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Calcineurin B-like interacting protein kinase OsCIPK23 functions in pollination and drought stress responses in rice (*Oryza sativa* L.)

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Abstract

Drought is very harmful to grain yield due to its adverse effect on reproduction, especially on pollination process in rice. However, the molecular basis of such an effect still remains largely unknown. Here, we report the role of a member of CBL (Calcineurin B-Like) Interacting Protein Kinase (CIPK) family, OsCIPK23, in pollination and stress responses in rice. Molecular analyses revealed that it is mainly expressed in pistil and anther but up-regulated by pollination, as well as by treatments of various abiotic stresses and phytohormones. RNA interference-mediated suppression of *OsCIPK23* expression significantly reduced seed set and conferred a hypersensitive response to drought stress, indicating its possible roles in pollination and drought stress. In consistent, overexpression of *OsCIPK23* induced the expression of several drought tolerance related genes. Taken together, these results indicate that *OsCIPK23* is a multistress induced gene and likely mediates a signaling pathway commonly shared by both pollination and drought stress responses in rice.

Keywords: *OsCIPK23*; water; pollination; drought; rice (*Oryza sativa* L.)

Introduction

In most of higher plants, pollination is initiated when a partially desiccated pollen grain contacts the stigma and acquires water from the stigma to hydrate (Ma, 2003). In rice, pollen grains are first released as a result of dehiscence of anthers, stick to the brush-shaped stigma and start germinating after hydration followed by the emergence of the tip of pollen tube from an aperture of a pollen grain, and the contents of the grain move into the pollen tube, including two sperm nuclei following a vegetative nucleus. Finally, the elongating tube begins to penetrate into the stigma through the transmitting tissues and eventually reaches the micropyle of ovule (Dai et al., 2006).

It has been found that pollination is a particular sensitive process to various environmental stresses including drought and high or low temperature (Schoper et al., 1987; Hedhly et al., 2005; Lan et al., 2005; Boavida and McCormick, 2007; Li et al., 2007). To adapt to the adverse abiotic stresses such as drought, wounding, high salinity, and low temperature, plants can initiate a number of molecular, cellular, and physiological changes and activate cascades of molecular networks (Albrecht et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005; Xiang et al., 2007). In addition, previous reports showed cross-talks between stress signaling pathways and pollination. For example, a recent study has revealed thirty-three genes specifically expressed at the anthers desiccation phase. Among them, six genes were both dehydration- and abscisic acid (ABA)-inducible (Hsu et al., 2007). Lan et al. (2005) reported that 53.8% (136/253) and 21% (57/253) of the pollination-related genes are regulated by dehydration and wounding, respectively, and they are likely associated with the necessary changes in osmotic tensions to facilitate the rapid pollen hydration and

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germination and/or establishing a gradient of diffusible signals required for pollen tube elongation to the ovule. Many stigma-specific genes have been shown to be related to dehydration and wounding, suggesting that they are probably involved in maintaining water homeostasis under the stress conditions to support the pollen tube growth (Li et al., 2007).

It has been emerged that an increased cytosolic free Ca^{2+} concentration as a secondary messenger transduces the cellular responses to extracellular stimuli and regulates various cellular and developmental processes (Albrecht et al., 2003; Berridge et al., 2003; Kim et al., 2003a, 2003b; Cheng et al., 2004; Kolukisaoglu et al., 2004). To date, several families of Ca^{2+} sensors have been identified in higher plants, such as the Calmodulin (CaM), CaM-related proteins, and CDPK (Calcium-Dependent Protein Kinase) proteins (Snedden et al., 1995; Cheng et al., 2004). Another important family of Ca^{2+} sensor proteins is referred to as Calcineurin B-like (CBL, also known as SCaBP, Skin Calcium Binding Protein) proteins (Kudla et al., 1999; Xu et al., 2006). The CBL proteins with necessary NAF domains facilitate the CIPKs (CBL-Interacting Protein Kinases, also known as PKS, protein kinase) to activate the kinase activity to transduce calcium signals by autophophorylating or phosphorylating downstream components to mediate Ca^{2+} signaling functions (Shi et al., 1999; Kim et al., 2000; Albrecht et al., 2003; Pandey et al., 2004; D'Angelo et al., 2006; Cheong et al., 2007; Batistic et al., 2008).

The *CBL* and *CIPK* genes have been studied extensively in *Arabidopsis* (Kudla et al., 1999; Albrecht et al., 2003; Kim et al., 2003a; Hwang et al., 2005; Lee et al., 2005; Cheong et al., 2007). In *Arabidopsis*, there are 25 CIPK proteins, which are also called SnRK3, PKS, or SOS2-like protein kinases (Kolukisaoglu et al., 2004), and 10 CBL proteins, which are also called SCaBPs or SOS3-like calcium-binding proteins. Many reported *Arabidopsis CBL* and *CIPK* genes could be induced by abiotic stresses and ABA (Albrecht et al., 2003; Berridge et al., 2003; Pandey et al., 2004). In rice, 30 members of CIPK and 10 CBL members were identified (Kolukisaoglu et al., 2004; Xiang et al., 2007; Gu et al., 2008). OsPK4/OsCIPK19 and OsPK7/OsCIPK12 were able to phosphorylate themselves and myelin basic proteins (Ohba et al., 2000). OsCK1/OsCIPK3 appears to be induced by diverse stimuli such as cold, light, salt, sugar, calcium, and cytokinin (Kim et al., 2003b). However, their functions remain largely undefined. Our previous rice Genchip analysis showed that *OsCIPK5*, *9*, *16*, *20*, and *30* are expressed highly in anther, whereas *OsCIPK1*, *17*, *19*, *24*, *28*, and *29* expressed highly in stigma (Li et al., 2007). In addition, the studies of CBL-CIPK calcium signaling pathways were reported in other species, such as maize, legume, wheat, and cotton (Ohba et al., 2000; Mahajan et al., 2006; Wang et al., 2007; Gao et al., 2008). In this study, we report the role of OsCIPK23 in pollination in rice. Our results showed that it is a multistress induced gene and likely mediates a signaling pathway involved in both pollination and stress responses.

Materials and methods

Plant materials and growth conditions

Two rice cultivars (*Oryza sativa* ssp. *Japonica* var. *Nipponbare* and var. *Zhonghua11*) were used in this study. Transgenic plants (RNAi T_0, T_1, and T_2 lines) and untransformed negative control were grown in the field under natural conditions within two growing seasons, respectively. The growing season, as well as their growth conditions, was described previously (Kong et al., 2006).

Real-time PCR

Total RNA from various tissues was isolated using the RNeasy Plant Minikit (Qiagen, Düsseldorf, Germany), and real-time PCR were performed as previously described (Li et al., 2007). In brief, reverse transcription was performed using TaqMan Reverse Transcription Regents kit (Applied Biosystems, Foster City, USA: ABI). The cDNA template samples were diluted to 5 ng/μL and 1.25 ng/μL. Triplicate quantitative assays were performed on 2 μL of each cDNA dilution using the SYBR GreenMaster Mix (ABI, PN 4309155) with an ABI 7900 sequence detection system according to the manufacturer's protocol (ABI). The gene-specific primers were designed by using PRIMER-EXPRESS software (ABI). The relative quantification method (Delta-Delta CT) was used to evaluate quantitative variation between replicates that were examined. The amplification of 18S rRNA was used as an internal control to normalize all data. Gene-specific primers for *OsCIPK23* were 5′-CAGACTTCAGTTGCCCGTCTT-3′ and 5′-TGGTGCTAGGATTAGGATCTAGTATTTTC-3′; for 18S rRNA were: 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-TGTCACTACCTCCCCGTGTCA-3′; for *DREB2A* were: 5′-AGAAGGAGCAAGGGATTGTAGAAAC-3′ and 5′-GGGCCAACCATAGTCTGCAA-3′; for *rd29A* were: 5′-TGGATCAAACAGAGGAACCA-3′ and 5′-CATCTTAGTCGCACCATTCTCA-3′; for *Rab18* were : 5′-TCGGTCGTTGTATTGTGCTTTT-3′ and 5′-CCAGATGCTCATTACACACTCATG-3′; for *NCED3* were: 5′-CCAGATTGCTTCTGCTTCCAT-3′ and 5′-TTAAGGGCATCAGCAATGTAGAGA-3′.

