Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation[1][C][W]

Sonia Campo[2], Patricia Baldrich, Joaquima Messeguer, Eric Lalanne, María Coca, and Blanca San Segundo*

Centre for Research in Agricultural Genomics, Consejo Superior de Investigaciones Científicas-Institut de Recerca i Tecnologia Agroalimentàries-Universitat Autònoma de Barcelona-Universitat de Barcelona, Campus UAB, Bellaterra, Cerdanyola del Valles, 08193 Barcelona, Spain (S.C., P.B., J.M., M.C., B.S.S.); and Oryzon Genomics, Cornella de Llobregat, 08940 Barcelona, Spain (E.L.)

The *OsCPK4* gene is a member of the complex gene family of calcium-dependent protein kinases in rice (*Oryza sativa*). Here, we report that *OsCPK4* expression is induced by high salinity, drought, and the phytohormone abscisic acid. Moreover, a plasma membrane localization of OsCPK4 was observed by transient expression assays of green fluorescent protein-tagged OsCPK4 in onion (*Allium cepa*) epidermal cells. Overexpression of *OsCPK4* in rice plants significantly enhances tolerance to salt and drought stress. Knockdown rice plants, however, are severely impaired in growth and development. Compared with control plants, *OsCPK4* overexpressor plants exhibit stronger water-holding capability and reduced levels of membrane lipid peroxidation and electrolyte leakage under drought or salt stress conditions. Also, salt-treated *OsCPK4* seedlings accumulate less Na^+ in their roots. We carried out microarray analysis of transgenic rice overexpressing *OsCPK4* and found that overexpression of *OsCPK4* has a low impact on the rice transcriptome. Moreover, no genes were found to be commonly regulated by *OsCPK4* in roots and leaves of rice plants. A significant number of genes involved in lipid metabolism and protection against oxidative stress appear to be up-regulated by *OsCPK4* in roots of overexpressor plants. Meanwhile, *OsCPK4* overexpression has no effect on the expression of well-characterized abiotic stress-associated transcriptional regulatory networks (i.e. *ORYZA SATIVA DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN1* and *ORYZA SATIVA No Apical Meristem, Arabidopsis Transcription Activation Factor1-2, Cup-Shaped Cotyledon6* genes) and *LATE EMBRYOGENESIS ABUNDANT* genes in their roots. Taken together, our data show that *OsCPK4* functions as a positive regulator of the salt and drought stress responses in rice via the protection of cellular membranes from stress-induced oxidative damage.

Plants have evolved diverse mechanisms for sensing and responding to adverse environmental conditions. If the acclimation potential is surpassed by environmental stress, plants encounter metabolic imbalances and disturbances of developmental processes, which might result in reduced productivity and, eventually, plant death. Therefore, the improvement of abiotic stress tolerance might increase actual yields in most crops.

Plants acquire tolerance to abiotic stress by reprogramming metabolism and gene expression (Rabbani et al., 2003; Matsui et al., 2008). Early events in most abiotic stress responses involve changes in the cellular redox status and uncontrolled redox reactions, these processes occurring in different phases depending on the stress intensity: redox-dependent deregulation in metabolism, development of oxidative damage, and cell death. Thus, the primary goal of cellular regulation during adaptation to abiotic stresses is the readjustment of metabolic processes under conditions of increasing redox imbalances.

Abiotic stress-responsive genes can be categorized into three groups in terms of their protein products. One group comprises genes encoding proteins involved in the protection of macromolecules and cellular structures (i.e. LATE EMBRYOGENESIS ABUNDANT [LEA] proteins) and enzymes responsible for the synthesis of osmolites. This group also includes genes playing a role in protection from damage produced by reactive oxygen species (ROS), such as peroxidase, superoxide dismutase, and glutathione *S*-transferase (GST), among others. A second group of abiotic stress-responsive genes

[1] This work was supported by the European Research Area-Plant Genomics Network project Trilateral Initiative for Enhancing Salt Tolerance in Rice (grant no. GEN2006–27794–C4–1–E from Ministerio de Ciencia e Innovación), Ministerio de Economía y Competitividad (grant nos. BIO2009–08719 and BIO2012–32838), the Consolider-Ingenio (grant no. CSD2007–00036 to the Centre for Research in Agricultural Genomics), and the Generalitat de Catalunya (Xarxa de Referencia en Biotecnología and grant no. SGR 09626).

[2] Present address: Donald Danforth Plant Science Center, St. Louis, MO 63132.

* Address correspondence to blanca.sansegundo@cragenomica.es.

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[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.113.230268

comprises regulatory genes involved in stress signal transduction (mitogen-activated protein kinases and protein phosphatases) and transcriptional control (transcription factors). Finally, the third group comprises genes that are involved in ion transport (aquaporins and ion transporters). It is difficult, however, to distinguish critical genes that determine the phenotype of tolerance to the plant from housekeeping genes that are activated as part of the stress-induced perturbation. The emerging picture also defines that, in addition to transcriptional changes, posttranscriptional and posttranslational mechanisms (i.e. protein phosphorylation) might represent additional layers of regulatory systems governing plant adaptation to abiotic stresses. It is also well established that the phytohormone abscisic acid (ABA) is an important signal that mediates plant responses to abiotic stresses (Hirayama and Shinozaki, 2007), although salt stress-inducible genes that are ABA independent have been described (Nakashima et al., 2009; Kumar et al., 2013).

Global transcript profiling reveals that many genes are coordinately regulated by salt and drought stress, supporting a high degree of cross talk between these types of stresses (Rabbani et al., 2003; Nakashima et al., 2009). In this respect, it is well known that salt stress disrupts the homeostasis of water potential (osmotic homeostasis) and ion distribution (ionic homeostasis). Then, the initial response to salt stress (the osmotic stress component) shares similarities with drought stress, although long-term exposure to high salinity introduces the ion toxicity component. The homeostasis of sodium (Na^+) and potassium (K^+) ions is of particular importance for plant salt tolerance.

Increasing evidence supports that calcium levels are altered in plant cells in response to abiotic stress (Boudsocq and Sheen, 2010). The stress-induced perturbation in cytosolic calcium ion (Ca^{2+}) levels is sensed by calcium-binding proteins (Ca^{2+} sensors). In plants, the Ca^{2+}-dependent protein kinase (CPKs or CDPKs) family represents a unique group of calcium sensors (Harper et al., 2004). Within a single polypeptide, CPKs contain a catalytic Ser/Thr kinase domain fused to a calmodulin domain. This unique structure allows the CPKs to recognize specific calcium signatures and transduce the signal into phosphorylation cascades. The existence of salt stress-induced Ca^{2+}-dependent phosphorylation events controlling cellular Na^+ homeostasis and salt tolerance is well illustrated by the SALT OVERLY SENSITIVE (SOS) signaling pathway (Quintero et al., 2002).

CPKs have been identified throughout the plant kingdom and in some protozoans, but not in animals (Harper et al., 2004). The available information on plant CPKs indicates that CPKs are encoded by multigene families consisting of 31 genes in rice (*Oryza sativa*) and 34 genes in Arabidopsis (*Arabidopsis thaliana*; Hrabak et al., 2003; Asano et al., 2005; Ray et al., 2007; Schulz et al., 2013). Whereas some of the *CPK* genes are ubiquitously expressed, others show a tissue-specific pattern of expression or are regulated by stress (wounding, salinity, cold, drought, and pathogen infection; Klimecka and Muszyńska, 2007; Coca and San Segundo, 2010). Microarray analysis of rice seedlings subjected to cold, drought, or salinity stress revealed the up-regulation of six *CPK* genes (*OsCPK4*, *OsCPK10*, *OsCPK12*, *OsCPK13*, *OsCPK15*, and *OsCPK21*) and the down-regulation of one gene (*OsCPK1*; Ray et al., 2007; Das and Pandey, 2010). Even though the expression of several rice *CPK* genes has been reported to be regulated by abiotic stresses, only a few members of this multigene family have been functionally characterized in rice (Saijo et al., 2000; Komatsu et al., 2007; Asano et al., 2011, 2012).

In this study, we report the functional characterization of *OsCPK4*, a member of the *CPK* gene family from rice. *OsCPK4* gene expression is up-regulated in response to salt and drought stress. Transient expression of the *OsCPK4-gfp* gene in onion (*Allium cepa*) epidermal cells showed that OsCPK4 localizes to the plasma membrane. Overexpression of *OsCPK4* leads to increased tolerance to salt and drought stress in rice plants. Even though *OsCPK4* overexpression has a low impact in the rice transcriptome, transcript profiling revealed the up-regulation of a significant number of genes involved in lipid metabolism and protection against oxidative stress in roots of *OsCPK4* overexpressor plants. Contrary to this, alterations in the expression of genes typically associated with the salt stress response did not occur in roots of *OsCPK4* rice plants. Compared with control plants, *OsCPK4* overexpressor plants showed reduced levels of membrane lipid peroxidation and electrolyte leakage under salt stress conditions. *OsCPK4* rice seedlings also accumulated less Na^+ in their roots. Collectively, these results suggest that the protective effect conferred by *OsCPK4* overexpression in rice might be mediated by an increased capacity of the transgenic plants to prevent oxidative damage and membrane lipid peroxidation under salt stress conditions.

