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Molecules and Cells

OsCIPK31, a CBL-Interacting Protein Kinase Is Involved in Germination and Seedling Growth under Abiotic Stress Conditions in Rice Plants

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Calcineurin B-like protein-interacting protein kinases (CIPKs) are a group of typical Ser/Thr protein kinases that mediate calcium signals. Extensive studies using *Arabidopsis* plants have demonstrated that many calcium signatures that activate CIPKs originate from abiotic stresses. However, there are few reports on the functional demonstration of CIPKs in other plants, especially in grasses. In this study, we used a loss-of-function mutation to characterize the function of the rice *CIPK* gene *OsCIPK31*. Exposure to high concentrations of NaCl or mannitol effected a rapid and transient enhancement of *OsCIPK31* expression. These findings were observed only in the light. However, longer exposure to most stresses resulted in down-regulation of *OsCIPK31* expression in both the presence and absence of light. To determine the physiological roles of *OsCIPK31* in rice plants, the sensitivity of *oscipk31*::*Ds*, which is a transposon *Ds* insertion mutant, to abiotic stresses was examined during germination and seedling stages. *oscipk31*::*Ds* mutants exhibited hypersensitive phenotypes to ABA, salt, mannitol, and glucose. Compared with wild-type rice plants, mutants exhibited retarded germination and slow seedling growth. In addition, *oscipk31*::*Ds* seedlings exhibited enhanced expression of several stress-responsive genes after exposure to these abiotic stresses. However, the expression of ABA metabolic genes and the endogenous levels of ABA were not altered significantly in the *oscipk31*::*Ds* mutant. This study demonstrated that rice plants use *OsCIPK31* to modulate responses to abiotic stresses during the seed germination and seedling stages and to modulate the expression of stress-responsive genes.

INTRODUCTION

Plants use multiple signal transduction pathways in response to external environments. The transient increase of cytosolic calcium concentration is a primary event in response to various signals including abiotic stress, phytohormones, light, and biotic stress (Baum et al., 1999; Evans et al., 2001; Knight and Knight, 2001; MacRobbie, 2000; Sanders et al., 1999). Higher plants express several major families of calcium sensors, which include calmodulin (CaM) and CaM-related proteins, CBL proteins, and calcium-dependent protein kinases (CDPKs) (Cheng et al., 2002a; Harmon et al., 2001; Luan et al., 2002; Snedden and Fromm, 2001; Zielinsik, 1998). CBL proteins trigger Ca^{2+}-mediated signal networks by activating their targets, i.e., the CBL-interacting protein kinases (CIPKs). Plant CIPKs contain a Ser/Thr protein kinase domain in their N terminal and a NAF domain in their C-terminal region. A systematic genome analysis revealed the existence of 25 *AtCIPK* genes in *Arabidopsis* and over 30 *OsCIPK* genes in rice (Kolukisaoglu et al., 2004; Xiang et al., 2007).

Arabidopsis has been extensively used to analyze the functions of CIPK proteins. Recently, knowledge of how CIPKs respond to salt stress, ABA, and nutrients such as low potassium and nitrogen has been greatly advanced. *AtCIPK3* negatively regulates ABA signaling during seed germination and stress-induced gene expression in the seedling stage (Kim et al., 2003a). Moreover, *PKS3* (*AtCIPK15*), which is homologous to *AtCIPK3*, interacts with protein phosphatases or transcription factors to modulate ABA signaling (Guo et al., 2002; Song et al., 2005). *SOS2* (*AtCIPK24*) specifically mediates salt-stress signaling and adaptation via an interaction with *SOS3* (*AtCBL4*) (Halfter et al., 2000; Liu et al., 2000; Sanchez-Barrena et al.,

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2005; Xiong et al., 2002a). *AtCIPK9* and *AtCIPK23* modulate low-potassium signaling. *AtCIPK23* directly interacts with, and positively regulates, the potassium transporter *AKT1* via a phosphorylation mechanism (Li et al., 2006; Pandey et al., 2007; Xu et al., 2006). In addition, the interaction of *AtCIPK23* with *AtCBL1* and *AtCBL9* regulates root potassium uptake and leaf transpiration (Cheong et al., 2007). *atcipk6* reduces auxin transport and tolerance to salt stress (Tripathi et al., 2009). *AtCIPK8* regulates the low-affinity phase of the primary nitrate response (Hu et al., 2009). Recently, it has been reported that *CBL5* may function as a positive regulator of salt or drought responses in plants (Cheong et al., 2010).

In rice, several reports based on expression patterns and biochemical data postulated the possible functions of *OsCIPK*s. *OsCIPK23* is suggested to act on both pollination and response to drought stress (Yang et al., 2008). *OsCIPK19* responds to light and nutrients (Ohba et al., 2000). The expression of *OsCK1* (*OsCIPK31*) is influenced by diverse signals, such as cold, salt, light, cytokinins, and sugars (Kim et al., 2003b). Overexpression of *OsCIPK31* (*OsCIPK3*) enhances cold tolerance (Xiang et al., 2007). Recently, *CIPK15* has shown to play a key role in O_2-deficiency tolerance in rice (Lee et al., 2009).

In this study, we used a loss-of-function mutation to examine the function of *OsCIPK31* (previously termed *OsCK1*). *Ds* element-inserted *OsCIPK31* mutants exhibited hypersensitive responses to abiotic stresses during seed germination and early seedling stages. In addition, the expression of stress-induced marker genes was altered in the *oscipk31*::*Ds* mutant.

MATERIALS AND METHODS

Isolation of *oscipk31*::*Ds* and generation of complementation lines

oscipk31::*Ds* was identified among the flanking sequence tags (FSTs) of a *Ds* population (Kim et al., 2004). *Ds*-flanking DNA was cloned by inverse PCR (iPCR). The four-base cutters *Nla*III, *Hae*III, and *Bfa*I were used to completely digest 1 μg of genomic DNA. Self-ligated templates (250 ng) were then subjected to sequential nested PCR. The primary and secondary products were loaded in adjacent lanes on a 3% agarose gel. The secondary products were expected to be shorter than the primary products. The iPCR primers used to amplify sequences flanking the 5′ and 3′ ends of *Ds* were as follows: Ds5-1, 5′-CCG TTT ACC GTT TTG TAT ATC CCG-3′; Ds5-2, 5′-CGT TCC GTT TTC GTT TTT TAC C-3′; Ds5-3, 5′-GTA CGG AAT TCT CCC ATC CT-3′; Ds502, 5′-ATA CGA TAA CGG TCG G-3′; Ds5I-1, 5′-TAA TCG GGA TGA TCC CGT TCG TT3′; Ds5I-2, 5′-ATG ACT GCA ATA TGG CCA GC-3′; Ds5I-3, 5′-TTC TAA TTC GGG ATG ACT GC-3′; Ds3I-105, 5′-AAA CGA ACG GGA TAA ATA CGG-3′; Ds3I-150, 5′-GGT TAA AGT CGA AAT CGG ACG-3′; Ds3-1, 5′-CCG ACC GGA TCG TAT CGG T-3′; Ds3-2, 5′-TTA ACC CGA CCG GAT CGT ATC GGT TTC G-3′; Ds3-3, 5′-GTT TCG TTA CCG GTA TAT CCC GTT TCG-3′; and Ds3-4, 5′-GTT AAA TAT GAA AAT GAA AAC GGT AGA GG-3′.