Southern blot analysis

Genomic DNA isolation and Southern blot analysis were performed as described previously (Lai et al., 2002).

In brief, 5 μg of genomic DNA was digested, separated on 0.8% agarose gel, and transferred onto Hybond Nt (Amersham, GeneQuant Pro, USA) membrane. Prehybridization, hybridization, and washing of the blot were performed as recommended by the manufacturers. *HPT* probe was labeled with ^{32}P by random priming using the Prime-a-Gene labeling system (Promega, Madison, USA). The *HPT* primers were 5′-GCAAGGAATCGGTCAATACAC-3′ and 5′-TCCACTATCGGCGAGTACTTC-3′.

Expression of OsCIPK23 in Escherichia coli and preparation of antibody

A full-length *OsCIPK23* cDNA was cloned into *pET-30a* bacterial expression vector between the *Bam*H I and *Sal* I sites in which the insert was fused to the C-terminal of HIS, and the primers used were 5′-CAGGATCCATGAGCGTGTCGGGCGGGAG-3′ and 5′-CAGTCGACTCACGGTGACCTCCGATGCT-3′. The resulting construct was introduced into protease-deficient *E. coli* strain *BL21*(*DE*3). When OD_{600} of the bacterial culture reached 0.6, 0.5 mmol/L IPTG was added to induce the recombinant protein product for 3 h at 37ºC. The collected bacterial cells were lysed by infiltration and the recombinant proteins were purified by electric elution. Purified recombinant OsCIPK23 was used to elicit polyclonal antisera production in rabbits. The OsCIPK23 polyclonal antibody was purified using the Protein A resin column according to the manufacturer's instruction (GenScript, Piscataway, USA).

Western blot analysis of transgenic plants

Preparation of plant total proteins from anthers of transgenic and wild type plants was performed as normal. Fresh materials were ground in liquid nitrogen and extracted in 50 mmol/L Tris buffer (pH 8.0) with 300 mmol/L NaCl, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 10 mmol/L DTT, and a protease inhibitor cocktail (Sigma, St Louis, USA) at a dilution of 1:100. The extraction mixtures were kept on ice for 15 min and then centrifuged for 10 min at 10,000 g to pellet cellular debris. Protein concentrations of the different fractions were estimated according to the method of Bradford. The SDS-PAGE, hybridization, washing, and detection were then performed as described previously (Qiao et al., 2004).

Plasmid constructs

OsCIPK23 RNAi construct

The specific 500 bp sequence of *OsCIPK23* cDNA was fused in the same orientation by *Sac* I and *Spe* I and reversed orientation by *Kpn* I and *Bam*H I under the *ubiquitin* promote of the vector *pTCK303*, respectively (Wang et al., 2004). The fragment of *OsCIPK23* was PCR amplified by Pyrobest DNA polymerase (TaKaRa, Dalian, China) using the wild-type genomic DNA as template. The construct was completely sequenced to ensure that it did not contain PCR or cloning errors. The PCR primers used were as follows: 5′-GGGGTACCACTAGTACGCTGTCGATTACTGTCA-3′ (*Kpn* I and *Spe* I site underlined) and 5′-CGGGATCCGAGCTCTTGGAGGCTGATATCCC-3′ (*Bam*H I and *Sac* I site underlined).

OsCIPK23 overexpression construct

The resulting PCR product of *OsCIPK23* CDS was digested using *Kpn* I and *Spe* I and inserted between the *ubiquitin* promoter and the nopaline synthase terminator of the vector *pTCK303* (Wang et al., 2004). The PCR primers used were as follows: 5′-CGGGTACCATGAGCGTGTCGGGCGGGAG-3′ (*Kpn* I site underlined) and 5′-GGACTAGTTCACGGTGACCTCCGATGCT-3′ (*Spe* I site underlined).

OsCIPK23-GFP fusion construct

The complete CDS of *OsCIPK23* was amplified using two primers 5′-CGGGATCCATGAGCGTGTCGGGCGGGAG-3′ (*Bam*H I site underlined) and 5′-CGCAAGCTTCGGTGACCTCCGATGCTGGA-3′ (*Hin*d III site underlined). The resulting PCR product was subcloned into a rebuilt vector *pCAMBIA1301* driven by maize *ubiquitin* promoter to generate *p1301-Ubi*:*OsCIPK23-GFP* to be transformed to rice and a rebuilt vector *pBI221* driven by CaMV *35S* promoter to generate *p221-35S*:*OsCIPK23-GFP* to be used for biolistic transformation to onion epidermis cells.

OsCIPK23-GUS fusion construct

A 2 K cDNA sequence upstream the ORF of *OsCIPK23* was cloned into *pCAMBIA1391* vector to generate *p1391-pOsCIPK23-GUS* between the *Hin*d III and *Bam*H I sites to be transformed to rice, and the primers used were 5′-CGCAAGCTTCACGCAAGCGGATAAGACTAAAT-3′ and 5′-CGGGATCCCAAGGAGGTGGGTGGGGGGT-3′.

Plant transformation

Plant transformation was performed as described previously (Kong et al., 2006). In brief, rice embryonic calli were induced on scutella from germinated seeds and transformed with strain EHA105 of *Agrobacterium tumefaciens* containing desired binary vector. Transgenic plants were selected in half-strength Murashige and Skoog medium containing 50 mg/L Hygromycin (Roche, Nutley, USA). Hygromycin-resistant plants from calli, defined as transgenic plants of T_0 generation, were transplanted into the field.

GUS staining

One milliliter of working GUS staining solution containing 100 mmol/L sodium phosphate (pH 7.0), 0.5 mL 0.1 mol/L EDTA-Na_2, 40 μL 0.1 mol/L $K_4[Fe(CN)_6]$ (potassium ferricyanide), 40 μL 0.1 mol/L K_6 $[Fe(CN)_6]$ (potassium ferrocyanide), 10 μL 100 mmol/L 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) and 1 μL Triton X-100. Rice tissues were put into the GUS staining solution in dark at 37°C overnight. After the chlorophyll was bleached with 95% ethanol, the tissues would be observed with somatotype microscope.

Rice seedling cultivation under stress conditions

Sterilized rice seeds were put onto MS media for two weeks and then were transferred to the MS media with 30% PEG, 8 μmol/L ABA, 15 mmol/L NaCl, and 5 μmol/L GA, respectively. The seedlings used to extract total RNA were obtained after 1 h, 3 h, 6 h, and 12 h, respectively.

Rice plants cultivation under drought conditions

Both the transgenic negative control plants and transgenic plants were treated by drought stress at the stage of flowering. After two weeks of drought stress and one week rewatering, the phenotype was observed. The plants were planted under natural conditions.

Observations of pollen viability

The detection of pollen viability was performed as described previously (Dai et al., 2006). Mature anthers (before flowering) of *OsCIPK23* RNAi lines and wild-type plants were harvested, and anthers before fertilization were dissected out from transgenic or wild type plants. Pollen grains were released on a glass slide by gently squashing anther in a drop of staining solution. I_2-KI solution containing 1% (v/v) of I_2 in 3% (v/v) KI was used to check starch accumulation to check pollen viability. Stained pollen grains were observed under an optical microscope.

Subcellular localization

The constructs of *p221-35S*:*OsCIPK23-GFP*, and *GFP* alone were transformed into onion epidermal cells using gene gun (Bio-Rad, Hercules, USA). Transformed materials were incubated in dark at 28°C. Propiolium iodide (50 μg/mL) was used as an indicator of nucleus. Expression and localization of OsCIPK23-GFP fusion protein and GFP were observed with a Zeiss LSM 510 META confocal microscope (Zeiss, Jena, Ge). The *p1301-Ubi*:*OsCIPK23-GFP* construct was transformed into wild-type rice plants. The root tip of the transgenic rice plant was sectioned longitudinally and visualized using a laser-scanning confocal microscope (Zeiss, LSM 510).

Alignment and phylogenetic analysis

Sequence alignment of the NAF domains of 30 members of CIPK family in rice was performed using ClustalW. Phylogenetic analysis of the 30 members of CIPK family in rice based on amino acid sequences was carried out using a neighbor-joining (NJ) method with MEGA version 3.0. NJ analysis was carried out with the 'complete deletion' option selected. Support for each node was tested using bootstrap analysis, 1,000 replicates for NJ, using random input order for each replicate.