RESULTS

OsCPK4 Expression Is Induced by Salt and Drought Stresses

The *OsCPK4* gene (Os02g03410) encodes a CDPK containing the four-domain structure typical of CDPKs: an N-terminal variable domain, a Ser/Thr kinase domain, a junction autoinhibitory domain, and a C-terminal calmodulin domain (Fig. 1A). When examining *OsCPK4* expression in rice plants at different developmental stages (14-, 28-, and 50-d-old plants), higher levels of expression were found in roots compared with leaves at all the developmental stages analyzed (Fig. 1B).

We examined *OsCPK4* expression in response to salt and drought stress and at different times of stress treatment. *OsCPK4* was rapidly and transiently activated upon exposure to salt stress in rice roots (approximately 5-fold increase after 1 h of salt treatment; Fig. 1C, left). Under drought stress imposed by either air-drying treatment or polyethylene glycol (PEG) treatment, induction of *OsCPK4* expression was also observed, its

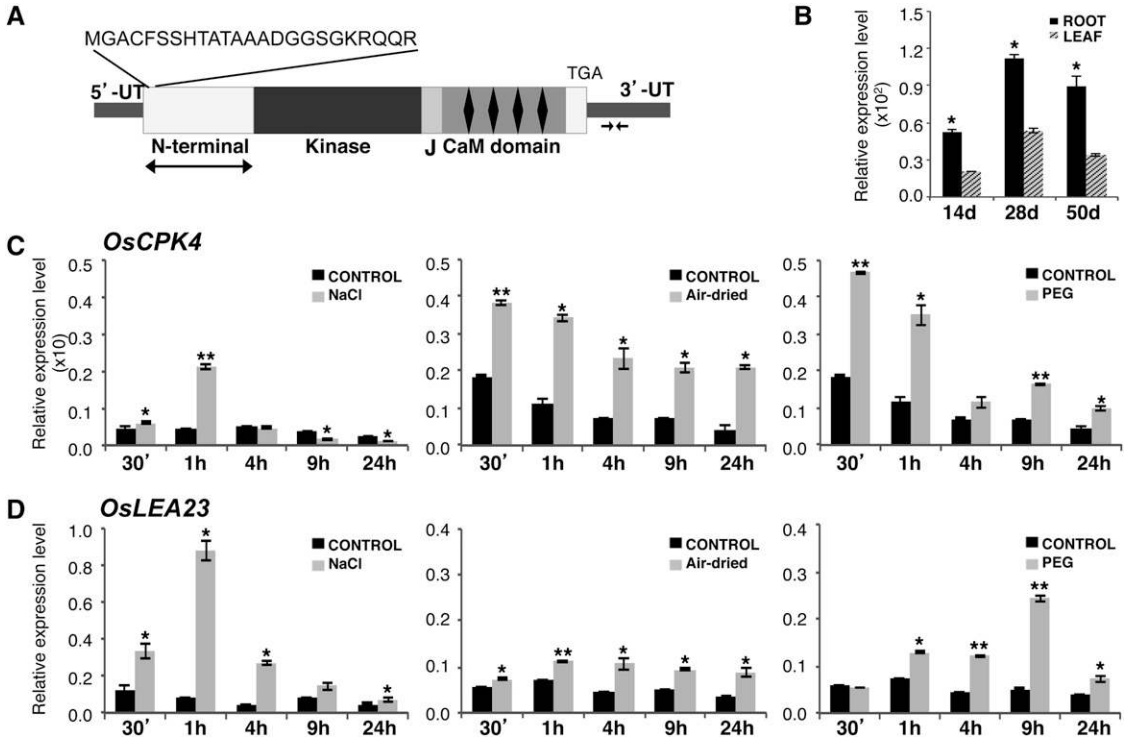

Figure 1. Expression of *OsCPK4* in response to salt and drought stress. Transcript levels were determined by qRT-PCR and normalized to *OsCYP* and *OsUBI* mRNAs. A, Structure of OsCPK4 showing the typical domains of plant CPKs: a kinase catalytic domain joined via a junction (J) domain sequence to a calmodulin-like domain (CaM) with four calcium-binding domains (rhombi in the calmodulin domain). The N-terminal amino acid sequence of OsCPK4 is indicated at the top. The bar indicates the DNA fragment used as a probe in northern-blot analyses of *OsCPK4* in Supplemental Figure S3. Arrows indicate the positions of primers used for qRT-PCR. UT, Untranslated region. B, Expression of *OsCPK4* in leaves and roots of rice plants at the indicated developmental stages. C and D, Expression of *OsCPK4* (C) and *OsLEA23* (D) in roots of rice plants in response to salt and drought stress. Seven-day-old seedlings were exposed to 100 mM NaCl and air dried or treated with 20% PEG 8000 for the indicated periods of time. The same RNA samples were used in C and D. Values represent means ± SE of three replicates (*$P \leq 0.05$, **$P \leq 0.001$).

activation being maintained during the entire period of stress treatment (Fig. 1C, middle and right). To assess the effectiveness of treatment, we analyzed the expression of the marker gene *OsLEA23*, a dehydrin gene also known as *OsDip1* (for *dehydration inducible protein1*; Fig. 1D). The expression of *OsLEA23/OsDip1* is known to be up-regulated by salt and drought stress (early-responsive gene; Rabbani et al., 2003). As expected, *OsLEA23/OsDip1* transcripts increased in response to high-salt conditions, with the highest levels recorded at 1 h of salt treatment and then decreasing at subsequent times of salt treatment (Fig. 1D, left). Air-drying and PEG treatment also induced *OsLEA23/OsDip1* expression (Fig. 1D, middle and right). When examining the salt responsiveness of *OsCPK4* expression in leaves of rice plants, a less intense and delayed activation of *OsCPK4* was observed compared with root tissues (Supplemental Fig. S1).

Together, this study indicated that *OsCPK4* gene expression is rapidly activated by salt and drought stress in the rice root. Moreover, *OsCPK4* and *OsLEA23/OsDip1* share similar expression patterns in response to salt stress, these two genes representing early salt stress-responsive genes.

OsCPK4 Expression Is Induced in Response to Treatment with ABA

ABA plays a major role in plant adaptation to high salinity and drought, and many genes that are induced by these stresses contain specific cis-regulatory elements in their promoters (Hirayama and Shinozaki, 2007). A detailed analysis of the *OsCPK4* promoter (2,000 bp upstream of the transcription start site) revealed the presence of multiple ABA-responsive cis-elements, including several ABREs (for ABA-responsive elements; PyACGTGGC), MYB recognition sites (TGGTTAG), and MYC recognition sites (CACATG; Fig. 2A; Supplemental Table S1). In particular, ABRE is considered a major cis-element in the promoter activity of genes regulated by ABA and osmotic stress (Yamaguchi-Shinozaki and Shinozaki, 2006). However, a single ABRE is not sufficient for an ABA response, and either additional ABREs

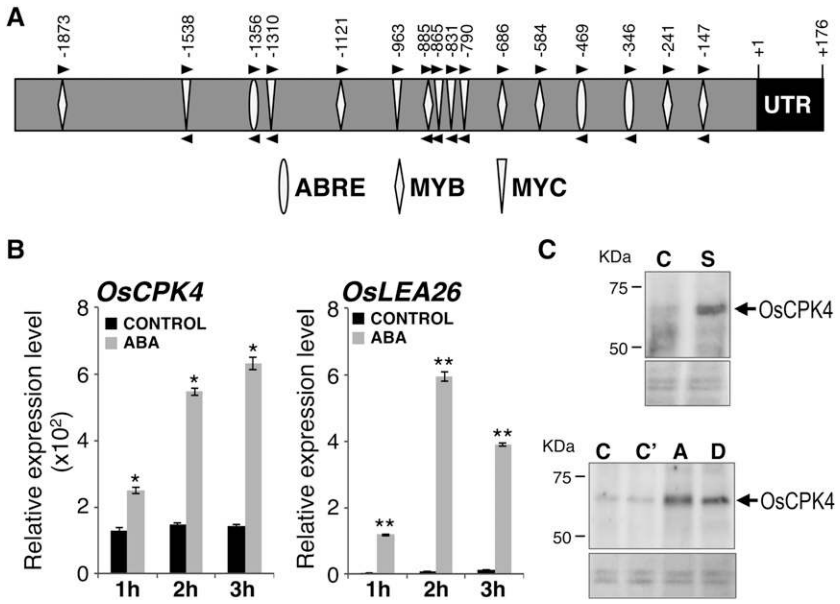

Figure 2. *OsCPK4* expression in response to ABA and accumulation of OsCPK4 in response to ABA treatment and salt and drought stress. A, Diagram of the *OsCPK4* promoter showing the distribution of abiotic stress-related cis-elements. UTR, Untranslated region. B, *OsCPK4* and *OsLEA26* expression in rice roots in response to ABA. The *OsCYP* and *OsUBI* mRNAs were used as internal controls for normalization. Significant differences are indicated (*$P < 0.05$, **$P < 0.001$). C, Accumulation of OsCPK4 in protein extracts of rice roots that were subjected to salt stress, ABA treatment, or drought stress (air drying) for 1 h. Protein extracts (20 μg each) were probed with an antiserum prepared against the N-terminal domain of OsCPK4. Top, protein extracts from control (C) and salt-stressed (S) roots. Bottom, protein extracts from control (C for ABA treatment, C′ for drought treatment), ABA-treated (A) and drought-stressed (D) rice roots. Ponceau S staining of western blots was used as the loading control.