For complementation of the *oscipk31*::*Ds* mutant, an 1.4 kb *OsCIPK31* coding region was amplified by PCR from reverse-transcribed cDNA using a forward primer (5′-AAG CTT ATG TAT AGG GCT AAG AGG GCT G-3′) and a reverse primer (5′-CAT GGC AAA AAC CAC GTT CAC-3′). The PCR product was cloned into the pBluescript KS(±) vector and sequenced. The 1.4 kb fragment was transferred to the pGA1611 binary vector to generate *pGA1611*::*OsCIPK31*. The T-DNA vector was transformed into the *Agrobacterium tumefaciens* strain LBA4404 and was introduced into rice calli of a *Japonica* Dongjin cultivar. Hygromycin-resistant transgenic seedlings were transplanted to soil and grown in the field to produce seeds. Over 10 transgenic lines were generated. Among them, two lines were crossed with *oscipk31*::*Ds* to create complementation plants. *oscipk31*::*Ds* plants containing the *CIPK31-1* transgene were used as complementation lines.

Alignment of protein sequences and phylogenetic analysis

The protein sequences of OsCIPK31 (Os03g20380), OsCIPK3 (Os07g48760), and AtCIPK3 (At2g26980) were aligned using the multiple-sequence alignment analysis website "CLUST W" http://www.ebi.ac.uk/Tools/clustalw2/. The alignment result was processed using "boxshade" at http://www.ch.embent.rog/. Phylogenetic analysis was performed using the Clustal X and MAGA 4 software.

Plant materials and germination assay

Wild-type, *oscipk31*::*Ds,* and *oscipk31*::*Ds*/*CIPK31-1* lines were of the *Japonica* type of rice (*Oryza sativa cv*. Dongjin). Seeds were surface-sterilized with 0.05% pesticide (SPOOTAC: *prochloraz* 25%) for 24 h and washed with flowing water over one day. After being surface-sterilized, thirty seeds each from the wild-type, *oscipk31*::*Ds*, and *oscipk31*::*Ds*/*CIPK31-1* lines were germinated in dishes (90 × 15 mm) containing ABA, NaCl, mannitol, or glucose. Samples were incubated in a growth chamber at 26°C for 16 h under light conditions (250 $\mu E/s \cdot m^2$) and at 22°C for 8 h in the dark. Incubation media were replaced with fresh medium every 12 h. Germination was scored every 12 h.

Stress treatments

After surface-sterilization (as described above), seeds were germinated in 0.25× MS medium. Seeds that germinated uniformly were sowed in transparent plastic boxes (25 × 18 × 9 cm) containing 0.25× MS medium and covered with the same plastic boxes. The sample boxes were incubated in a growth chamber at 26°C for 16 h in light conditions (250 $\mu E/s \cdot m^2$) and at 22°C for 8 h in the dark. Incubation media were replaced with fresh medium every 24 h. Ten day-old seedlings were used for stress treatments. For polyethylene glycol, NaCl, mannitol, and ABA treatments, 15% or 30% polyethylene glycol (PEG) 6000, 50 mM or 250 mM NaCl, 100 mM or 300 mM mannitol, and 10 uM or 100 μM (±)-*cis*,*trans*-ABA, respectively, were made with 0.25× MS solution. Seedlings were incubated in a growth chamber at 26°C for 16 h under light conditions (250 $\mu E/s \cdot m^2$) and at 22°C for 8 h in the dark.

Isolation of RNA, Northern hybridization, and reverse transcriptase PCR (RT-PCR)

Samples were ground in liquid nitrogen immediately after harvesting. Total cellular RNA was extracted using the RNAiso Plus reagent (TaKaRa, Japan) and the RNeasy plant mini kit (QIAGEN, Germany), according to the manufacturers' instructions. For Northern hybridization, formaldehyde (1.3%) gels were prepared in MOPS/EDTA buffer (0.5 M MOPS, pH 7.0; 0.01 M Na_2EDTA, pH 7.5). Twenty microgram of each vacuum-dried RNA sample was dissolved and heat-denatured in formaldehyde/formamide buffer. After electrophoresis, gels were washed with water and 10× SSC and blotted onto a Hybond N^+ membrane (Amersham Pharmacia Biotech, UK) prewetted in 10× SSC. rRNA was stained with EtBr and used as a loading control. Hybridization was performed at 65°C in Church buffer (1% BSA, 200 μM EDTA, 0.5 M sodium phosphate, 7% SDS) containing a ^{32}P-labeled probe. The membranes were autora-