Accession number

Sequence used in this study can be identified in NCBI GenBank data libraries under accession no. CR291522.

Results

Identification of OsCIPK23

The cDNA encoding candidate kinases involved in pollination and fertilization were initially screened in 10 K unique rice cDNA database (http://plantbiol.genetics.ac.cn) (Lan et al., 2004). Among them, a cDNA encoding a CBL (Calcineurin B-Like) protein interaction protein kinase (CIPK) was discovered and designated as *OsCIPK23*. Database search in GenBank (http://www.ncbi.nlm.nih.gov) revealed that it is a gene with 15 exons and 14 introns (Supplemental Fig. 1A). Further sequence analysis revealed that it has 1,353 base pairs in the predicted ORF encoding a polypeptide of 450 amino acids in length with a calculated molecular mass of 49.5 kDa. It encompassed a putative serine/threonion domain, a NAF domain, and a C-terminal domain (Supplemental Fig. 1B). Database search showed that *OsCIPK23* is localized on chromosome 7. Amino acid alignments revealed a high degree of sequence homology of NAF domains among 30 members of the CIPK family in rice (Supplemental Fig. 2). Phylogenetic analysis showed that OsCIPK23 belonged to a subfamily with a close evolutionary relationship with OsCIPK9, OsCIPK3, OsCIPK8, and OsCIPK24 (Supplemental Fig. 3). These results showed that *OsCIPK23* encodes a rice kinase protein with NAF domain.

Expression profiles of OsCIPK23

To examine the expression of *OsCIPK23*, seven cDNA samples were used for real-time quantitative PCR analysis (Fig. 1A). The results showed that the expression level of *OsCIPK23* was highest in pistil just 3 h after anthesis and

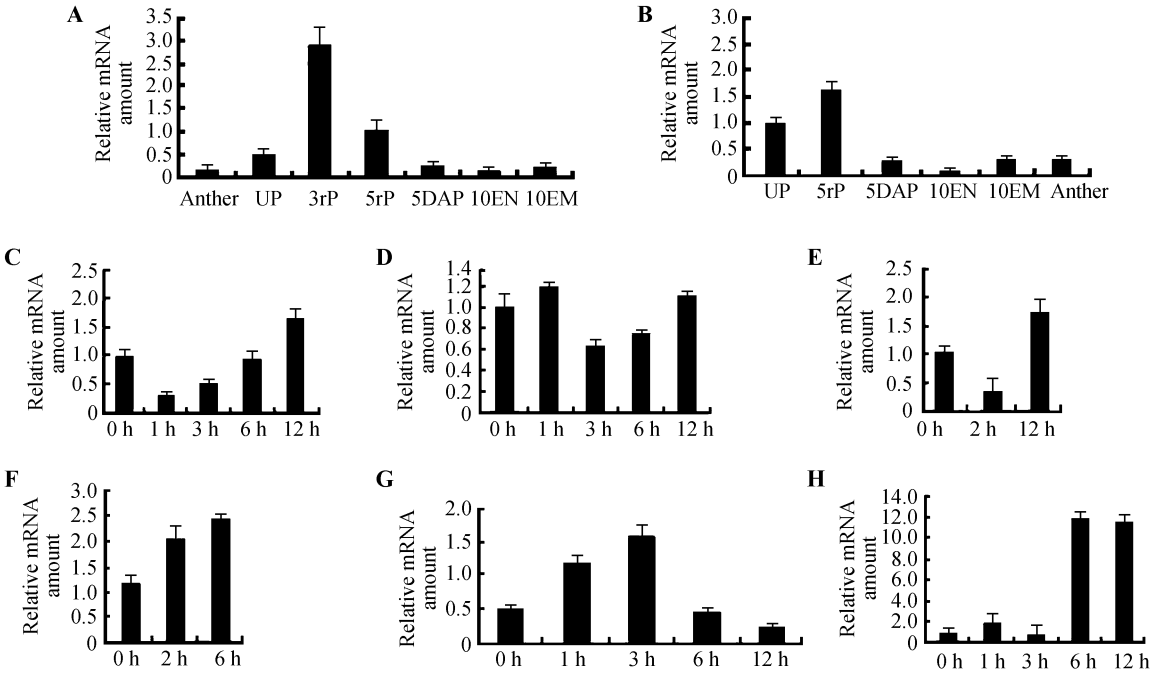

Fig. 1. Expression patterns of *OsCIPK23*. **A:** expression analysis of *OsCIPK23* by qRT-PCR assay. Total RNA were from UP, unpollinated pistil; 3rP, pistil collected 3 h after pollination; 5rP, pistil collected 5 h after pollination; 5DAP, embryo collected 5 days after pollination; 10 EN, endosperm collected 10 days after pollination and 10 EM, embryo collected 10 days after pollination. Error bars indicate standard errors of the mean (n = 6). **B:** expression of *OsCIPK23* based on cDNA microarray hybridization. The data were derived from a 10 K rice cDNA microarray (Lan et al., 2004). Error bars indicate standard errors of the mean (n = 3). **C** and **D:** expression analysis of *OsCIPK23* under 150 mmol/L sodium chloride and 4°C cold by qRT-PCR assay, respectively. Error bars indicate standard errors of the mean (n = 6). **E** and **F:** expression analysis of *OsCIPK23* under drought and wounding stress treatment by cDNA microarray hybridization, respectively. The data were derived from a 10 K rice cDNA microarray (Lan et al., 2004). Error bars indicate standard errors of the mean (n = 3). **G** and **H:** expression analysis of *OsCIPK23* under 5 μmol/L GA and 8 μmol/L ABA, respectively. Error bars indicate standard errors of the mean (n = 6).

pollination and decreased 5 h after pollination. Further analyses revealed that *OsCIPK23* was expressed strongly in the pistil and up-regulated during pollination (Fig. 1B), which is consistent with our previous finding (Lan et al., 2004).

Previous analysis revealed that most of the genes in volved in pollination also appeared to be responsive to various stress responses (Lan et al., 2005). To examine the expression of *OsCIPK23* under stresses, cDNA samples of seedlings under stress treatments were used for real-time quantitative PCR analysis. The results showed that *OsCIPK23* was induced by 150 mmol/L sodium chloride 6 h after treatments and 4°C cold 1 h after treatment (Fig. 1, C and D). Microarray analysis showed that *OsCIPK23* was induced 12 h after drought treatment and 2 h after wounding treatment (Fig. 1, E and F) (Lan et al. 2005). It is likely that an early response of *OsCIPK23* could be different from a late response (Fig. 1, C and E) (Ladrera et al., 2007). Also, the results of qRT-PCR showed that *OsCIPK23* was up-regulated after 5 μmol/L gibberellin treatment for 1 h and 8 μmol/L abscisic acid 6 h after treatments (Fig. 1, G and H), consistent with our previous finding (Wang et al., 2005b).

Taken together, the results indicated that *OsCIPK23* is mainly expressed in pistil and anther and up-regulated by pollination, abiotic stresses, and phytohormones, suggesting that it is likely involved in both pollination and stress responses.