or coupling elements are required. In line with this, several ABRE motifs were identified in the *OsCPK4* promoter region examined here (Fig. 2A). Consistent with the observation that the *OsCPK4* promoter contains cis-acting elements for ABA-dependent gene expression, a marked increase in *OsCPK4* transcripts occurred in rice plants in response to ABA (Fig. 2B, left). *OsLEA26/RAB16B* (for *responsive to ABA*) was used as a marker gene to show the effectiveness of the ABA treatment (Fig. 2B, right).

In this study, we also examined OsCPK4 protein accumulation in rice roots in response to treatment with ABA, high salt, and drought stress. Toward this end, polyclonal antibodies were generated against the N-terminal domain of OsCPK4 (Met-1 to Arg-58), a region that is known to be highly variable among CPK proteins (Supplemental Fig. S2, A and B). In agreement with real-time PCR data, western blotting with the anti-OsCPK4 protein showed an important increase in the accumulation of this protein after ABA, NaCl, and drought treatment (Fig. 2C). No serological reaction occurred when the preimmune serum was used to probe protein extracts from roots of either control or stressed rice plants (data not shown). Together, the results presented here indicate that the transcriptional activation of *OsCPK4* expression in response to salt and drought stress or ABA treatment is accompanied by the accumulation of the OsCPK4 protein in rice roots.

OsCPK4 Localizes to the Plasma Membrane

CPK proteins have been shown to localize in many different cellular compartments, including the nucleus, cytosol, peroxisome, plasma membrane, oil bodies, mitochondria, and endoplasmic reticulum, as well as in association with actin filaments (Dammann et al., 2003; Klimecka and Muszyńska, 2007; Campos-Soriano et al., 2011). To localize OsCPK4 in the plant cell, we transiently expressed an *OsCPK4-GFP* fusion gene in onion epidermal cells. Confocal laser scanning microscopy revealed an OsCPK4-specific labeling in the cell periphery, likely the plasma membrane (Fig. 3A). As expected, onion cells expressing the *GFP* gene alone showed a distribution of green fluorescence throughout the cell (Fig. 3B). Next, *OsCPK4-GFP*-transformed onion cells were also incubated in a hypertonic solution (15 min in 750 mM mannitol) to induce plasmolysis. In plasmolyzed onion cells, the OsCPK4-GFP displayed a pattern consistent with its location in the plasma membrane of the shrunken protoplasm (Fig. 3C). Under these conditions, protoplasts pull away from the cell wall, leaving large numbers of thin plasma membrane bridges, known as Hechtian strands, firmly anchored to the cell wall (Fig. 3D), thus confirming a plasma membrane localization for the OsCPK4 protein. Consistent with its subcellular localization site, a myristoylation site and a potential site for palmitoylation (Cys-4) are present at the N-terminal end of OsCPK4 (Fig. 1A).

Overexpression of *OsCPK4* Improves Tolerance to Salt and Drought Stress

Transgenic rice lines overexpressing *OsCPK4* were generated. Northern- and western-blot analyses confirmed the transgene expression and OsCPK4 protein accumulation (Supplemental Fig. S3). Under normal growth conditions, there were no obvious phenotypical differences between homozygous *OsCPK4* plants (T2–T4 generations) and wild-type plants (data not shown).

The transgenic rice plants overexpressing *OsCPK4* and transgenic rice lines expressing the empty vector (five independent homozygous *OsCPK4* lines and three

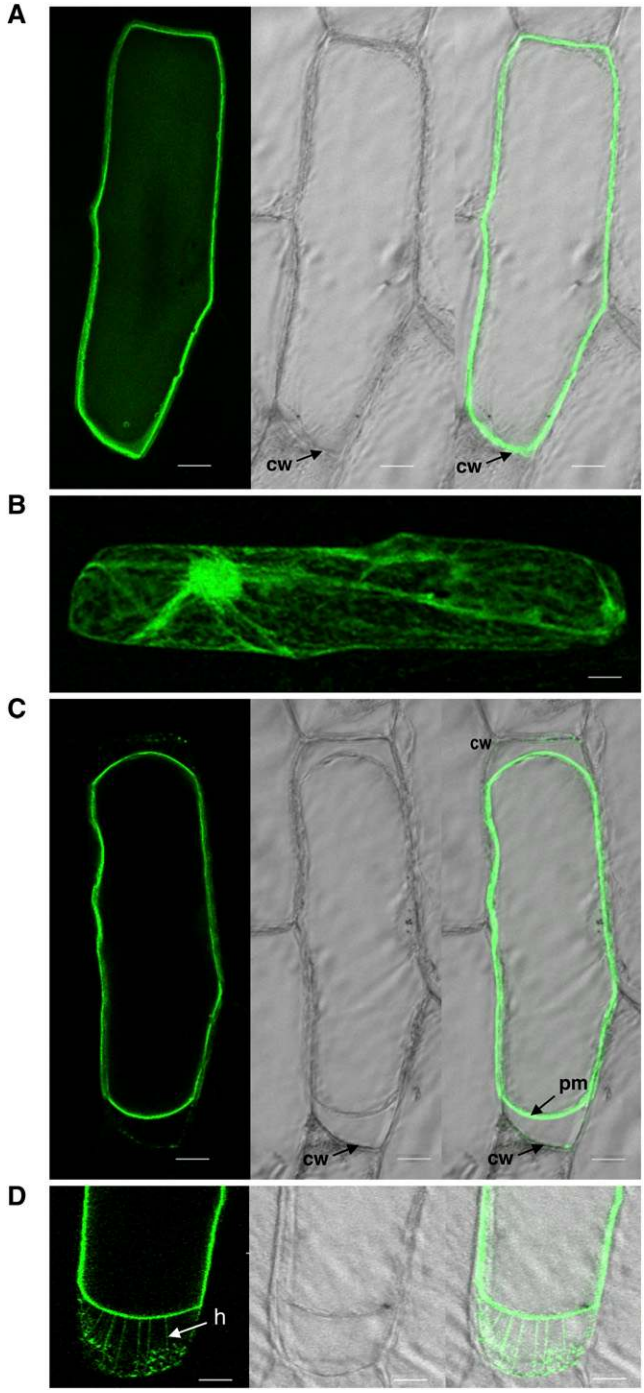

Figure 3. Plasma membrane localization of OsCPK4. Onion epidermal cells were transformed with the *OsCPK4-GFP* gene via particle bombardment. Confocal images were taken 5 h after bombardment. Projection (A and B) and individual (C and D) sections are shown. A, Localization of the OsCPK4 fusion protein. Fluorescence and transmission images are shown at left and middle, respectively. A merged image of the green fluorescence channel with the corresponding light micrographs is shown at right. B, Onion epithelial cell expressing *GFP*. C, Onion cell transformed with the *OsCPK4-GFP* gene after plasmolysis with mannitol (15 min of treatment). Light micrographs show the shrinkage of the protoplast. D, Higher magnification of a plasmolyzed onion cell showing the Hechtian strands (h). Treatment with mannitol renders the Hechtian strands attaching the plasma membrane (pm) to the cell wall (cw). Bars = 20 μm.

independent homozygous empty vector lines) as well as wild-type (cv Nipponbare) plants were tested for salt tolerance. The rice plants were grown hydroponically until they reached the three-leaf stage (Fig. 4, A and B, D0). Then, plants were exposed to salt stress (60 mM NaCl). Phenotypical differences between *OsCPK4* and control plants were evident by 11 d of salt treatment (data not shown). By 18 d of salt treatment, *OsCPK4* plants showed a clear phenotype of tolerance to salt stress compared with both vector control and wild-type plants (Fig. 4B, D18). Survival of *OsCPK4* transgenic lines after 25 d in the presence of salt was much higher than in control plants, the highest *OsCPK4*-expressing line consistently exhibiting the highest level of tolerance (line 1, 60% survival; Fig. 4, C and D). Under the same experimental conditions, only 2% to 12% of the control plants survived. From these results, it is concluded that overexpression of *OsCPK4* in transgenic rice results in improved tolerance to salt stress.