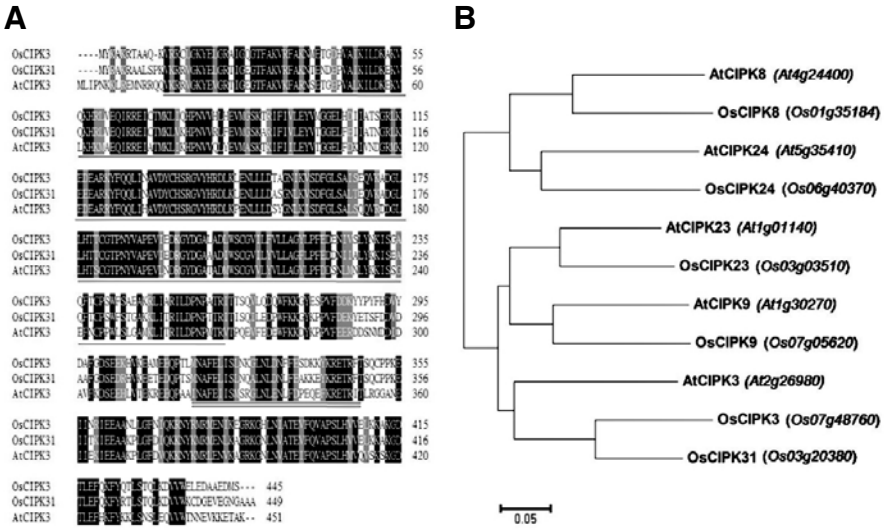

Fig. 1. Sequence alignment of OsCIPK31 with OsCIPK3 and AtCIPK3 (A) and phylogenetic analyses of *Arabidopsis* and rice CIPK proteins (B). (A) The deduced amino acid sequence of *OsCIPK31* was aligned with the AtCIPK3 and OsCIPK3 proteins. Amino acids were numbered on the right. Identical residues and conservative replacements were shown with black and gray shading, respectively. The N-terminal kinase catalytic domain (underline) was highly conserved and the C-terminal regulatory domain contained the conserved NAF motif (double underline). The accession numbers of *AtCIPK3*, *OsCIPK3*, and *OsCIPK31* are *At2g26980*, *Os07g48760*, and *Os03g20380*, respectively. (B) Phylogenetic relationship among multiple-intron and exon. of the subfamily of *CIPK* genes in *Arabidopsis* and rice. The OsCIPK31 protein is phylogenetically close to AtCIPK3 and OsCIPK3. The accession number of each gene was presented in parenthesis.

diographed using Fuji X-ray film. The photographs were scanned using a high-resolution scanner and processed using Adobe Photoshop (Mountain View, USA). Probe DNAs specific for the stress-responsive genes *OsRAB21* (*Os11g26790*), *OsDip1* (*Os02g44870*), *OsSalT* (*Os01g24710*) and the stress-inducible transcription factors *OsSNAC1* (*Os03g60080*) and *OsNAC6* (*Os01g66120*), and the ABA metabolic genes *OsABA8ox1* (*Os02g47470*), *OsZEP* (*Os04g37619*), and *OsNCED1* (*Os02g47510*), were obtained by PCR amplification from genomic DNA or reverse-transcribed cDNA using the following gene-specific primers: *OsRAB21*, forward 5′-AGC AAG AGC TAA GTG AGC TAG CCA C-3′ and reverse 5′-CGG CTA GCT CAT CTT ATT ATT CAG-3′; *OsDip1*, forward 5′-ATG GAG GAT GAG AGG AAC ACG GAG-3′ and reverse 5′-CCG GGC AGT TTC TCC ATG ATC TTG-3′; *OsSalT*, forward 5′-ACG AAG AGT ATG ACG CTG GTG AAG-3′ and reverse 5′-ATA TTA AAA AGT CGA CTG GGA CAA C-3′; *OsSNAC1*, forward 5′-TTG GAT GAT TGG GTG CTG TGT C-3′ and reverse 5′-TCA GAA CGG GAC CAT GCC CAT G-3′; *OsNAC6*, forward 5′-GTT GGA TGA TTG GGT GCT GTG-3′ and reverse 5′-CTA GAA TGG CTT GCC CCA GTA C-3′; *OsABA8ox1*, forward 5′-CAT GAG CTT CAC CCG CAA GA-3′ and reverse 5′-AAC TCC TCT GCT CCT CCT CT-3′; *OsZEP*, forward 5′-TGC ATT TCA TAA GGA ACC TGC TG-3′ and reverse 5′-TGG CTG ACT GAA GTC TCT CGT TC-3′; and *OsNCED1*, forward 5′-TGG AGC ACA TGG AGC TAG TG-3′ and reverse 5′-CGG TCT CGA AGT ATG TGT GCA C-3′. For RT–PCR, RNA samples were treated with DNase at 37°C for 30 min and at 65°C for 20 min. The first-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (TOYOBO, Japan) in 20 μl of a reaction mixture containing 3 μg total RNA and an oligo $(dT)_{25}$ primer, according to the manufacturer's instructions. One microliter of this reaction mixture was used for PCR amplification using gene-specific primers, which was performed via an initial denaturation at 94°C for 3 min, followed by 25 cycles at 94°C for 30 s, 58°C for 40 s and 72°C for 1 min, and a final extension at 72°C for 10 min. *Actin* expression levels were used as a quantitative control. The following primers were used for RT-PCR: *OsCIPK31RT*, forward 5′-CCT TCA TTT GTT GGA AGA TG-3′, reverse 5′-AGG CTG ATC TTC AGT TTC-3′; *OsCIPK3RT*, forward 5′-TCT GCT TGT GTG GAC TCT TGG AG-3′, reverse 5′-CTAA TTTGTTCTAG CATTGCCAG-3′; and *Actin*, forward 5′-CGC AGT CCA AGA GGG GTA TC-3′, reverse 5′-TCC TGG TCA TAG TCC AGG GC-3′. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized via ethidium bromide staining under UV light.

Measurement of endogenous ABA

To measure endogenous ABA levels, plant samples were extracted using a solution containing isopropanol, glacial acetic acid, and [(±)-3,5,5,7,7,7-d6]-ABA. Subsequent treatments were performed according to the methods published previously by Kang et al. (2005). For quantification, the Lab-Base (ThermoQuset, UK) data system software was used to monitor responses to ions with an *m*/*e* of 162 and 190 for Me-ABA and 166 and 194 for Me-[2H6]-ABA.]

RESULTS

Expression characteristics of *OsCIPK31*

The 449 amino acid OsCIPK31 protein contained a catalytic Ser/Thr protein kinase domain in its N-terminal region and a regulatory domain with a NAF motif at its C terminus, which is in keeping with the typical CIPK structural characteristics. OsCIPK31 showed high sequence homology with OsCIPK3 in rice (79% identity) and with AtCIPK3 in *Arabidopsis* (71% identity). The protein Ser/Thr kinase domain and NAF motif were highly conserved (Fig. 1A). Previously, the expression patterns of *OsCIPK31* were characterized under the name of *OsCK1* (Kim et al., 2003b). The *OsCIPK31* mRNA is abundant in leaves, but not in roots (Kim et al., 2003b; Fig. 2A). In addition, *OsCIPK31* is highly induced upon light exposure (Kim et al., 2003b).

