific cDNA from *Kleinia odora* define a class of receptor-like proteins required for the normal accumulation of cuticular waxes. Plant Physiol **113:** 1091–1100

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa L.*) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J **6:** 271–282

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res **27:** 297–300

Hofer AM, Brown EM (2003) Extracellular calcium sensing and signalling. Nat Rev Mol Cell Biol **4:** 530–538

Hrabak EM, Chan CW, Gribskov M, Harper JF, Choi JH, Halford N, Kudla J, Luan S, Nimmo HG, Sussman MR, et al (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. Plant Physiol **132:** 666–680

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 377–403

Ishitani M, Liu J, Halfter U, Kim CS, Shi W, Zhu JK (2000) SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding. Plant Cell **12:** 1667–1678

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol **47:** 141–153

Jayasekaran K, Kim KN, Vivekanandan M, Shin JS, Ok SH (2006) Novel calcium-binding GTPase (AtCBG) involved in ABA-mediated salt stress signaling in Arabidopsis. Plant Cell Rep **25:** 1255–1262

Kim KN, Cheong YH, Grant JJ, Pandey GK, Luan S (2003a) CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. Plant Cell **15:** 411–423

Kim KN, Lee JS, Han H, Choi SA, Go SJ, Yoon IS (2003b) Isolation and characterization of a novel rice Ca^{2+}-regulated protein kinase gene involved in responses to diverse signals including cold, light, cytokinins, sugars and salts. Plant Mol Biol **52:** 1191–1202

Kishor P, Hong Z, Miao GH, Hu C, Verma D (1995) Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol **108:** 1387–1394

Knight H, Knight MR (2001) Abiotic stress signalling pathways: specificity and cross-talk. Trends Plant Sci **6:** 262–267

Kolukisaoglu U, Weinl S, Blazevic D, Batistic O, Kudla J (2004) Calcium sensors and their interacting protein kinases: genomics of the Arabidopsis and rice CBL-CIPK signaling networks. Plant Physiol **134:** 43–58

Kudla J, Xu Q, Harter K, Gruissem W, Luan S (1999) Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci USA **96:** 4718–4723

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods **25:** 402–408

Liu J, Ishitani M, Halfter U, Kim CS, Zhu JK (2000) The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. Proc Natl Acad Sci USA **97:** 3730–3734

Liu J, Zhu JK (1997) Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of Arabidopsis. Plant Physiol **114:** 591–596

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W (2002) Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. Plant Cell (Suppl) **14:** S389–S400

Ludwig AA, Saitoh H, Felix G, Freymark G, Miersch O, Wasternack C, Boller T, Jones JD, Romeis T (2005) Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. Proc Natl Acad Sci USA **102:** 10736–10741

Mahajan S, Sopory SK, Tuteja N (2006) Cloning and characterization of CBL-CIPK signalling components from a legume (*Pisum sativum*). FEBS J **273:** 907–925

Mori IC, Murata Y, Yang Y, Munemasa S, Wang YF, Andreoli S, Tiriac H, Alonso JM, Harper JF, Ecker JR, et al (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. PLoS Biol **4:** e327

Nagae M, Nozawa A, Koizumi N, Sano H, Hashimoto H, Sato M, Shimizu T (2003) The crystal structure of the novel calcium-binding protein AtCBL2 from Arabidopsis thaliana. J Biol Chem **278:** 42240–42246

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol **56:** 165–185

Narusaka Y, Nakashima K, Shinwari ZK, Sakuma Y, Furihata T, Abe H, Narusaka M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis rd29A* gene in response to dehydration and high-salinity stresses. Plant J **34:** 137–148

Nozawa A, Koizumi N, Sano H (2001) An *Arabidopsis* SNF1-related protein kinase, AtSR1, interacts with a calcium-binding protein, AtCBL2, of which transcripts respond to light. Plant Cell Physiol **42:** 976–981

Ohba H, Steward N, Kawasaki S, Berberich T, Ikeda Y, Koizumi N, Kusano T, Sano H (2000) Diverse response of rice and maize genes encoding homologs of WPK4, an SNF1-related protein kinase from wheat, to light, nutrients, low temperature and cytokinins. Mol Gen Genet **263:** 359–366

Pagnussat GC, Fiol DF, Salerno GL (2002) A CDPK type protein kinase is involved in rice SPS light modulation. Physiol Plant **115:** 183–189

Pandey GK, Cheong YH, Kim KN, Grant JJ, Li L, Hung W, D'Angelo C, Weinl S, Kudla J, Luan S (2004) The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. Plant Cell **16:** 1912–1924