Next, we examined whether the overexpression of *OsCPK4* affects tolerance to drought stress in rice. Transgenic plants and vector control plants were grown for 15 d under a fully watered regime (Fig. 5, A and B, D0). Plants were deprived of irrigation for 17 d and then returned to the regular watering conditions to allow their recovery. By 14 d without water, vector control plants showed visual symptoms of drought-induced damage, such as leaf rolling and wilting, whereas transgenic *OsCPK4* plants remained green (data not shown). On day 17 of drought treatment, all the control plants were severely affected, while *OsCPK4* plants remained healthy and vigorous (Fig. 5B, D17). After 9 d of rewatering, only 18% of the control plants still contained green tissues (Fig. 5, B, RW, and C). Under the same experimental conditions, approximately 90% of the *OsCPK4* rice plants recovered from the stress. None of the control plants survived drought treatment.

It is generally assumed that plants with higher water retention ability can better survive under drought stress conditions. Accordingly, we checked whether *OsCPK4* overexpression has an effect on the water loss rate and water retention in the rice plant. Under drought conditions, overexpression of *OsCPK4* reduced water losses in leaves of these plants (Fig. 5D). Leaves from *OsCPK4* overexpressor plants also showed higher water retention ability than control plants (Fig. 5E).

Altogether, the results obtained in salt and drought tolerance assays demonstrated that *OsCPK4* overexpression confers tolerance to salt and drought stress in rice plants.

OsCPK4 Knockout Results in Severe Growth Inhibition

To further investigate the function of *OsCPK4*, we searched for loss-of-function mutants. Two transfer DNA (T-DNA) insertion mutants of *OsCPK4* were identified in

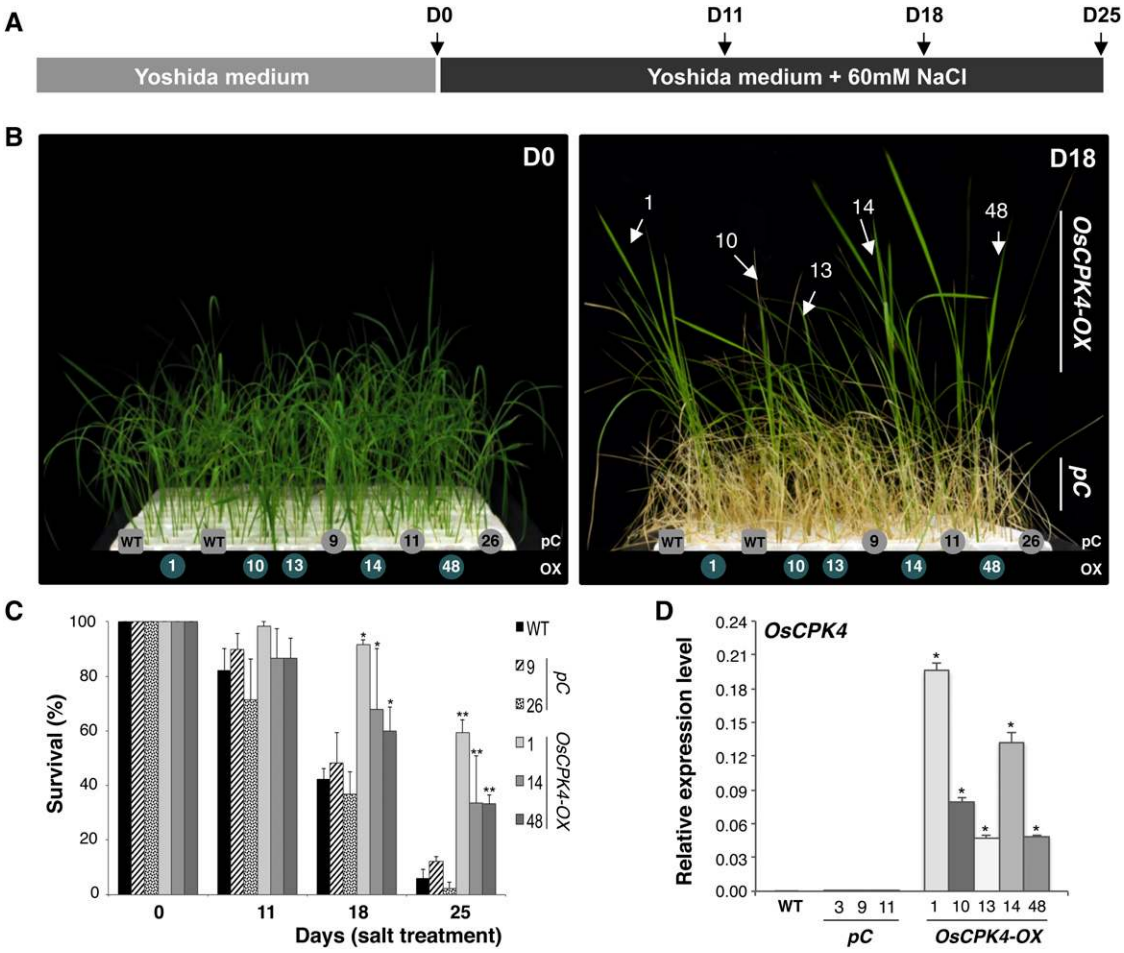

Figure 4. Salt tolerance of rice plants overexpressing *OsCPK4*. A, Diagram showing the experimental design for salt tolerance assays. Salt stress was imposed on both transgenic and control plants grown in hydroponic culture until they reached the three-leaf stage (D0). Plants were then transferred to nutrient solution containing 60 mM NaCl or to fresh nutrient solution. In each experiment, wild-type (WT; cv Nipponbare), vector control (pC; three independent lines), and *OsCPK4* overexpressor (*OsCPK4-OX*; five independent lines) plants were assayed. B, Phenotypes of *OsCPK4-OX* rice plants under salt stress conditions. Whereas control plants (wild type and vector control) grew poorly and exhibited chlorosis, the *OsCPK4-OX* lines grew in the presence of NaCl. Photographs were taken after 18 d of salt stress (D18). C, Survival rates of *OsCPK4-OX* and vector control plants at different times of salt treatment. D, *OsCPK4* expression in roots of transgenic and control plants used in salt tolerance assays as determined by qRT-PCR analysis. Specific primers for the analysis of *OsCPK4* transgene expression were used (Supplemental Table S4). Samples were taken at the D0 time point (onset of salt treatment). Salt tolerance assays were carried out three times and in two successive generations (T2 and T3). In each experiment, at least 30 plants per line were assayed. Asterisks denote significant differences between the *OsCPK4-OX* and control (wild type and vector control) groups (*$P \leq 0.05$, **$P \leq 0.001$). [See online article for color version of this figure.]

the POSTECH collection: 2D-00040 (cv Dongjin background) and 1D-03351 (cv Hwayoung background). Both of them contained the T-DNA insertion in the kinase domain of the *OsCPK4* gene (Fig. 6A). Results obtained for the 2D-00040 *cpk4* mutant are presented in Figure 6, B to D (similar results were obtained for the second *cpk4* mutant, 1D-03351; data not shown). PCR-based genotyping with combinations of different gene-specific and T-DNA primers allowed the identification of homozygous and heterozygous individuals for the *cpk4* mutants, and quantitative reverse transcription (qRT)-PCR confirmed *OsCPK4* suppression in homozygous mutant plants (Fig. 6, B and C). To note, the homozygous *cpk4* mutant plants were severely affected in their growth and development compared with the azygous plants (Fig. 6D). These findings support that *OsCPK4* disruption has a strong impact on plant growth, this gene being required for the normal growth and development of rice plants.