In this study, *OsCIPK31* expression was examined under various stress conditions in the presence or absence of light. In seedlings, *OsCIPK31* expression was induced rapidly and transiently following exposure to NaCl (250 mM) or mannitol (300 mM). However, no significant induction was observed with either ABA (100 μM) or dehydration (Fig. 2B). It is interesting to note that the transient enhancement of abiotic stress-induced *OsCIPK31* expression was detected only in the presence of light. Under dark conditions, *OsCIPK31* expression decreased after exposure to the same stresses (Fig. 2C). However, it

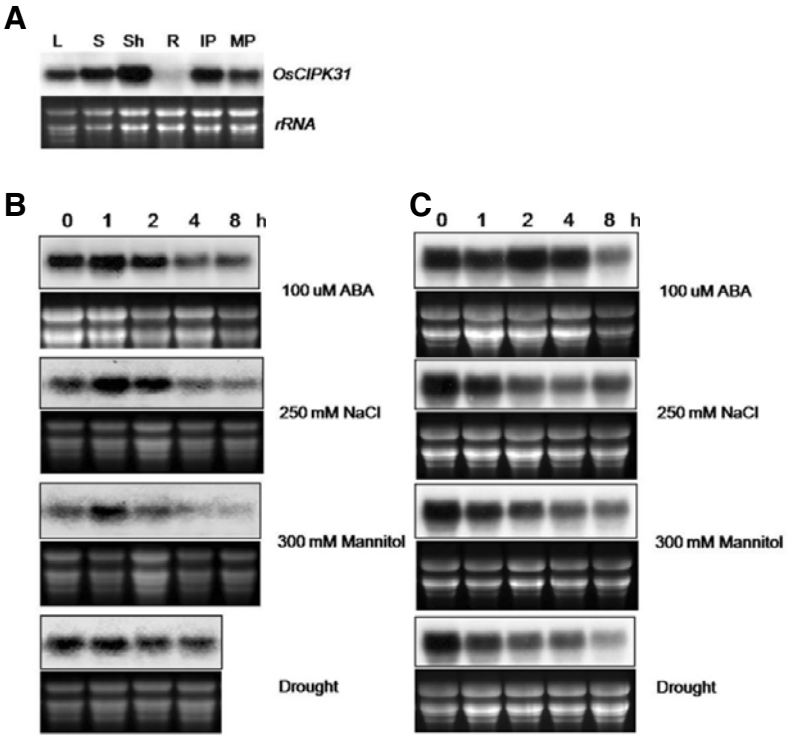

Fig. 2. Northern blot analyses of *OsCIPK31* transcript levels in various tissues (A) and under stress conditions in the presence or absence of light (B, C). (A) Northern blot analysis was performed using total RNA from various tissues of 10-day-old plants (L, leaf; S, SAM; Sh, shoot; and R, root) and floral organs (IP, immature panicles; MP, mature panicles). The RNA gel was EtBr-stained to ensure equal RNA loading (bottom gel). (B and C) The expression levels of the *OsCIPK31* transcript were measured in 10-day-old seedlings subjected to various stress conditions, in light conditions (B) or in the dark (C). For ABA, NaCl, and mannitol treatment, seedlings grown for 10 days on 0.25× MS medium were transferred to 0.25× MS medium containing 100 μM ABA, 250 mM NaCl, and 300 mM mannitol. For drought treatment, seedlings grown for 10 days on 0.25× MS medium were transferred to paper towels. After 4 h of the drought treatment, samples were severely damaged; therefore, they were not examined for gene expression. Samplings were made at five or four different time points.

should be noted that long exposure (> 2 h) to all the stresses led to the eventual down-regulation of *OsCIPK31* expression in both the presence and absence of light. As light and the various stresses increased the levels of *OsCIPK31* mRNA in shoots, we then examined the pattern of *OsCIPK31* expression in roots exposed to these conditions. We found that roots did not exhibit detectable levels of *OsCIPK31*, even under abiotic stresses and in the presence of light (Supplementary Fig. 1).

Identification of *oscipk31*::*Ds* mutants and complementation lines

An *oscipk31*::*Ds* mutant was isolated from *Ds* transposon gene-trap populations of the *Japonica* type rice cultivar (*Oryza sativa cv*. Dongjin) (Kim et al., 2004). Sequencing of the genomic DNA flanking the insertion site revealed that the *Ds* element was located in the fourth exon of *OsCIPK31* (Fig. 3A). Northern blot analysis using a gene-specific probe showed that *OsCIPK31* transcripts were not detected in the *oscipk31*::*Ds* plants (Fig. 3B). To generate complementation lines, we constructed a binary vector containing an *OsCIPK31* cDNA fused to the ubiquitin promoter. This vector (*pGA1611*::*OsCIPK31*) was transformed into Japonica rice (*cv*. DongJin) calli using tissue culture. Over 10 transgenic lines were generated. Among them, two transgenic lines were crossed with the *oscipk31*::*Ds* mutant. Crossed F1 plants were selfed to obtain *oscipk31*::*Ds* plants carrying the *OsCIPK31* transgenes. They were named *oscipk31*::*Ds*/*OsCIPK31-1* and *oscipk31*::*Ds*/*OsCIPK31-2* and were used as complementation stocks in this study (Fig. 3C). *OsCIPK31* mRNA levels in two complementation lines were similar to those of wild-type plants.

***oscipk31*::*Ds* mutants exhibited hypersensitivity to abiotic stresses during germination and at the early seedling stage**

To detect physiological roles of *OsCIPK31*, *oscipk31*::*Ds* was compared with its wild type siblings that were derived from selfed progeny of *oscipk31*::*Ds*/+. These wild type siblings were named wild-type plants in the following text. Under normal growth conditions in the field, greenhouses, and growth chambers, no significant differences in growth rates and morphological phenotypes were detected between *oscipk31*::*Ds* mutants and wild-type siblings. Next, we examined the abiotic stress-sensitivity of *oscipk31*::*Ds* mutants. Several assays were performed to examine abiotic stress responses during the germination and seedling stages. Seeds from wild-type plants, from the *oscipk31*::*Ds* mutant, and from the complementation line (*oscipk31*::*Ds*/*CIPK31-1*) were surface-sterilized for one day in dH_2O. Imbibed seeds were incubated in 0.25× MS medium containing various concentrations of ABA (1-5 μM), NaCl (50-200 mM), mannitol (100-400 mM), and glucose (2-8%), under light conditions. Glucose was used in this experiment, since it is well-known that exogenous glucose affects germination by modulating endogenous ABA levels (Price et al., 2003). The rates of germination were measured on the third day after the onset of the incubation. Germination rates are shown in Fig. 3D. At 5 μM ABA, more than 40% of wild-type seeds germinated within three days; however, the germination rate of mutant seeds was only 7.5% at the same time point. NaCl at a concentration of 200 mM led to a germination rate for wild-type and *oscipk31*::*Ds* mutant seeds of 20% and 1.6%, respectively. Similarly, the germination rates of wild-type and *oscipk31*::*Ds* were 25% and 2.5% (for 400 mM mannitol) and 18% and 0.8% (for 8% glucose), respectively. Therefore, the germination rates of *oscipk31*::*Ds* were sensitive to ABA, NaCl, mannitol and glucose. Increased concentrations of ABA, NaCl, mannitol and glucose led to increasingly dramatic retardations of germination in mutants compared with wild-type plants. The germination rates of wild-type and mutant specimens exposed to the indicated concentrations of the chemicals were subsequently measured everyday for five days after the onset of incubation (Fig. 3E). Compared to one of wild type the germination rates of *oscipk31*::*Ds* were significantly delayed in the presence of 5 μM