Subcellular localization of OsCIPK23

To examine the subcellular localization of OsCIPK23, we constructed a translation fusion between OsCIPK23 and a synthetic green fluorescent protein (sGFP). The *OsCIPK23-sGFP* fusion and s*GFP* alone, both driven by the *35S* promoter, were introduced into onion epidermal cells by particle bombardment. The OsCIPK23-GFP fusion protein appeared to be strong in the nucleus and weak in the cytosol, whereas the control sGFP was uniformly distributed throughout the cell (Fig. 2, A and B). Furthermore, the subcellular localization of the OsCIPK23 protein *in vivo* were examined using a construct of a *OsCIPK23-sGFP* fusion driven by the *ubiquitin* promoter for transformation of *O. sativa* ssp. *Japonica* var. *Nipponbare*, and a total of six transgenic T_0 lines were generated (data not shown). The OsCIPK23-GFP fusion protein was detected predominantly in the nucleus of root cells of the transgenic plants (Fig. 2C), and the colocalization of OsCIPK23-GFP

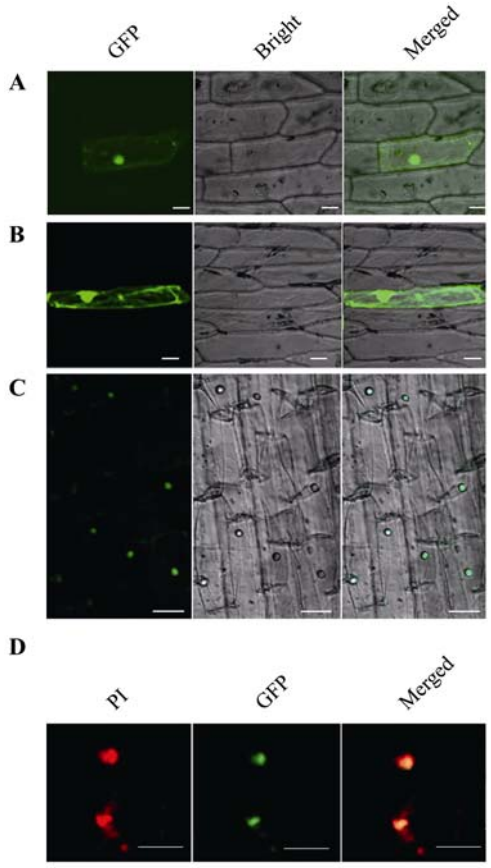

Fig. 2. Subcellular localization of the OsCIPK23 protein. **A** and **B:** onion epidermal cells expressing the OsCIPK23-green fluorescent protein (GFP) fusion protein and GFP alone driven by the *35S* promoter, respectively. Bars = 50 μm. **C:** expression of the OsCIPK23-GFP fusion protein driven by the *Ubiquitin* promoter in the root cells in the transgenic rice plant. Bars = 10 μm. **D:** the green fluorescence of OsCIPK23-GFP fusion overlapped with the red fluorescence signal of propiolium iodide (PI), which formed yellow fluorescence. Bars = 10 μm.

fusion protein and propidium iodide (PI) stain showed the similar result (Fig. 2D), suggesting that the OsCIPK23 protein is likely a nuclear protein. However, it is possible that OsCIPK23 is also weakly expressed in the cytosol.

Promoter analysis of OsCIPK23

To further examine the expression profiles of *OsCIPK23 in vivo*, a construct of *pOsCIPK23-GUS* fusion driven by the native promoter of *OsCIPK23* was cloned into *pCAMBIA1391*, which was transformed into *O. sativa* ssp. *Japonica* var. *Nipponbare* (Fig. 3A), and a total of six transgenic T_0 lines were generated (data not shown). The results indicated that the pOsCIPK23-GUS fusion protein was detected predominantly in the stigma and lodicules, glume, pollen grains, anther, and root of the transgenic plants (Fig. 3, B–F), confirming its expression in the reproductive tissues.

Molecular and phenotypic analyses of the T_0 and T_1 OsCIPK23 RNAi transgenic plants

To investigate the function of *OsCIPK23 in vivo*, a construct of *pTCK303-Ubi:OsCIPK23-RNAi* driven by the maize *ubiquitin* promoter was transformed into *O. sativa* ssp. *Japonica* var. *Nipponbare* (Fig. 4A), and a total of nine independent T_0 lines (*i-1* to *i-9*) were generated. Southern blot analysis showed that all of them were positive transgenic rice plants, and among them, lines *i-2*, *i-4*, and *i-5* had two copies and other seven lines contained single copy of the transgene insertion, and all of the nine plants were independently transformed (Fig. 4B). To examine the transcription levels of *OsCIPK23* in the RNAi transgenic T_0 plants (*i-6*, *i-7*, *i-8*, and *i-9*), real-time quantitative PCR

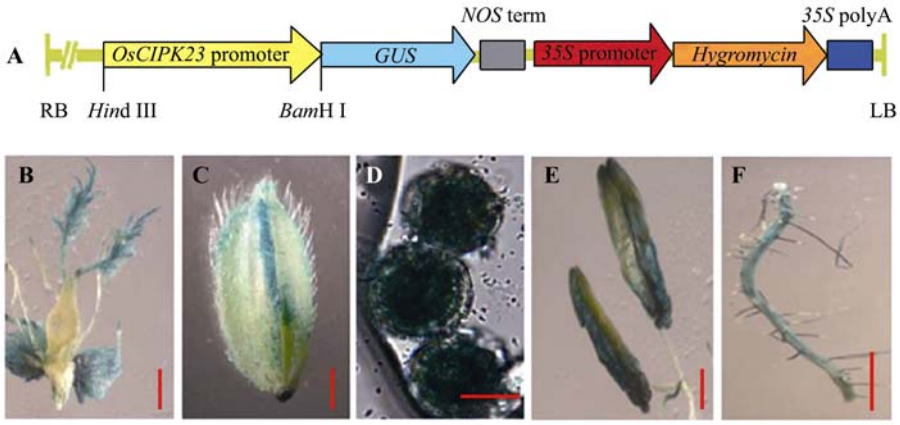

Fig. 3. Promoter analysis of *OsCIPK23*. **A:** a schematic representation of the *pOsCIPK23*::*GUS* construct. The 2 K sequence upstream of the *OsCIPK23* coding region was fused with *GUS* gene and cloned into *pCAMBIA1391*. *Hin*d III and *Bam*H I are resctriction sites used for subcloning. **B–F:** detection of the *OsCIPK23* expression in stigma and lodicules, glume, pollen grains, anther, and root, respectively. Bars = 6 mm (B), 10 mm (C), 50 μm (D), 6 mm (E), and 1 cm (F), respectively.