Transcript Profiling of Rice Plants Overexpressing *OsCPK4*

Knowing that *OsCPK4* overexpression enhances salt and drought tolerance, we initially hypothesized an

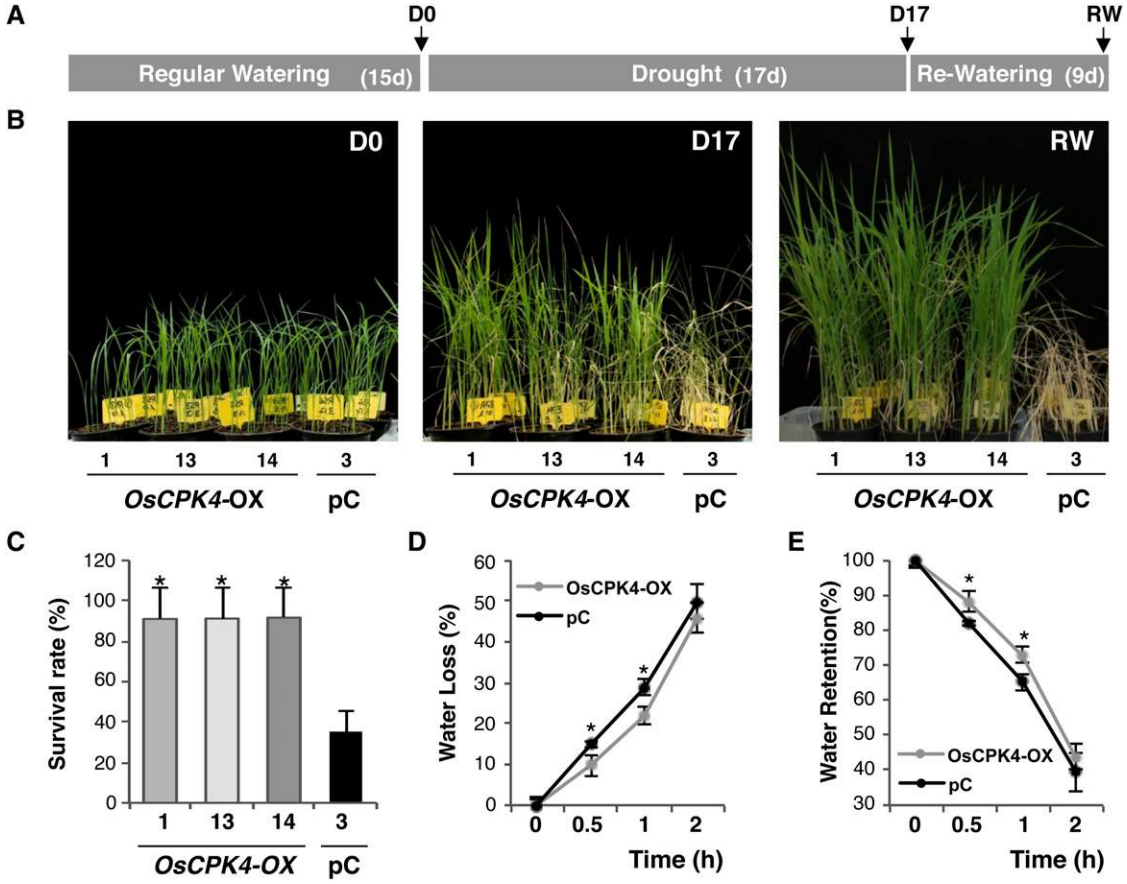

Figure 5. Drought tolerance of rice plants overexpressing *OsCPK4*. A, Diagram showing the experimental design for drought tolerance assays. Transgenic and control (wild-type and transgenic lines expressing the empty vector) plants were grown for 2 weeks (D0), subjected to 17 d of drought stress (D17), followed by 9 d of rewatering (RW) in the greenhouse. B, Phenotypes of *OsCPK4* overexpressor (*OsCPK4-OX*) and vector control (pC) plants during drought stress. Photographs were taken at day 0 (onset of drought treatment), at 17 d of drought stress (D17), and after 9 d of rewatering (RW). C, Survival rates of *OsCPK4-OX* and vector control plants exposed to drought stress. D and E, Water loss (D) and water retention rates (E) of detached leaves of *OsCPK4-OX* and vector control plants at the three-leaf stage. Drought tolerance assays were repeated three times using five independent *OsCPK4* transgenic lines and two independent vector control lines (at least 30 plants per line). Values represent means ± SE of three replicates (*$P \leq 0.05$). [See online article for color version of this figure.]

OsCPK4-mediated activation of genes with a known role in conferring tolerance to abiotic stresses. We examined the expression level of rice *LEA* genes in *OsCPK4* plants, including dehydrin genes (group 2 of LEA proteins). Because the expression of many dehydrins is induced by ABA, they are also referred as RAB proteins (Wang et al., 2007). However, overexpression of *OsCPK4* does not result in a significant increase in transcript accumulation of either *LEA* genes (*OsLEA19a* and *OsLEA21*) or dehydrin genes (*OsLEA23*, also named *OsDip1*, *OsLEA24*, and *OsLEA27*; Supplemental Fig. S4A).

Several major regulons are known to be active in the plant response to abiotic stress, such as the DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN1/C-REPEAT BINDING FACTOR (DREB1/CBF) and DREB2 regulons, the ABA-RESPONSIVE ELEMENT BINDING PROTEIN/ABRE BINDING FACTOR regulon, and the NAC regulon (Todaka et al., 2012). In particular, overexpression of *OsDREB1A*, *OsNAC6*, and *OsNAC45* in rice or Arabidopsis plants has been shown to confer drought and salt tolerance, whereas overexpression of DREB2B enhances drought tolerance and thermotolerance (Dubouzet et al., 2003; Ito et al., 2006; Sakuma et al., 2006; Nakashima et al., 2007; Zheng et al., 2009; Matsukura et al., 2010). In this work, we examined the expression of transcription factors belonging to one or another of the salt/drought-associated regulons in roots of *OsCPK4* rice plants (in all cases, plants were grown under nonstress conditions). They were *OsDREB1A* and *OsDREB2B* (DREB1/CBF and DREB2 regulons, respectively), *OsNAC6* (NAC regulon), and *OsNAC45*. Although the expression of these genes varied among the individual transgenic lines, none of the transcription factor genes assayed here was found to be significantly misregulated (overexpressed or underexpressed) in any of the *OsCPK4* lines compared with vector control plants (Supplemental Fig. S4B). There was, however, the possibility that the salt-associated genes

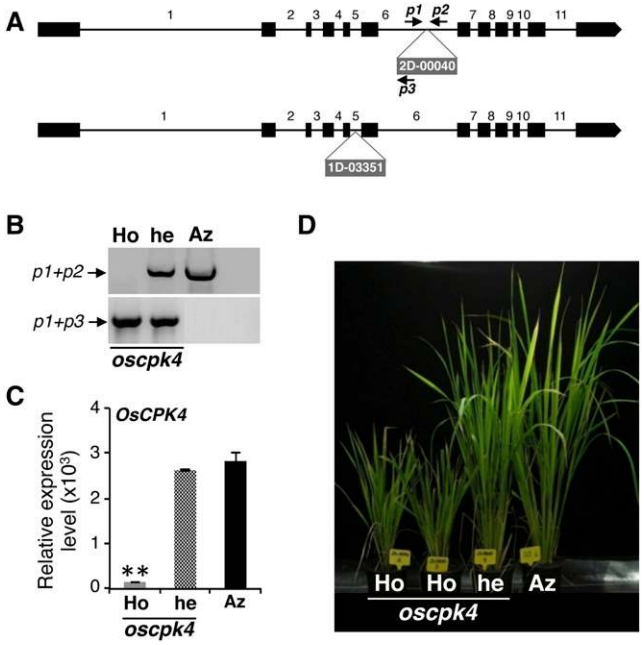

Figure 6. Analysis of *cpk4* mutants. A, *OsCPK4* gene structure showing the T-DNA insertion sites for the *cpk4* mutants identified in the POSTECH collection (2D-00040 and 1D-03351 mutants). Exons and introns are indicated by black boxes and lines, respectively. Gene-specific primers used for the analysis of T-DNA integration are indicated by arrows. B, Analysis of T-DNA integration in the 2D-00040 *cpk4* mutant. Ho, he, and Az indicate homozygous, hemizygous, and azygous plants, respectively. C, qRT-PCR analysis of *OsCPK4* expression in leaves of 2D-00040 *cpk4* mutant plants. Asterisks denote significant differences (**$P \leq 0.001$). D, Appearance of the homozygous and hemizygous 2D-00040 *cpk4* mutant plants. Similar results were obtained for the 1D-03351 *cpk4* mutant. [See online article for color version of this figure.]

examined (*LEA* and transcription factors) might show stronger differential regulation in transgenic plants only under salt stress conditions (as opposed to being constitutively activated in the transgenic plants). However, when examining the expression of these genes in salt-stressed *OsCPK4* and vector control transgenic plants (1 h of treatment at 60 mM NaCl), no differences in the intensity of the response to salt stress could be observed for any of them (data not shown).

To obtain further insights into the molecular mechanisms by which *OsCPK4* mediates tolerance to salt stress in rice plants, gene expression profiles in roots and leaves of *OsCPK4* overexpressor plants and vector control plants were compared. Plants were grown under nonstressed conditions (two independent *OsCPK4* lines and two independent control plants were analyzed). Differentially expressed genes were selected based on fold change (fold change ≥ 2.0) and *P* value ($P \leq 0.05$). Only 62 genes were found to be up-regulated in roots of the transgenic lines relative to vector control plants, supporting that *OsCPK4* overexpression has a low impact on the root transcriptome of rice plants. Using the same criteria, genes showing down-regulation in roots of *OsCPK4* plants were not identified (fold change ≤ -2.0 and $P \leq 0.05$).