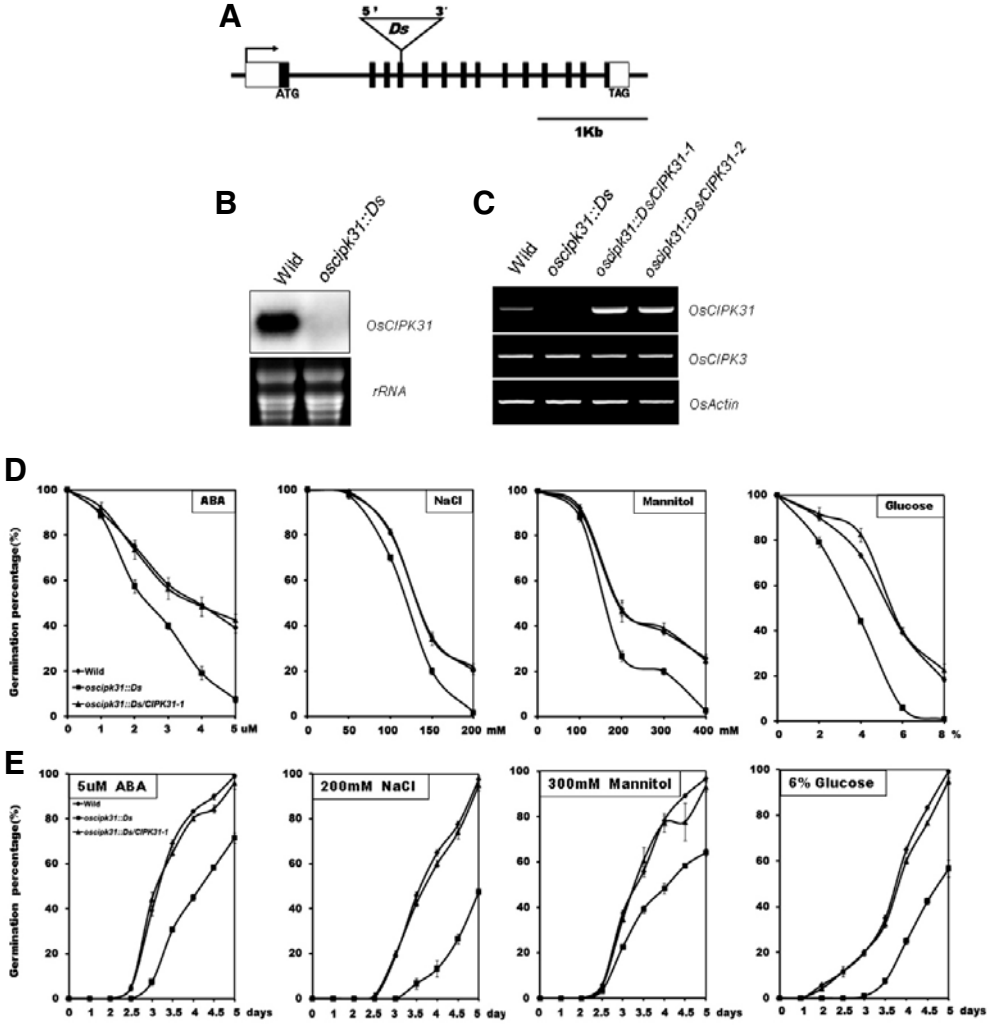

Fig. 3. Genomic structure, expression, and germination rates of the *oscipk31*::*Ds* mutation lines. (A) The *OsCIPK31* gene contains 14 exons (boxes) and 13 introns (lines). The *Ds* element was located in the fourth exon of the *OsCIPK31* gene. (B) Northern hybridization was performed using total RNA from two-week-old seedlings of wild-type and *oscipk31*::*Ds* plants. A gene-specific probe was used to detect the *OsCIPK31* mRNA. rRNA was visualized in the gel by staining with EtBr and was used as an equal-loading control. (C) RT-PCR analysis was performed using total RNA from wild-type, *oscipk31*::*Ds*, and two different complementation lines, *oscipk31::Ds/OsCIPK31-1* and *oscipk31::Ds/OsCIPK31-2* that were obtained from crosses of *oscipk31::Ds* and transgenic *OsCIPK31-1* or *OsCIPK31-2,* respectively. *OsCIPK3* and *actin* were used as controls. (D) The germination frequencies of wild-type (open squares), *oscipk31*::*Ds* mutant (closed squares), and complementation (*oscipk31/CIPK31-1*; open circles) plants were measured on the third day after onset of incubation in varying concentrations of ABA, NaCl, mannitol, and glucose. (E) The germination time course of wild-type, *OsCIPK31*::*Ds* mutant, and complementation (*oscipk31/CIPK31-1*) plants was measured in plants grown in 0.25× MS medium containing 5 μM ABA, 200 mM NaCl, 300 mM Mannitol, or 6% glucose. Average values and standard errors were measured from three experiments.

ABA, 200 mM salt, 300 mM mannitol, and 6% glucose. These results suggested that the *oscipk31*::*Ds* mutation is associated with a hyper-sensitivity of germinating seeds to ABA, high salt, osmotic stress, and glucose.

Next, the post-germination growth of *oscipk31*::*Ds* mutant seedlings was examined under similar abiotic stress conditions (Fig. 4A). Wild-type and *oscipk31*::*Ds* mutant seeds were surface-sterilized for one day and were germinated in 0.25× MS solution for two days. Seeds that germinated uniformly were transferred to 0.25× MS medium containing 4 μM ABA, 150 mM NaCl, 300 mM mannitol, or 6% glucose. The length of shoots and roots was measured everyday for six days after incubation with the chemicals. Figures 4B and 4C show the growth rates of shoots and roots, respectively. Compared with wild-type seedlings, *oscipk31*::*Ds* mutant seedlings exhibited a clear retardation in the growth of both shoots and roots under ABA, salt, and osmotic stress conditions. The ratios of shoot and root length of wild-type plants versus *oscipk31*::*Ds* mutant plants are shown in Table 1. The shoots and roots of mutant plants were 25-35% shorter than those of wild-type plants under abiotic stress conditions. These results suggested that *OsCIPK31* was involved in physiological responses to abiotic stresses during the germination and seedling development stages in rice.