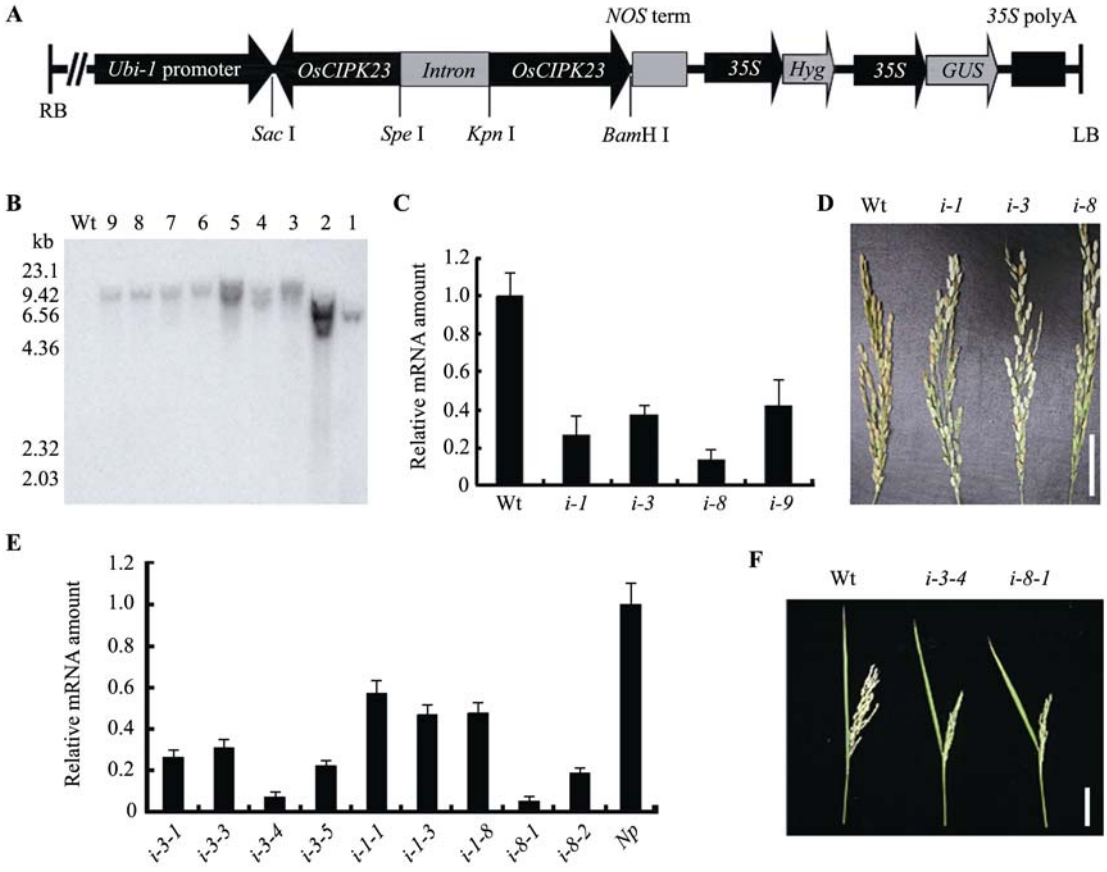

Fig. 4. Molecular and phenotypic analyses of the T_0 and T_1 *OsCIPK23* RNAi transgenic lines. **A:** a schematic representation of the *OsCIPK23* RNAi construct. The 500 bp sequence of *OsCIPK23* cDNA was fused in the same orientation by *Sac*I and *Spe* I and reversed orientation by *Kpn* I and *Bam*H I under the *Ubiquitin* promoter, respectively. **B:** genomic DNA gel blot analysis of the T_0 *OsCIPK23* RNAi transgenic lines and wide type (Wt). Lines 1–9 represented nine independent T_0 lines (*i-1* to *i-9*). Genomic DNA was isolated from leaves and digested using *Eco*R I. A PCR fragment derived from the *Hygromycin* gene of the construct was used as the probe. **C:** Histogram of the reduced *OsCIPK23* expression in T_0 *OsCIPK23* RNAi lines. Total RNA samples of unpollinated pistil, the four independent T_0 RNAi plants, and the wild type control were used for qRT-PCR analysis. Error bars indicate standard errors of the mean (n = 6). **D:** gross morphology of panicles of wild type and T_0 *OsCIPK23* transgenic lines at the ripening stage. Bar = 2 cm. **E:** histogram of the reduced *OsCIPK23* expression in the T_1 *OsCIPK23* RNAi lines. Total RNA samples of unpollinated pistil, the four independent T_0 RNAi plants, and the wild type control were used for qRT-PCR analysis. **F:** gross morphology of panicles of wild type and the T_1 *OsCIPK23* transgenic lines at the ripening stage. Bar = 5 cm.

analysis was conducted using anther total RNA from the T_0 positive transgenic lines and wild type. The results showed that its transcription in all of the four transgenic lines was reduced substantially (Fig. 4C). Phenotypic analyses showed that no significant morphological differences were observed during vegetative development stages between the wild type and the T_0 *OsCIPK23* RNAi transgenic lines, suggesting that its knockdown had no apparent effect on vegetative growth. However, a poor seed set in the T_0 *OsCIPK23* RNAi transgenic lines were observed, especially in *i-1*, *i-3*, and *i-8* (Fig. 4D), indicating that a reduction of OsCIPK23 had an adverse effect on the pollination process.

To further examine the phenotypes of the transgenic lines, a total of forty-five T_1 progeny of the *OsCIPK23* RNAi lines derived from the T_0 lines *i-1*, *i-3*, and *i-8*, respectively, were grown in the field under similar conditions for the phenotypic analysis, and GUS staining assay and PCR were used to detect the positive RNAi transgenic plants. The transcription levels of the target *OsCIPK23* in the T_1 RNAi transgenic plants were reduced substantially as revealed by real-time quantitative PCR analysis (Fig. 4E). Further genetic analysis showed that the T_1 progeny of all the three T_0 lines containing a single copy insertion conformed to a segregation ratio of 3:1 because the ratios of the positive to negative by GUS staining were 34:11, 35:10, and 29:11, respectively. The T_1 transgenic lines appeared normal during the vegetative growth, but their seed set after self-pollination was drastically reduced, especially the progeny derived from the T_0 RNAi transgenic lines *i-3* and *i-8* (*i-3*: 34.01%, *i-8*: 32.96%, and Wt: 95.49%) (Fig. 4F). Taken together, the results indicated that the reduction of *OsCIPK23* in the transgenic lines was stably inherited into their T_1 progeny, and its knockdown

significantly affected seed set.

Molecular and phenotypic analyses of the T_2 OsCIPK23 RNAi transgenic plants

To further examine the RNAi effect on *OsCIPK23*, a total of five T_2 homozygous transgenic lines were obtained after examination by using PCR and GUS staining screening (data not shown). Among them, one single copy insertion line was derived from *i-1* (*i-1-1*), three from *i-3* (*i-3-1*, *i-3-4*, and *i-3-5*), and one from *i-8* (*i-8-1*). Furthermore, Southern blot analysis with the *hygromycin* probe of the T_2 *OsCIPK23* RNAi transgenic plants revealed that the *OsCIPK23* RNAi transgene were stably inherited (Supplemental Fig. 4A). To examine the transcription levels of *OsCIPK23* in the RNAi transgenic T_2 plants, real-time quantitative PCR analysis was conducted using anther total RNA from T_2 homozygous transgenic lines (*i-3-4* and *i-8-1*) and one line of the T_2 null segregant progeny (*i-3-N*) as a negative control. The results showed that its transcription in all of the two transgenic lines was reduced (Supplemental Fig. 4B). It is often the case that RNAi shows a variability in inhibiting the target gene expression (Wang et al., 2005a), which might be the reason why the reduced expression of *OsCIPK23* was not significant in the T_2 progeny of transgenic lines. And Western blot analysis showed that the expression of OsCIPK23 in *i-1-1*, *i-3-4*, and *i-8-1* was reduced by various degrees (Supplemental Fig. 4C). To detect the expression of three *OsCIPK* genes similar to *OsCIPK23*, real-time quantitative PCR analysis was conducted using anther total RNA from the RNAi transgenic T_2 plants *i-3-4*, *i-8-1*, and *i-3-N*. The results showed that their transcription in all of the three lines had no difference, suggesting the specific inhibition on *OsCIPK23* (Supplemental Fig. 4D). These results demonstrated that the stable *OsCIPK23* RNAi transgenic lines were generated with a specific reduction of the *OsCIPK23* transcripts.

Phenotypic observation again showed that there were no significant morphological differences between the T_2 *OsCIPK23* RNAi homozygous transgenic lines (*i-3-4* and *i-8-1*) and the negative control *i-3-N* during vegetative development stages (data not shown). The two T_2 homozygous transgenic lines showed a decreased seed set of various levels (*i-3-N*: 98.99%, *i-3-4*: 93.56%, and *i-8-1*: 91.27%), whereas the seed sets of T_2 transgenic lines were increased compared with those of T_0 and T_2 transgenic lines, resulting from a reduced efficiency of RNAi inhibition on *OsCIPK23*.

To further examine the role of OsCIPK23 on pollen fertility, we stained the pollen grains of the T_2 RNAi transgenic lines *i-3-4* and *i-8-1* by using I_2-KI solution staining and found that they were irregularly shaped or pear-shaped and stained weakly and uneven, containing a large central empty vacuole without any starch granule, typical for sterile pollen in rice, whereas almost all of the pollen grains of