Examination of the functional distribution of genes showing up-regulation in roots of *OsCPK4* plants revealed that the majority of these genes grouped into the category Metabolism (46.77% of the total number of differentially expressed genes), most of them being involved in Lipid Metabolism and Protein Metabolism (16.13% and 14.52%, respectively; Fig. 7A; Table I). Based on the molecular function, genes encoding proteins with oxidoreductase activity were represented in different subcategories of Metabolism (Table I). We also noticed that genes involved in oxidative stress and redox regulation, such as peroxidase, *THIOREDOXIN*, GST, and *LACCASE*, were present in the set of *OsCPK4*-regulated genes (6.45% of the total number of *OsCPK4*-regulated genes; Fig. 7A; Table I). In this respect, it is well recognized that salt and drought stress induce ROS generation and that antioxidant enzymes play an important role in tolerance to abiotic stress in plants, including rice plants (Dionisio-Sese and Tobita, 1998; Miller et al., 2010). Consistent with the data obtained by microarray analysis, qRT-PCR analysis confirmed the up-regulation of *THIOREDOXIN* and *LACCASE* genes in roots of *OsCPK4* overexpressor plants (Fig. 7B). In the category Lipid Metabolism, genes encoding proteins with lipid-binding activities and lipid transfer proteins, as well as lipases, were found to be up-regulated in roots of *OsCPK4* plants (Table I).

The category Signal Transduction and Intracellular Trafficking was also highly represented in the set of genes that are up-regulated in *OsCPK4* plants (12.9%; Fig. 7A), comprising several protein kinases, receptor-like protein kinases, and calmodulin-binding proteins. To note, our comparative transcript profiling analysis of root tissues confirmed that *OsCPK4* overexpression does not result in up-regulation of the major transcription factors that function under salt and drought stress, such as DREB or NAC transcription factors (as seen previously by qRT-PCR analysis of transgenic plants). Only the cold-inducible *OsMyb4* gene was identified among the differentially expressed transcription factor genes in OsCPK4 rice plants (Table I). *OsMyb4* overexpression in rice has been shown to reduce membrane injury under stress conditions (Laura et al., 2010). Finally, the expression of genes encoding ion transporters, namely a cation antiporter and a potassium transporter, was found to be up-regulated in *OsCPK4* plants (Table I).

We also investigated whether *OsCPK4* overexpression has an impact on the leaf transcriptome. Microarray analysis showed that only 94 genes were differentially expressed in leaves of *OsCPK4* plants relative to control plants, of which 22 and 72 genes were up-regulated (fold change ≤ 2.0 and $P \leq 0.05$) and down-regulated (fold change ≤ -2.0 and $P \leq 0.05$), respectively (Supplemental Table S2). The functional categories of the differentially expressed genes in leaves of *OsCPK4* plants are presented in Figure 7C. The majority of the misregulated genes grouped into the categories Metabolism (31.91%) and Transcription and RNA Binding (22.34%). Validation of microarray gene expression for selected genes, namely the *OsWRKY71* and *OsWRKY76* transcription factors (up-regulated and down-regulated,

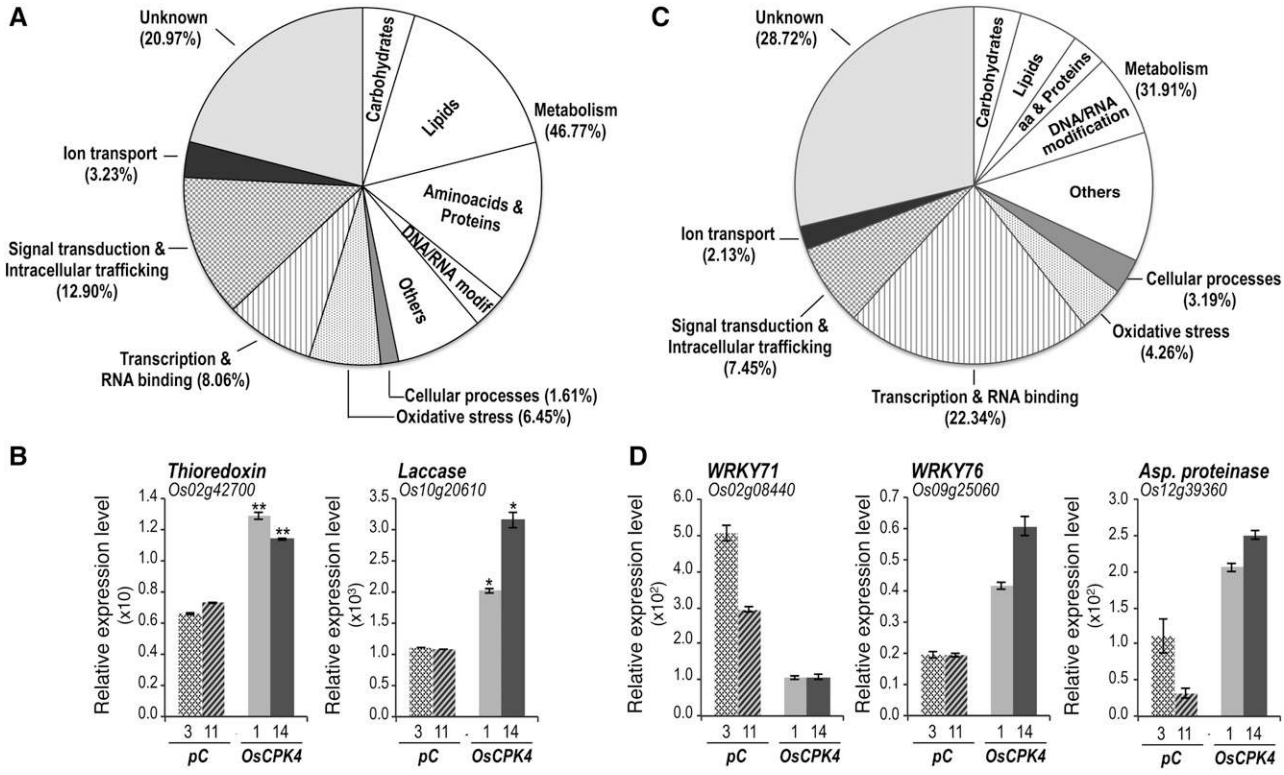

Figure 7. Differentially expressed genes in *OsCPK4* rice plants. A, Functional categories of up-regulated genes in roots of *OsCPK4-OX* plants as determined by microarray analysis. B, Expression of *THIOREDOXIN* and *LACCASE* genes in roots of *OsCPK4-OX* plants. C, Functional categories of misregulated genes in leaves of *OsCPK4-OX* plants. D, Expression of *WRKY71*, *WRKY76*, and *ASPARTIC PROTEINASE* genes in leaves of *OsCPK4-OX* plants. Transcript levels were determined by qRT-PCR using total RNA. Representative results are shown for two *OsCPK4* lines (lines 1 and 14) and two vector control lines (lines 3 and 11) from a total of five and three independent *OsCPK4* and vector control lines, respectively, analyzed in this work.

respectively) and *ASPARTIC PROTEINASE*, is presented in Figure 7D. Intriguingly, overexpression of *OsCPK4* in rice causes a reduction in the expression of the salt-associated *DREB1A*, *DREB1B*, and *LEA21* genes in leaves, whereas the expression of these genes was not affected in root tissues. A trehalose-6-phosphate phosphatase (*OsTPP*) gene was down-regulated in leaves of *OsCPK4* plants, this gene being involved in the biosynthesis of trehalose. Although trehalose has been proposed to act as an osmoprotectant during desiccation, it is also true that trehalose does not accumulate in plants in quantities high enough to directly protect against abiotic stress as a compatible solute. Similarly, overexpression of *OsTPP1* in rice rendered the plant more tolerant to salinity and cold even though no increase in the trehalose content could be observed (Ge et al., 2008).

Collectively, these findings indicated that overexpression of *OsCPK4* results in few transcriptional changes in either root or leaf tissues. No genes were found to be commonly regulated by *OsCPK4* in leaves and roots of rice plants. In root tissues, *OsCPK4* positively regulates the expression of genes involved in metabolic processes, mainly lipid metabolism, as well as genes involved in protection against oxidative stress. Because roots are the first organs that encounter excess salinity, they are also the first sites of potential damage. Presumably, the up-regulation of genes involved in lipid metabolism and protection against oxidative stress in root tissues might be relevant in conferring salt tolerance to the rice plant. Noticeably, no significant alterations in the expression of salt-associated genes appear to occur in roots of *OsCPK4* rice plants, whereas a reduction in the expression of certain salt-associated genes occurs in leaves of *OsCPK4* plants.

Effect of Salt Stress on Membrane Lipid Peroxidation and Electrolyte Leakage in *OsCPK4*-Overexpressing Rice Plants

The control of membrane integrity and membrane-associated functions is crucial for salt tolerance (López-Pérez et al., 2009). As mentioned previously, salt stress induces ROS production, which, in turn, might cause oxidative damage on membrane lipids and perturbation of cell membrane functioning. Salt stress-induced membrane damage also results in electrolyte leakage (Verslues et al., 2006). As lipid peroxidation is the symptom mostly ascribed to oxidative damage, it is often used as an indicator of damage to cell membranes in plants under salt stress conditions.