***oscipk31*::*Ds* plants exhibited alterations in the expression of stress-induced marker genes in shoots, but not in roots**

Compared to wild-type plants, *oscipk31*::*Ds* mutant plans exhibited retardation in the growth of seedlings under abiotic stress conditions. To characterize the involvement of *OsCIPK31* in abiotic stress responses further, the expression of stress-induced marker genes was examined under stress conditions. Previous studies showed that *OsRAB21*, *OsDip1*, and *OsSalT* are strongly induced by drought, high salt, osmotic shock, and ABA (Oh et al., 2005; Rabbani et al., 2003). Ten-day-old wild-type and *oscipk31*::*Ds* seedlings were exposed to ABA, NaCl, mannitol, and PEG. The expression of *OsRAB21*, *OsDip1*, and *OsSalT* was examined in plants exposed to two different concentrations of each stress reagent. In the presence of 300 mM mannitol, *OsRAB21*, *OsDip1,* and *OsSalT* were clearly induced in mutant plants when compared with wild-type plants. In addition, *OsRAB21* and *OsDip1* were induced more quickly in mu-

Table 1. Shoot and root lengths of wild-type and *oscipk31*::*Ds* plants under different abiotic stresses.

	Genotype	[a]0.25× MS	[b]4 uM ABA	[b]150 mM NaCl	[b]300 mM Mannitol	[b]6% Glucose
Shoot length (mm)	Wild type	46.0 ± 2.5	23.3 ± 1.7	21.5 ± 1.1	7.3 ± 0.7	19.9 ± 1.7
	oscipk31::Ds	45.5 ± 2.5	15.9 ± 3.7	17.2 ± 1.3	5.2 ± 0.8	14.3 ± 1.6
	Ratio (%)	98.9	68.2	80	71.2	71.8

	Genotype	[c]0.25× MS	[a]4 uM ABA	[a]150 mM NaCl	[b]300 mM Mannitol	[b]6% Glucose
Root length (mm)	Wild type	32.9 ± 3.7	33.3 ± 3.3	29.5 ± 3.6	20.7 ± 1.5	10.1 ± 1.9
	oscipk31::Ds	32.5 ± 2.0	22.9 ± 3.0	22.3 ± 3.6	16.1 ± 2.9	8.40 ± 1.9
	Ratio (%)	98.9	68.7	75.6	77.8	83.2

[a]The length of shoots and roots was measured on the fifth day.
[b]The length of shoots and roots was measured on the sixth day.
[c]The length of roots was measured on the third day. Values shown are the means ± SD from 15-20 plants.

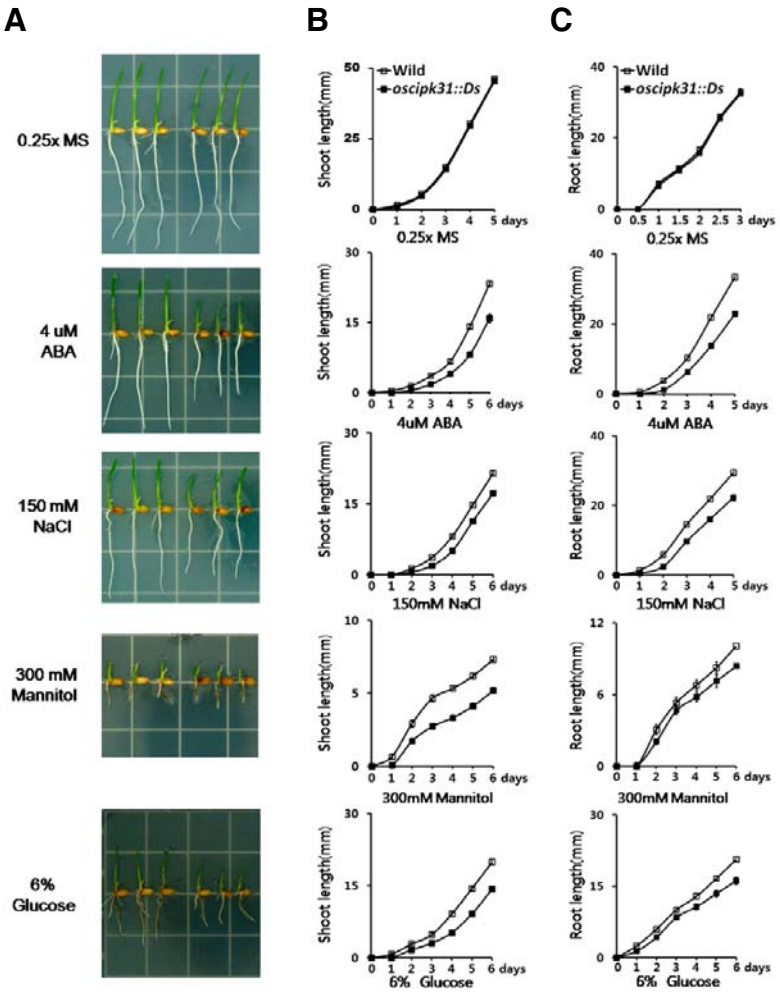

Fig. 4. Shoot and root growth under ABA, NaCl, mannitol, and glucose stress after germination. (A) Germinating wild-type and *oscipk31*::*Ds* mutant seeds were transferred to 0.25× MS liquid medium or 0.25× MS medium containing 4 μM ABA, 150 mM NaCl, 300 mM mannitol, and 6% glucose. Images were acquired on the fifth (0.25× MS with 4 μM ABA and 150 mM NaCl) or sixth (0.25× MS with 300 mM mannitol and 6% glucose) day after the transfer of seeds. (B and C) The length of shoots (B) and roots (C) was measured every day after germinating seeds were transferred to 4 μM ABA, 150 mM NaCl, 300 mM mannitol, and 6% glucose solutions. Wild-type plants were denoted as opened squares and *oscipk31*:: *Ds* mutant plants were depicted as closed squares. Average values and standard errors were measured from 15-20 plants.