Pla M, Vilardell J, Guiltinan MJ, Marcotte WR, Niogret MF, Quatrano RS, Pages M (1993) The cis-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene rab28. Plant Mol Biol **21:** 259–266

Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK (2002) Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc Natl Acad Sci USA **99:** 8436–8441

Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS, Zhu JK (2004) Regulation of vacuolar Na^+/H^+ exchange in *Arabidopsis thaliana* by the salt-overly-sensitive (SOS) pathway. J Biol Chem **279:** 207–215

Quintero FJ, Ohta M, Shi H, Zhu JK, Pardo JM (2002) Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Na^+ homeostasis. Proc Natl Acad Sci USA **99:** 9061–9066

Romeis T, Ludwig AA, Martin R, Jones JD (2001) Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J **20:** 5556–5567

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J **23:** 319–327

Sanchez-Barrena MJ, Martinez-Ripoll M, Zhu JK, Albert A (2005) The structure of the *Arabidopsis thaliana* SOS3: molecular mechanism of sensing calcium for salt stress response. J Mol Biol **345:** 1253–1264

Sanders D, Brownlee C, Harper JF (1999) Communicating with calcium. Plant Cell **11:** 691–706

Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiya A, Nakajima M, Enju A, Sakurai T, et al (2002a) Monitoring the expression pattern of around 7,000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray. Funct Integr Genomics **2:** 282–291

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, et al (2002b) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J **31:** 279–292

Sheen J (1996) Ca^{2+}-dependent protein kinases and stress signal transduction in plants. Science **274:** 1900–1902

Shi H, Ishitani M, Kim C, Zhu JK (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. Proc Natl Acad Sci USA **97:** 6896–6901

Shi J, Kim KN, Ritz O, Albrecht V, Gupta R, Harter K, Luan S, Kudla J (1999) Novel protein kinases associated with calcineurin B-like calcium sensors in *Arabidopsis*. Plant Cell **11:** 2393–2405

Shinozaki K, Yamaguchi-Shinozaki K (1997) Gene expression and signal transduction in water-stress response. Plant Physiol **115:** 327–334

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochem Biophys Res Commun **250:** 161–170

Tabuchi A, Kikui S, Matsumoto H (2004) Differential effects of aluminium on osmotic potential and sugar accumulation in the root cells of Al-resistant and Al-sensitive wheat. Physiol Plant **120:** 106–112

Tanaka H, Onouchi H, Kondo M, Hara-Nishimura I, Nishimura M, Machida C, Machida Y (2001) A subtilisin-like serine protease is required for epidermal surface formation in *Arabidopsis* embryos and juvenile plants. Development **128:** 4681–4689

Tran PO, Hinman LE, Unger GM, Sammak PJ (1999) A wound-induced Ca^{2+} increase and its transcriptional activation of immediate early genes is important in the regulation of motility. Exp Cell Res **246:** 319–326

Trewavas AJ, Malho R (1998) Ca^{2+} signalling in plant cells: the big network! Curr Opin Plant Biol **1:** 428–433

Troll W, Lindsley J (1955) A photometric method for the determination of proline. J Biol Chem **215:** 655–660

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci USA **97:** 11632–11637

Xia Y, Nikolau BJ, Schnable PS (1997) Developmental and hormonal regulation of the Arabidopsis *CER2* gene that codes for a nuclear-localized protein required for the normal accumulation of cuticular waxes. Plant Physiol **115:** 925–937

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell (Suppl) **14:** S165–S183

Xiong L, Yang Y (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell **15:** 745–759

Xu X, Dietrich CR, Delledonne M, Xia Y, Wen TJ, Robertson DS, Nikolau BJ, Schnable PS (1997) Sequence analysis of the cloned *glossy8* gene of maize suggests that it may code for a beta-ketoacyl reductase required for the biosynthesis of cuticular waxes. Plant Physiol **115:** 501–510

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251–264

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci **10:** 88–94

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol **57:** 781–803

Zhang T, Wang Q, Chen X, Tian C, Wang X, Xing T, Li Y, Wang Y (2005) Cloning and biochemical properties of CDPK gene *OsCDPK14* from rice. J Plant Physiol **162:** 1149–1159

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53:** 247–273

Zhu JK, Liu J, Xiong L (1998) Genetic analysis of salt tolerance in *Arabidopsis*. Evidence for a critical role of potassium nutrition. Plant Cell **10:** 1181–1191