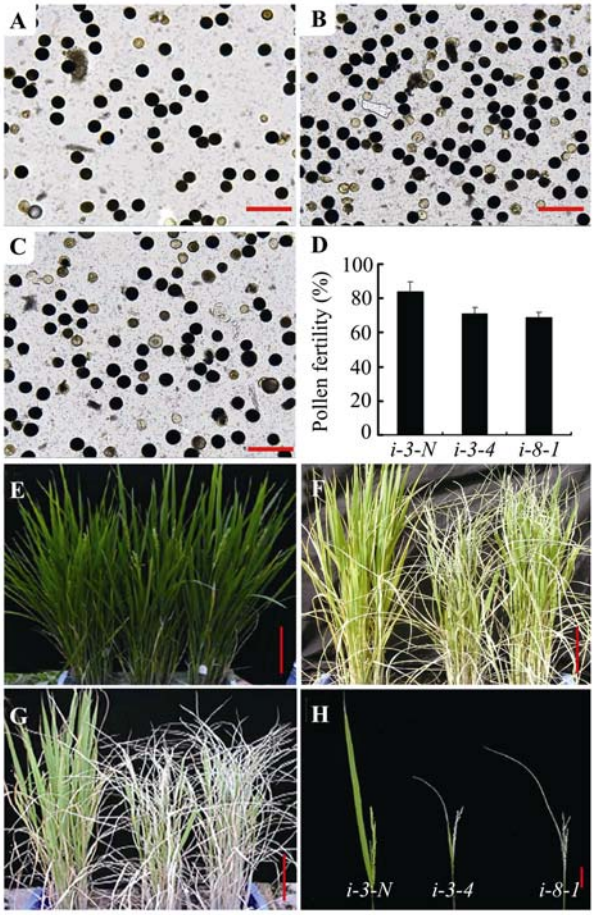

Fig. 5. Phenotypic analysis of the T_2 *OsCIPK23* RNAi transgenic lines under drought stress. **A–C:** examination of pollen fertility of the transgenic T_2 lines. I_2-KI solution staining of the mature pollen grains from *i-3-N* (A), *OsCIPK23* RNAi transgenic line *i-3-4* (B), and *i-8-1* (C) were collected just before anthesis and used for in vitro pollen germination assay. The sterile pollen grains failed to be stained or stained weakly, indicating that they did not contain starch or irregularly contained distributed starch, whereas the viable pollen grains were stained deep brown. Bars = 50 μm. **D:** histogram of the frequencies of pollen staining by I_2-KI solution *in vitro*. Error bars indicate standard errors of the mean (n = 10). **E:** gross morphology of the *OsCIPK23* RNAi T_2 progenies at the flowering stage before drought stress. The rice plants were from *i-3*-N, *i-3-4*, and *i-8-1*. **F:** gross morphology of the same plants as A after two weeks' drought stress. **G:** gross morphology of the same plants as B after one week of rewatering. Bar = 20 cm. **H:** the magnification of the panicles showed decreased seed set after drought stress. Bar = 6 cm.

line *i-3-N* were round and dyed deep brown (Fig. 5, A–C). And the statistical results were *i-3-N*, 84.6%; *i-3-4*, 71.2%; *i-8-1*, 68.5% (Fig. 5D).

To further elucidate the function of *OsCIPK23* during pollination under drought stress, the two T_2 RNAi transgenic lines (*i-3-4* and *i-8-1*) and the negative control plant of *i-3-N* were treated by drought stress at the stage of flowering (Fig. 5E). After two weeks of drought stress, both the RNAi transgenic lines and the *i-3-N* null segregant line were wilted (Fig. 5F). However, after rewatering,

some of the wilted leaves and stalks of the *i-3-N* were restored to green, whereas there was only little green tissue restored in the *i-3-4* and *i-8-1* (Fig. 5G), indicating that the RNAi transgenic lines were hypersensitive to drought stress. Although both of the seed set of *i-3-N* and RNAi transgenic lines were reduced, the effects of drought on the *i-3-4* (38.03%) and *i-8-1*(35.72%) were significantly more severe than on the *i-3-N* (47.71%) (Fig. 5H). These results indicated that *OsCIPK23* plays a role in pollination, especially under drought stress.

Molecular and phenotypic analyses of the T_0 OsCIPK23 overexpressing transgenic plants

To further examine the function of *OsCIPK23 in vivo*, an overexpression construct of *pTCK303-Ubi:OsCIPK23* driven by the maize *ubiquitin* promoter was transformed into *O. sativa* ssp. *Japonica* var. *Zhonghua11* (Fig. 6A), and a total of six T_0 lines (*OX3*, *OX4*, and *OX6* to *OX9*) were generated. Southern blot analysis showed that all of them were positive transgenic rice plants, among them, the lines *OX3* and *OX4,* as well as the *OX6* and *OX7*, were derived from two independent identical transformation events, and the four independent lines all contained a single copy of the transgene insertion (Fig. 6B). To examine the transcription levels of *OsCIPK23* in the overexpressing transgenic T_0 plants (*OX6* and *OX9*), real-time quantitative PCR analysis was conducted using anther total RNA from the T_0 positive transgenic lines and wild type (*Zhonghua 11*). The results showed that its transcription in all of the two transgenic lines was increased substantially (Fig. 6C). Western blot analysis showed that the expression of OsCIPK23 in the *OX6* and *OX9* was increased by various degrees (Fig. 6D). However, phenotypic analyses showed that no significant morphological and seed set differences were observed during vegetative development stages between the wild type and the T_0 *OsCIPK23* overexpressing transgenic lines. These results suggested that the expression of *OsCIPK23* is increased in overexpressing transgenic plants likely involved in drought stress response.

Expression patterns of drought tolerance related genes in the transgenic plants

To examine the drought related phenotype of the *OsCIPK23* transgenic plants, real-time quantitative PCR

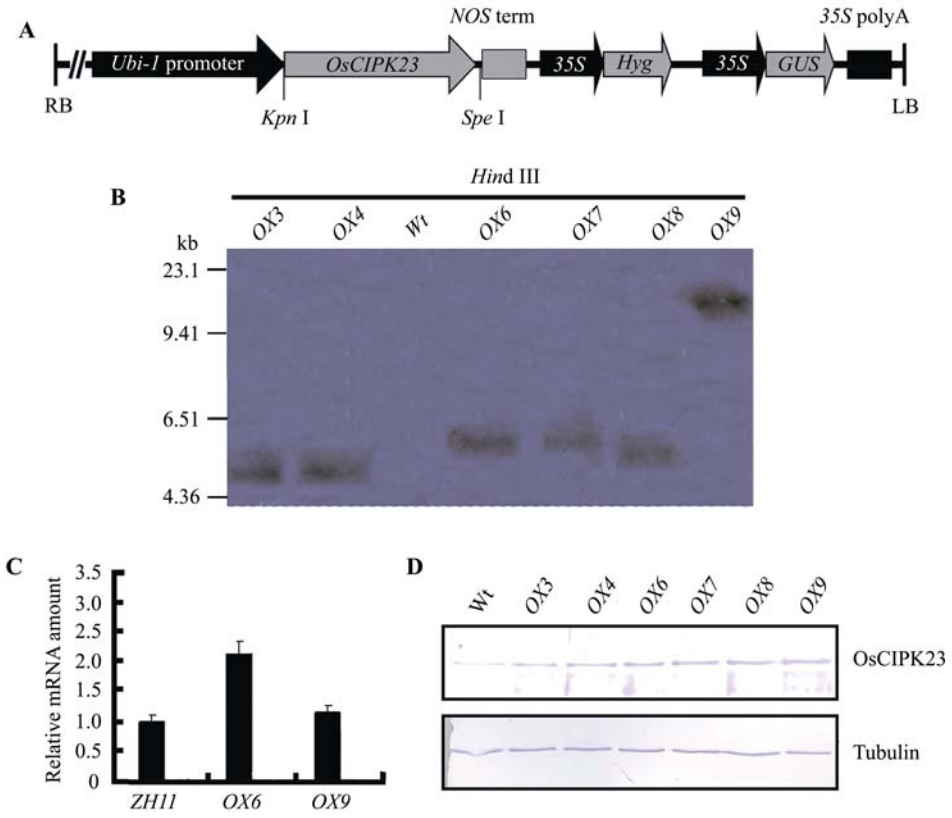

Fig. 6. Molecular analyses of the T_0 *OsCIPK23* overexpressing transgenic lines. **A:** a schematic representation of the *OsCIPK23* overexpressing construct. The full length of *OsCIPK23* cDNA was fused in the same orientation by *Kpn* I and *Spe* I under the *Ubiquitin* promoter. **B:** genomic DNA gel blot analysis of the T_0 overexpressing transgenic lines and wide type (Wt: *Japonica* var. *ZhongHua11*). **C:** Histogram of the increased *OsCIPK23* expression in T_0 *OsCIPK23* overexpressing lines. Error bars indicate standard errors of the mean (n = 6). **D:** western blot assay of the OsCIPK23 protein expression in four overexpressing transgenic and wild type plants with an antibody against OsCIPK23. Bottom panel shows an immunoblot of tubulin as loading control.