Table I. *Genes up-regulated in roots of plants overexpressing OsCPK4 relative to vector control plants, identified by microarray analysis*

Asterisks denote genes involved in oxidation-reduction processes. Indicated are genes with 2-fold or greater change in *OsCPK4* plants. Genes with $P \leq 0.05$ were considered. GDSL, Glycine, aspartic acid, serine, and leucine; BPI, bactericidal/permeability-increasing; FACT, facilitates chromatin transcription; SPT, Suppressor of Ty; OBG, *Bacillus subtilis* spo0B stage 0 sporulation-associated GTP-binding protein; DUF, domain of unknown function.

Biological Function	Gene Identifier	Description	Fold Change
Metabolism			
Carbohydrates	LOC_Os01g62420	Triose phosphate isomerase, cytosolic	2.20
	LOC_Os03g36530	FAD-binding and arabinolactone oxidase domain-containing protein*	2.03
	LOC_Os10g32980	Cellulose synthase	2.23
Lipids	LOC_Os01g46250	Lipase	2.00
	LOC_Os03g64170	GDSL-like lipase/acyl hydrolase	2.06
	LOC_Os05g36000	FabA-like domain-containing protein	2.36
	LOC_Os05g46580	Polyprenyl synthetase (similar to farnesyl diphosphate synthase)	2.21
	LOC_Os06g20430	Lipid-binding protein (BPI/LBP family protein), At3g20270 precursor	2.01
	LOC_Os06g29650	CDP-diacylglycerol-inositol 3-phosphatidyltransferase1	2.03
	LOC_Os07g07930	Lipid transfer protein (LTPL78), protease inhibitor/seed storage/LTP family	2.65
	LOC_Os10g25400	GDSL-like lipase/acylhydrolase	2.20
	LOC_Os10g40530	Lipid transfer protein (LTPL146), protease inhibitor/seed storage/LTP family	2.00
	LOC_Os12g16640	Similar to cyclopropane fatty acid synthase*	2.10
Amino acids and proteins	LOC_Os01g47410	Aspartic proteinase oryzasin1 precursor	2.00
	LOC_Os01g48740	Aspartyl protease family protein	2.19
	LOC_Os01g69040	Zinc finger, C3HC4-type domain-containing protein (ubiquitination)	2.04
	LOC_Os04g27850	Oxidoreductase*	2.09
	LOC_Os04g49160	Zinc finger, C3HC4-type domain-containing protein (ubiquitination)	2.51
	LOC_Os05g41010	Oxidoreductase*	2.00
	LOC_Os05g41630	Translation initiation factor eIF3 subunit	2.14
	LOC_Os01g60790	40S ribosomal protein S26	2.03
	LOC_Os11g24240	Sinapoyl Glc choline sinapoyl transferase	2.05
DNA/RNA modification	LOC_Os01g01400	Polynucleotidyl transferase, RNase H fold domain-containing protein	2.14
	LOC_Os02g47560	DNA helicase	2.14
Others	LOC_Os02g30630	Thiamine pyrophosphate enzyme, C-terminal TPP-binding domain	2.18
	LOC_Os04g33670	Carbonic anhydrase, eukaryotic family protein	2.07
	None	Cytochrome P450* (AK106236)	2.48
	LOC_Os07g28400	CDGSH iron sulfur domain-containing protein1	2.01
	LOC_Os07g37420	Short-chain dehydrogenase/reductase*	2.16
Cellular processes			
	LOC_Os09g29100	Cyclin	2.07
Oxidative stress and redox regulation			
	LOC_Os02g14160	Peroxidase*	2.19
	LOC_Os02g42700	Thioredoxin*	2.00
	LOC_Os10g20610	Laccase*	2.19
	LOC_Os07g07320	GST*	2.00
Transcription and RNA binding			
	LOC_Os01g50110	MYB family transcription factor (OsMYB4)	2.02
	LOC_Os03g14860	G-patch domain-containing protein	2.00
	LOC_Os04g25550	FACT complex subunit SPT16	2.32
	LOC_Os04g57010	Zinc finger C-x8-C-x5-C-x3-H-type family protein	2.33
	LOC_Os11g06010	Helix-loop-helix DNA-binding protein	2.11
Signal transduction and intracellular trafficking			
	LOC_Os03g23960	Isoleucine glutamine calmodulin-binding motif family protein	2.00
	LOC_Os04g49530	GTP1/OBG domain-containing protein	2.28

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Biological Function	Gene Identifier	Description	Fold Change
	LOC_Os04g53998	S-locus receptor-like kinase RLK13	2.25
	LOC_Os06g36400	Haloacid dehalogenase superfamily phosphatase	2.26
	LOC_Os07g44330	Kinase	2.33
	LOC_Os09g02410	S-domain receptor-like protein kinase	2.37
	LOC_Os11g44310	Calmodulin-binding protein	2.00
	LOC_Os12g06630	OsFBT14, F-box and tubby domain	2.06
Ion transport			
	LOC_Os02g20330	Cation antiporter	2.00
	LOC_Os08g39950	Potassium transporter	2.01
Unknown			
	LOC_Os01g26370	Expressed protein	2.17
	LOC_Os01g39080	Expressed protein	2.07
	LOC_Os01g55160	Expressed protein	2.04
	LOC_Os01g74170	Expressed protein	2.09
	LOC_Os04g39660	OsFBDUF22, F-box and DUF domain-containing protein	2.03
	LOC_Os05g01210	Expressed protein	2.24
	LOC_Os05g03410	Expressed protein	2.00
	LOC_Os05g39980	Protein of unknown function, DUF3615 domain-containing protein	2.34
	LOC_Os06g05470	Expressed protein	2.03
	LOC_Os06g22410	Expressed protein	2.30
	LOC_Os07g02360	Expressed protein	2.34
	LOC_Os11g31680	Retrotransposon protein	2.01
	LOC_Os11g42970	Membrane-associated DUF588 domain-containing protein	2.13

To further investigate the mechanism underlying salt tolerance in *OsCPK4* rice plants, we examined lipid peroxidation levels in *OsCPK4* overexpressor plants with or without salt treatment. Lipid peroxidation was measured as malondialdehyde (MDA) content, MDA being a typical breakdown product of peroxidized polyunsaturated fatty acids in plant membranes (Weber et al., 2004). Under normal conditions (no NaCl treatment), the MDA levels were lower in *OsCPK4* rice plants than in vector control plants (Fig. 8A). Most importantly, under salt stress conditions, the MDA levels were significantly higher in vector control plants than in *OsCPK4* plants, indicating a lower degree of lipid peroxidation due to salt stress in *OsCPK4* plants.

Next, we measured electrolyte leakage in unstressed and salt-stressed *OsCPK4* plants and control plants. Both the *OsCPK4* transgenic plants and vector control plants showed similar levels of relative electrolyte leakage under nonstress conditions. However, the presence of NaCl induced an increase in electrolyte leakage in vector control plants but not in OsCPK4 rice plants (Fig. 8B). Altogether, these observations suggest that *OsCPK4* overexpression prevents salt stress-induced lipid peroxidation and electrolyte leakage in cellular membranes under salt stress conditions.

OsCPK4 Regulates Na^+ Accumulation

Growth under salt stress conditions results in increased levels of cytoplasmic Na^+ accumulation (Munns and Tester, 2008), which, in turn, causes membrane injury and affects MDA accumulation in the cell. Fluorescent indicators of Na^+, such as CoroNa-Green, are valuable tools for nondestructive monitoring of the spatial and temporal distribution of Na^+ in plant tissues. In order to measure the relative concentration of Na^+ in *OsCPK4* overexpressor and control plants, roots were incubated with NaCl, stained with CoroNa-Green, and analyzed by confocal fluorescence microscopy. Root cells were visualized by propidium iodide staining. This study revealed that roots from vector control plants had much stronger fluorescence than roots from the *OsCPK4* plants (Fig. 8C). The difference in Na^+ accumulation was quantified from confocal CoroNa-Green data, this analysis confirming that Na^+ levels were substantially lower in the *OsCPK4* overexpressor roots ($P \leq 0.001$; Fig. 8D). These findings further support that, under salt stress conditions, the *OsCPK4* transgenic plants accumulate less Na^+ in their roots than control plants.