tant specimens when compared with wild-type plants (Fig. 5A). Mutant seedlings that were exposed to 250 mM NaCl contained higher amounts of *OsRAB21*, *OsDip1*, and *OsSalT* mRNA when compared with wild-type seedlings. We noted that the basal level of expression of *OsDip1* in mutants was slightly higher than that of the wild type. However, the overall induction kinetics of these genes were similar between wild-type and mutant seedlings (Fig. 5B). Plants exposed to 30% PEG exhibited expression patterns that were almost identical to those observed in plants exposed to high NaCl concentrations (Fig. 5C). Treatment with 15% PEG led to a slight increase in the basal level of *OsSalT* in mutants when compared with wild-type plants. Under ABA stress, there was no significant difference in the mRNA levels of *OsRAB21*, *OsDip1* and *OsSalT* between wild-type and mutant seedlings (Fig. 5D). It is worth noting that a negative correlation could be observed between the induction

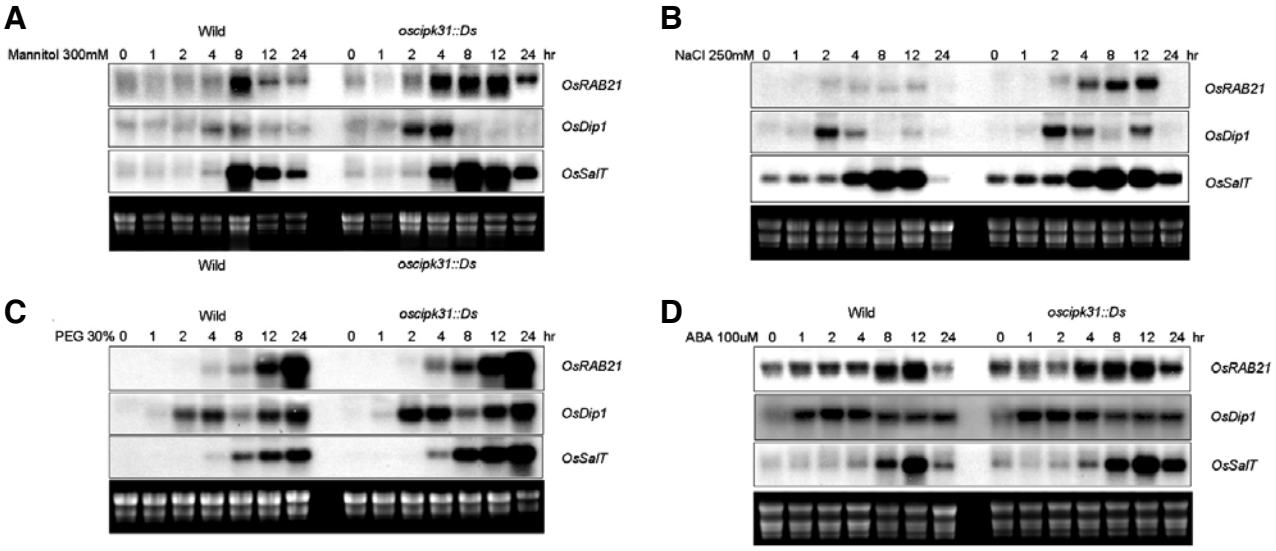

Fig. 5. Expression of the stress-responsive marker genes *OsRAB21*, *OsDip1*, and *OsSalT* in wild-type and *oscipk31*::*Ds* plants under various stress conditions. The levels of expression of the *OsRAB21*, *OsDip1* and *OsSalT* transcripts were measured using Northern analysis of 10-day-old seedlings exposed to 300 mM mannitol (A), 250 mM NaCl (B), 30% PEG (C), and 100 μM ABA (D) for different periods. All fourteen lanes in each RNA gel were transferred to the same blot, which were analyzed using the same hybridization conditions. It should be noted that the basal expression level of *OsSalT* in mutants was slightly higher than its value in wild-type plants. rRNA was visualized in the gel by staining with EtBr and was used as an equal-loading control.

kinetics of *OsRAB21*, *OsDip1* and *OsSalT* in mutants, and the expression pattern of *OsCIPK31* in wild-type plants under the same conditions. Following rapid induction during the first hour of stress treatment, *OsCIPK31* mRNA levels decreased gradually (Fig. 2B).

Transcription factors play an important role in the response to abiotic stresses (Nakashima et al., 2007). In particular, genes of the *NAC* family are induced by several abiotic stresses. *OsSNAC1* and *OsNAC6* were induced by drought, salt, cold, and ABA stresses and may regulate stress tolerance in rice (Hu et al., 2006; Nakashima et al., 2007). The expression of *OsSNAC1* and *OsNAC6* was examined in plants exposed to PEG, salt, mannitol, and ABA. Subtle differences in the levels of the *OsSNAC1* and *OsNAC6* mRNAs were observed between *oscipk31*::*Ds* mutants and wild-type plants under PEG (30%), salt (250 mM), mannitol (300 mM), and ABA (100 μM) stress (Supplementary Fig. 3). Significant differences in the levels of the *OsSNAC1* and *OsNAC6* mRNAs were not observed between *oscipk31*::*Ds* mutants and wild-type plants under (Supplementary Fig. 2).

Measurement of endogenous ABA levels and expression of ABA metabolic genes in *oscipk31*::*Ds*

Environmental stresses activate ABA biosynthesis and subsequently enhance the levels of endogenous ABA. As the *oscipk31*::*Ds* mutant was sensitive not only to ABA, but also to abiotic stresses, during germination and seedling growth, we measured the endogenous levels of ABA in plants exposed to drought. Two-week-old wild-type, *oscipk31*::*Ds* mutant, and complementation plants were grown on soil in a growth chamber. ABA levels were measured in over-ground tissues for five days after holding the water supply (Supplementary Fig. 4). There were no differences in ABA levels among the three lines.

The following rice genes encoding ABA metabolic enzymes have been identified: a single-zeaxanthin epoxidase (*ZEP*) gene, several putative nine-*cis*-epoxycarotenoid dioxygenase (*NCED*) genes, and three ABA 8′-hydroxylases (*ABA8ox*) genes (Agrawal et al., 2001; Saika et al., 2007). To examine whether different abiotic stresses might affect ABA metabolism in *oscipk31*::*Ds* mutants, the expression of ABA synthetic genes was measured in 10-day-old seedlings exposed to 30% PEG, 250 mM NaCl, 300 mM mannitol, and 100 μM ABA. The levels of *OsABA8ox1*, *OsZEP* (*OsABA1*), and *OsNCED* mRNA were examined using Northern hybridization (Supplementary Fig. 3). Wild-type and *oscipk31*::*Ds* mutant plants exhibited similar overall expression patterns and mRNA levels of these genes. However, slightly elevated levels of *OsZEP* and *OsABA8ox* were detected in mutants grown under 250 mM NaCl or 300 mM mannitol.