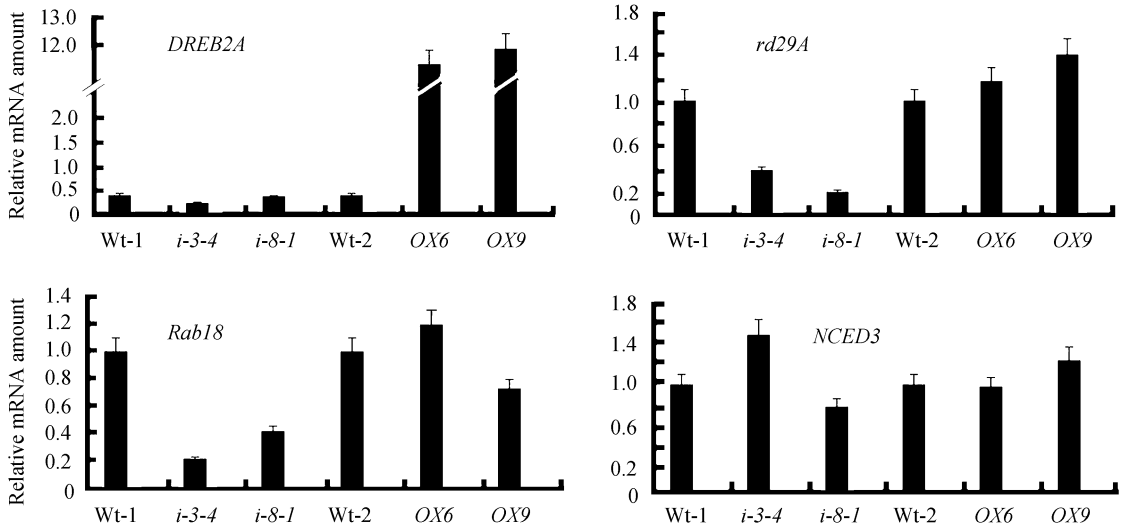

Fig. 7. Expression patterns of four drought marker genes in the transgenic plants. Histogram of the expression levels of four drought marker genes in the RNAi and overexpressing transgenic plants. Error bars indicate standard errors of the mean (n = 6). Wt-1, the rice variety *Japonica* var. *Nipponbare* and Wt-2, the *Japonica* var. *Zhonghua11*.

analysis of four drought tolerance related genes was conducted using anther total RNA from the T_2 positive RNAi lines (*i-3-4* and *i-8-1*), the T_0 positive overexpressing transgenic lines (*OX6* and *OX9*), and wild type (*Nipponbare* and *Zhonghua11*). The results showed that the expressions of *DREB2A*, *rd29A,* and *Rab18* were reduced in the lines *i-3-4* and *i-8-1*, whereas the expression of *NCED3* was increased in the line *i-3-4* and reduced in the line *i-8-1*. In contrast, *DREB2A*, *rd29A,* and *NCED3* were increased in the lines *OX6* and *OX9,* whereas the expression of *Rab18* was increased in the line *OX6* but reduced in the line *OX9* (Fig. 7). These results showed that the drought hypersensitive and tolerant phenotypes conferred by reducing and overexpressing *OsCIPK23*, respectively, were highly correlated with the expression of the drought related genes.

Discussion

OsCIPK23 responses to multiple abiotic stresses and hormones

Although *OsCIPK23* was previously shown to be only induced by drought and PEG treatments but not by ABA and sodium chloride (Xiang et al., 2007), our results revealed that it is in fact induced by both ABA and various stresses including sodium chloride, cold, drought, and wounding treatment (Fig. 1, C–F and H), indicating that it is likely involved in both hormone and abiotic stress responses. Several ABA-(*ABRE*), drought-(*DRE*), and cold-(*LTRE*) response elements have been detected in the upstream regions of rice *CIPK* genes (Xiang et al., 2007), but some of them such as *OsCIPK7*, *8*, *12*, and *20* without reported stress responsive *cis*-elements also were induced by abiotic stress signals (Xiang et al., 2007). Despite only one *DRE* is found in the upstream region of *OsCIPK23*, the fact that it could be induced by different stress signals shows the existence of some other regulatory elements for those stress signals. Previous studies have shown that genes induced by ABA also could be induced by drought and salt stress and *vice versa*, indicating a cross-talk between different abiotic stress signaling pathways (Pandey et al., 2004). Thus, it is likely that the multiple responses of *OsCIPK23* to various stress signals and hormones indicate that it could act as a molecular integrator of the cross-talks in either the ABA-dependent or ABA-independent manners or both.

In rice, both the stigma-specific genes and those related to abiotic stresses shared similar GCC-box *cis*-elements. In *Arabidopsis*, two transcription factors, AtERF4 and AtERF7, bind to the GCC-box region of target genes and overexpressing *AtERF7* showed a reduced sensitivity to drought stress (Yang et al., 2005). In rice, OsBIERF3 could bind specifically to the GCC-box sequence and was induced by salt, cold, drought, and wounding (Cao et al., 2006). Twenty-four GCC-boxes were detected in the upstream region of *OsCIPK23*, supporting its role in integrating multiple stress responses.

However, it remains unclear how *OsCIPK23* functions in abiotic stress responses. Plants appear to adopt three different mechanisms to fight against drought stress: to induce the expression of drought-response genes, to trigger an ABA-dependent signaling pathway, and to adjust the stomata opening-closure to control water loss rate. Our results showed that the expression levels of *DREB2A*, *rd29A*, *Rab18*, and *NCED3* were altered in both the *OsCIPK23* RNAi and the overexpressing transgenic plants

(Fig. 7). In addition, *DREB2A*, *NCED3*, and several *Rab* and *rd* genes were previously found to be expressed in stigma, and it is possible that *OsCIPK23* could also regulate these drought-response genes during pollination. However, our results of leaf water loss assay showed that no significant difference was detected between the RNAi and the overexpressing transgenic positive and negative plants (data not shown), suggesting that the overexpressing *OsCIPK23* alone could not confer a drought tolerance phenotype. Interestingly, our results showed that OsCIPK23 likely has a dual role in both stress tolerance and signal transduction.

A possible shared role of OsCIPK23 in abiotic stress and pollination responses

Several protein kinases are known to be expressed in reproductive tissues and play important roles in pollination, such as two pollen-specific kinases LePRK1 and LePRK2 and one pollen-specific receptor-like cytoplasmic kinase OsRLCK1 (Tang et al., 2002; Kong et al., 2007). Our expression profile analyses showed that *OsCIPK23* is expressed in both pistil and anther (Fig. 1, A and B; Fig. 3, B–E), indicating its role in pollination. In consistent with this view, its knock-down reduced the pollen fertility and seed set (Fig. 4, D and F; Supplemental Figs. 5). However, it is also expressed in other tissues like root, indicating that it must have additional roles.

GA is known to be critical for pollen development (Chhun et al., 2007), and many genes related to both pollen development and GA action were identified (Wang et al., 2005b). Twenty-four cDNAs out of 253 ESTs initially identified to be regulated by pollination also were responsive to GA treatment, suggesting that the GA response pathway has a role in pollination (Lan et al., 2005; Wang et al., 2005b). PsGA3ox1 was hardly detectable in unpollinated pericarps and ovules in pea, but increased dramatically after pollination, indicating an important role in pollination (Ozga et al., 2003). The induction of *OsCIPK23* by both drought stress and GA (Fig. 1, E and G), suggests that it could act as a regulator for pollination.

It is widely known that potassium (K^+) acts as an important regulator in pollination. For example, K^+ is an essential constituent of the pollen-germinating medium and is detected only at the aperture area of the mature pollen and anther dehiscence area of mature anthers to regulate anther dehiscence, pollen imbibition, and papillae hydration leading to pollination (Rehman and Yun, 2006). The physiological importance of K^+ in *Arabidopsis* pollen germination and tube growth was reported (Fan et al., 2001). Interestingly, twenty-seven putative K^+ transporters were detected to be highly expressed in stigma (our unpublished data), indicating the important roles of K^+ in pollination.

However, it is unclear how *OsCIPK23* acts during the pollination. In our results, *OsCIPK23* is induced by drought stress and ABA (Fig. 1, E and H), it is possible that it could mediate a stress-responsive ABA signaling function during pollination, but its mechanism needs further investigation. It is known that plant stress responses often mimic certain normal developmental processes, and there are genes which must be coregulated by both environmental factors and developmental cues to mount a coordinated response (Cooper et al., 2003). Thus, it is not surprising that a number of genes induced by pollination were also induced by drought and wounding stress, as well as several stigma-specific genes by stresses (Lan et al., 2005; Li et al., 2007). Nevertheless, OsCIPK23 appears to act as a shared molecular signaling component in mediating both pollination and abiotic stress responses.