DISCUSSION

This study used the loss-of-function mutation to elucidate the following functional roles of *OsCIPK31* in rice plants: 1) *OsCIPK31* is involved in germination and seedling growth under abiotic stress conditions, and 2) under abiotic stress conditions, *OsCIPK31* modulates the expression of the stress-inducible genes *OsRAB21*, *OsDip1*, and *OsSalT*. During germination, ABA antagonizes GA function in grain aleurones, which inhibits seed germinating processes (Gilroy, 1996). In order to examine the possibility that *OsCIPK31* may affect sensitivity to GA, the germination and early growth of *oscipk31*::*Ds* mutant plants were tested at different concentrations of GA. However, there was no significant difference in the rates of germination and growth of shoots and roots between mutant and wild-type plants (data not shown). In addition, mutant plants exhibited a normal growth response of second leaf sheaths to exogenous GA (data not shown). Therefore, slow germination in the presence of ABA appeared to result from failure of the cellular responses to abiotic stresses. Another characteristic of *oscipk31*::*Ds* was that rice stress-inducible genes, such as *OsRAB21*, *OsDip1* and *OsSalT*, were expres-

sed at higher levels in mutants than in wild-type plants under the same stress conditions. These findings were rather unexpected, since *OsCIPK31* expression decreases under the same stress conditions. It is possible that *oscipk31* is more sensitive to abiotic stresses than wild type and that such hyper-sensitivity might lead to early induction of stress-response genes in mutant plants. However, further work will be required to elucidate the mechanisms underlying these observations. It is worth noticing that hyper-induction of stress genes has been reported previously in *Arabidopsis cbl*/*cipk* mutants and stress marker genes have shown higher induction levels in *atcbl9* mutant plants. It should be noted that the enhancement of the expression of these genes under the stresses tested here was observed only in shoots. The analysis of stress-induced genes in roots under abiotic stress conditions revealed almost identical expression levels and patterns between *oscipk31*::*Ds* and wild-type plants. As *OsCIPK31* was not expressed in roots, even when exposed to light, it is reasonable to argue that the enhancement of the expression of stress-inducible genes may be a direct consequence of the loss of *OsCIPK31* function in shoots. However, the mutation affected growth of both roots and shoots. Therefore, it is tempting to speculate that slow root growth may result from a failure of shoots to cope with stress conditions. However, further work will be required to confirm this hypothesis. Additional evidence that *OsCIPK31* was sensitive to environmental conditions could be inferred from the observation that abiotic stress-mediated short induction of *OsCIPK31* took place only in light conditions. One might speculate that light induces the second message and triggers inductive expression. One of the candidates may be reactive oxygen species (ROS) or Ca^{2+}, which can be pulsed upon exposure to light (Mohammad et al., 2009).

This study also examined the expression of two *NAC* (*OsSNCA1, OsNAC6)* genes and three ABA metabolic genes. Overexpression of *OsSNCA1* and *OsNAC6* in rice plants significantly enhanced resistance to drought and salinity. These genes were also induced by abiotic stresses, which included drought, high salt, and ABA (Hu et al., 2006; Nakashima et al., 2007). ABA synthetic genes of *Arabidopsis*, such as *AtABA1 (ZEP), AtABA3, AtAAO3*, and *AtNCED3*, are upregulated by ABA exposure and osmotic stress in *Arabidopsis* (Cheng et al., 2002b; Xiong et al., 2002a). However, there were no distinct changes in the expression of these *NAC* and ABA metabolic genes. Therefore, *oscipk31* plants exposed to abiotic stresses did not exhibit a broad spectrum of stress symptoms.

Previous studies have reported that *OsCIPK31* (*OsCK1*) is highly induced by cold stress (Kim et al., 2003b) and that rice plants that overexpress *OsCIPK31* (*OsCIPK03*) are more tolerant to cold stress than wild-type plants (Xiang et al., 2007). However, the *oscipk31*::*Ds* knockout did not show any alteration in cold resistance (Supplementary Fig. 5). Instead, *oscipk31*::*Ds* mutants exhibited a slow germination rate and retardation of seedling growth under abiotic stresses, which included ABA treatment. The most plausible explanation for this discrepancy may be gene redundancy. A phylogenetic analysis revealed that *OsCIPK31* is most closely related to *OsCIPK3* and *AtCIPK3* (Fig. 1B) (Kolukisaoglu et al., 2004; Xiang et al., 2007). Therefore, rice genomes encode two genes that are homologous to *AtCIPK3.* Similar to *oscipk31*::*Ds* plants, *atcipk3* mutants show no significant phenotypic changes under normal conditions (Gou et al., 2004; Kim et al., 2003a). In *Arabidopsis*, two related CBL/CIPK calcium-signaling proteins (AtCBL9 and AtCIPK3) modulate seed germination and young seedling growth via similar ABA signal transduction pathways. *AtCBL9* modulates ABA signaling under drought, high salt, and osmotic stresses at the seedling stage, whereas *AtCIPK3* affects salt-induced ABA-dependent signaling and cold-induced ABA-independent signaling during seed germination (Kim et al., 2003a; Pandey et al., 2004). *AtCBL9* may interact with *AtCIPK3* to regulate ABA sensitivity during seed germination. As observed for the *oscipk31*::*Ds* mutants, hyperinduction of stress marker genes was also detected in *atcbl9* mutant plants. These genes included *RD29A, RD29B, KIN1, RAB18*, and *COR47* (Pandey et al., 2004). However, the expression of the stress marker genes *RD29A*, *RD29B*, and *KIN1* in the *atcipk3* mutant was decreased under salt and exogenous ABA stress conditions. In contrast, the expression of these genes was not altered by drought and PEG stresses (Kim et al., 2003a).

In summary, the phenotypes and the expression pattern of stress marker genes observed in *oscipk31*::*Ds* plants are closest to those of *Arabidopsis atcbl9* mutant plants, among all *Arabidopsis* CBL/CIPK genes. However, the expression of ABA metabolic genes was not significantly changed, and endogenous ABA levels were not altered, in *oscipk31*::*Ds* plants. Additional work will be required to address the possibility that *OsCIPK31* may be involved in ABA-mediated stress signaling pathways during the development of rice plants. In particular, this possibility can be thoroughly examined using double mutants for the *OsCIPK31* and *OsCIPK3* genes, if they become available.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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