In conclusion, our results show that OsCIPK23 is able to show response to pollination, multiple abiotic stresses, and phytohormones and has a dual role in regulating both pollination and drought stress in rice. It is likely that OsCIPK23 acts in a signaling pathway commonly shared by pollination and drought stress responses. Further studies including identification of OsCIPK23 targets will contribute to our understanding of the molecular basis of this shared function.

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Supplementary Data

Supplemental Figs. 1–4 associated with the article can be found in the online version at www.jgenetgenomics.org.

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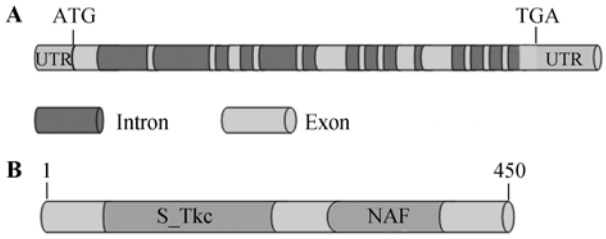
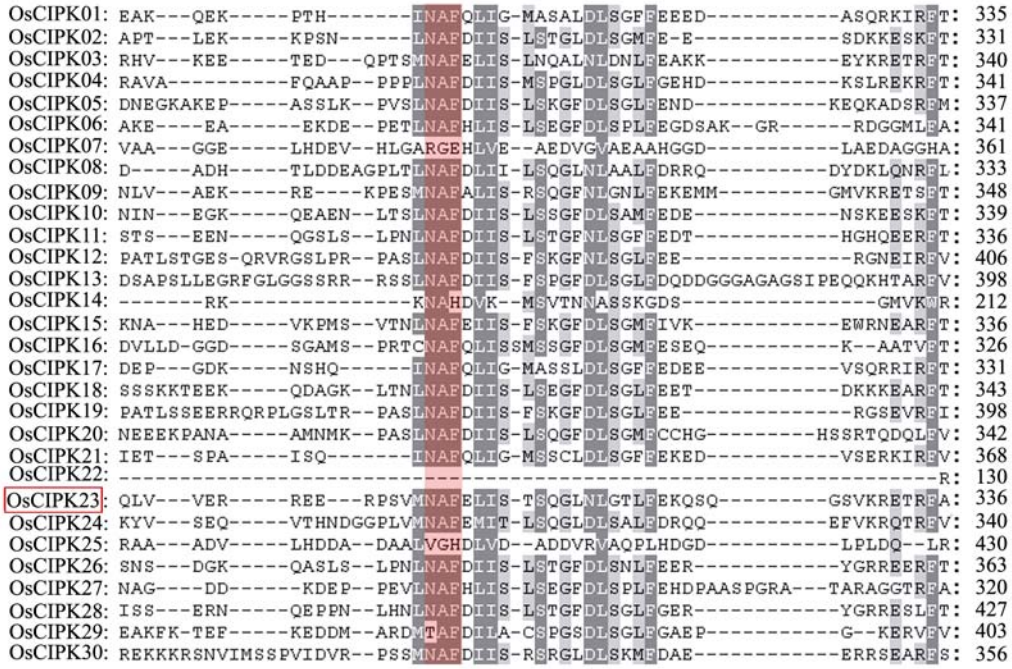
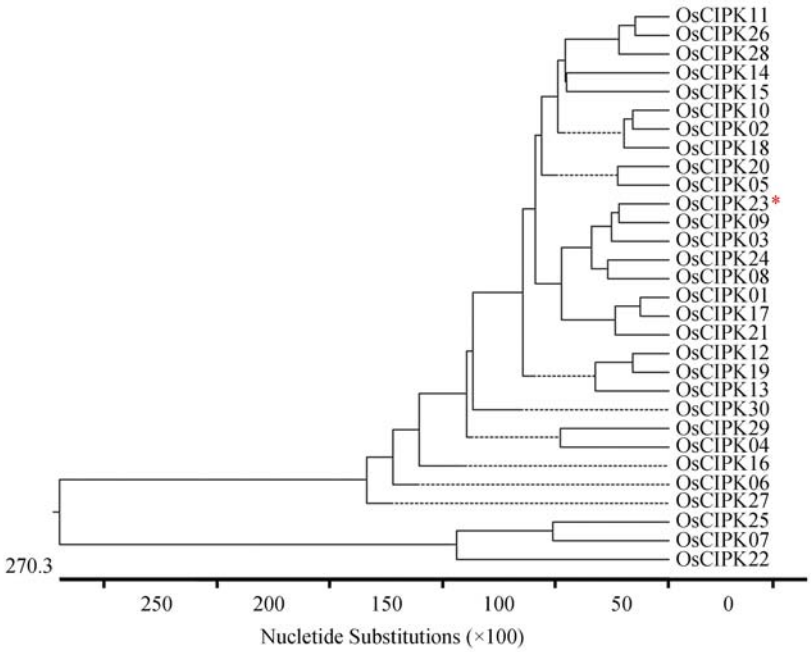
Supplemental Figures



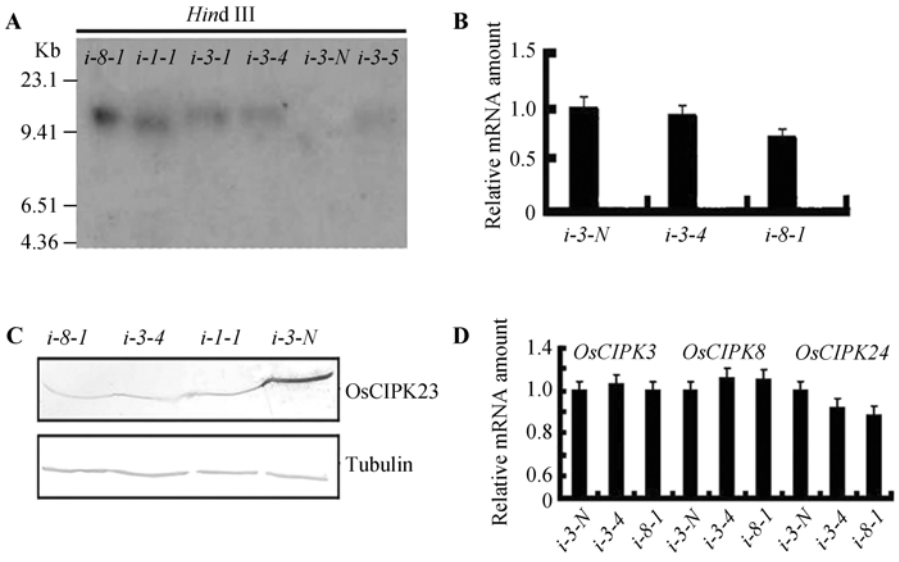
Supplemental Fig. 1. Identification and structure features of *OsCIPK23.* **A:** the structure of *OsCIPK23.* The dark and light gray boxes represent introns and exons, respectively. **B: t**he structure of OsCIPK23. The gray box represents S_Tkc and NAF domain.



Supplemental Fig. 2. Alignment of NAF domain of CIPK proteins. A ClustalW alignment of the deduced NAF domain of OsCIPK23 together with NAF domains from other members of CIPK family in rice. Identical and similar amino acids are shaded with dark and light gray, respectively. The red area represents NAF domain, and OsCIPK23 is marked with red rectangle.



Supplemental Fig. 3. Phylogenetic analysis of predicted NAF domains of 30 members of OsCIPK family in rice. The red asterisk shows the OsCIPK23.



Supplemental Fig. 4. Molecular analyses of the T_2 *OsCIPK23* RNAi transgenic lines. **A:** genomic DNA gel blot analysis of the positive and negative T_2 plants derived from the T_0 *OsCIPK23* RNAi transgenic lines. **B:** histogram of the reduced *OsCIPK23* expression in the T_2 *OsCIPK23* antisense lines. Error bars indicate standard errors of the mean (n=6). **C:** western blot assay of the OsCIPK23 protein expression in the T_2 transgenic and wild type plants with an antibody against OsCIPK23. Bottom panel shows an immunoblot of tubulin as loading control. **D**: expression analyses of the OsCIPK genes with high evolutionary relationship with *OsCIPK23* in T_2 *OsCIPK23* RNAi lines. Error bars indicate standard errors of the mean (n=6).