DISCUSSION

In this study, we show that *OsCPK4* is induced by high salinity and drought stress as well as by ABA treatment. Transcriptional activation of *OsCPK4* is consistent with the presence of various abiotic stress-related cis-elements in the *OsCPK4* promoter, including the ABRE, MYB, and MYC motifs. Overexpression of *OsCPK4* enhances salt and drought tolerance in transgenic rice. In addition to its involvement in abiotic stress tolerance, *OsCPK4* plays a key role in controlling plant development, as inferred from the severe growth inhibition

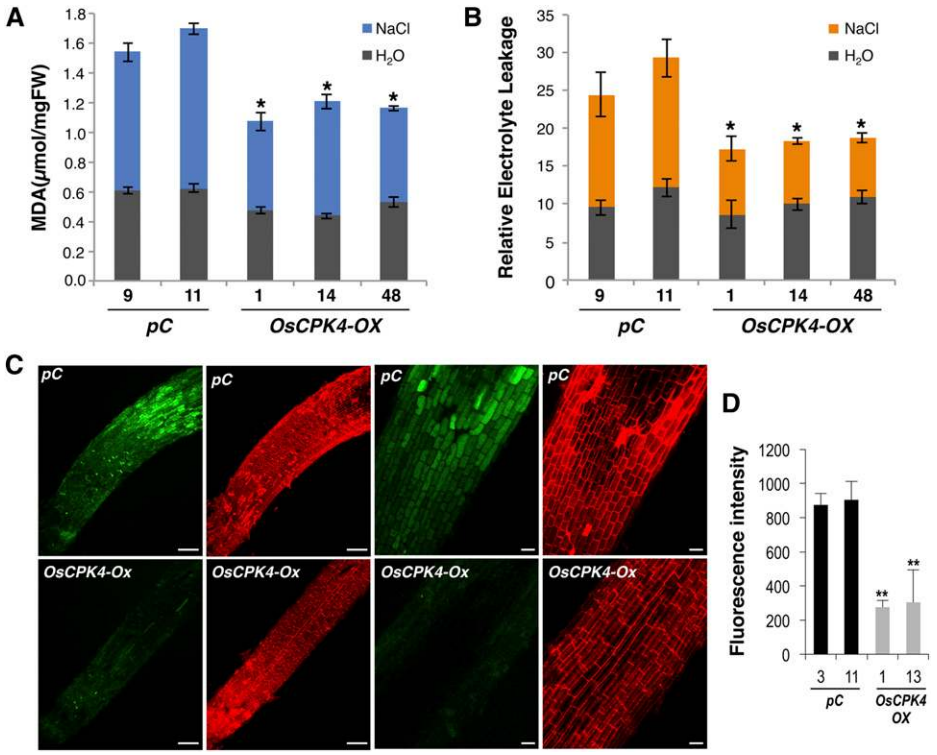

Figure 8. Effect of salt treatment on lipid peroxidation, electrolyte leakage, and Na^+ content. A and B, Detection of lipid peroxidation (A) and electrolyte leakage (B) in leaves of 3-week-old plants, both vector control (pC; two independent lines) and *OsCPK4* overexpressor (*OsCPK4-OX*; three independent lines) plants, treated with 100 mM NaCl for 14 d. Data shown correspond to unstressed (gray bars) and salt-stressed (blue and orange bars) *OsCPK4*-OX and vector control lines. FW, Fresh weight. C, Imaging of Na^+ content in salt-stressed vector control (top row) and *OsCPK4-OX* (bottom row) roots. Confocal images from roots of 5-d-old seedlings that had been treated with 150 mM NaCl for 16 h and stained with CoroNa-Green (green) and propidium iodide (red) are shown. Bars = 20 μm. D, Quantitative comparison of CoroNa-Green fluorescence intensities. Two independent lines and three individual plants per line were examined for each genotype. Values represent means ± SE of average intensities of root sections at 700 μm distance to the tip. Asterisks indicate significant differences (**$P \leq 0.001$, ANOVA).

observed in *cpk4* mutants. At present, however, the function of *OsCPK4* in controlling developmental processes remains to be solved.

Transcript profiling indicated that *OsCPK4* overexpression has a low impact on the transcriptome of rice plants growing under normal conditions. Moreover, no significant differences in the expression of the typical salt/drought-associated genes were observed in roots of OsCPK4 rice plants. Interestingly, an important number of genes involved in lipid metabolism and protection against oxidative stress were found to be up-regulated in roots of *OsCPK4* rice plants compared with control plants (i.e. peroxidase, *THIOREDOXIN*, *LACCASE*, and GST genes). In this respect, it has long been recognized that the expression of genes encoding antioxidant enzymes is activated by salt and drought stress and that overexpression of these genes is an effective way of protecting plants from abiotic stress-induced oxidative damage (Edwards et al., 2000; Roxas et al., 2000; Liang et al., 2006; Csiszár et al., 2012). ROS induce oxidative stress by reacting with several macromolecules. When ROS target lipids, they initiate a lipid peroxidation process, resulting in cell damage. An interesting observation arising from this study was that the *OsCPK4* rice plants exhibited a lower rate of membrane lipid peroxidation than control plants under salt stress conditions. It is known that salt-tolerant rice genotypes have a lower lipid peroxidation rate compared with salt-sensitive cultivars (Demiral and Turkan, 2005; Khan and Panda, 2008). Moreover, a relationship between the expression of antioxidant genes, lipid peroxidation, and abiotic stress tolerance is also documented in different plant species (Oberschall et al., 2000; Roxas et al., 2000; Katsuhara et al., 2005). Thus, the up-regulation of genes involved in protection against oxidative stress in roots of *OsCPK4* rice plants might prevent salt stress-induced lipid peroxidation and oxidative damage in cellular membranes. The protection from oxidative damage of membrane lipids will also positively influence membrane permeability to ions, neutral solutes, and water. Along with this, *OsCPK4* overexpressor plants accumulate less Na^+ in their roots compared with control plants. The up-regulation of ion transporter genes in roots of *OsCPK4* rice plants could be, at least in part, responsible for the observed lower level of Na^+ accumulation in roots of *OsCPK4* rice plants. Furthermore, *OsCPK4* overexpressor

plants exhibited stronger water-holding capability and lower levels of electrolyte leakage in their leaves under stress conditions. Presumably, *OsCPK4* overexpression promotes salt tolerance by reducing membrane lipid peroxidation, which, in turn, reduces electrolyte leakage and Na^+ content under salt stress conditions.

Genes involved in lipid metabolism, including lipid-binding genes and *LTP* genes, were highly represented among the set of genes that are up-regulated in roots of *OsCPK4* rice plants. As for LTPs, these proteins have been implicated in the regulation of cell functions where the movement of lipids is thought to be important, including the maintenance of cell membrane stability under abiotic stress conditions (Wu et al., 2004). The expression of *LTP* genes has also been shown to respond to salt and drought stress in different plant species (Hughes et al., 1992; Guo et al., 2013). We also noticed up-regulation of several lipases and glycine, aspartic acid, serine, and leucine (GDSL)-like lipases in *OsCPK4* overexpressor plants, the expression of these genes being regulated by abiotic stress conditions in different plant species (Hong et al., 2008). Presumably, this set of *OsCPK4*-regulated genes might also contribute to the phenotype of salt tolerance that is observed in *OsCPK4* rice. Recently, a role of lipid metabolism in the acquisition of desiccation tolerance in the resurrection plant *Craterostigma plantagineum* has been proposed (Gasulla et al., 2013). It is also accepted that changes in membrane integrity and the modulation of lipid composition are key factors in the primary sensing of salt and drought (López-Pérez et al., 2009). Clearly, the maintenance of membrane integrity in root tissues, the first site at which salt stress is perceived, is important to protect the cell from abiotic stress injuries and, accordingly, to confer tolerance in rice plants to abiotic stress.

Collectively, the results presented here suggest that the protective effects of *OsCPK4* overexpression in conferring salt and drought tolerance might rely more on the protection against oxidative damage of membranes and the activation of genes involved in lipid metabolism rather than on the activation of the typical salt/drought stress-associated transcriptional networks in root tissues.

The finding that the expression of *OsDREB1A* and *OsDREB1B* genes is down-regulated in leaves of *OsCPK4* rice plants is paradoxical, because these genes are positive regulators of abiotic stress tolerance. Here, it should be mentioned that, whereas the involvement of *DREB* genes in conferring tolerance to abiotic stress is well documented in Arabidopsis, the contribution of these genes in abiotic stress tolerance in rice is not fully understood. In Arabidopsis, *DREB1* and *DREB2* distinguish two different signal transduction pathways, in which *DREB1* preferentially functions in cold stress tolerance and *DREB2* is mainly involved in drought and salt stress but not in cold stress (Nakashima et al., 2009; Akhtar et al., 2012). Different results, however, are found in the literature concerning transgenic rice overexpressing *OsDREB1* genes. Whereas Ito et al. (2006) described tolerance to cold, drought, and high-salt stresses in *DREB1* rice plants, Lee et al. (2004) did not observe any protective effect on tolerance to either cold or drought stress in rice constitutively expressing DREB1. Different rice cultivars with different levels of tolerance to abiotic stresses (cv Dongjin and Kita-ake) were used for the transgenic expression of *DREB1* genes, this fact being proposed as a possible explanation for the different responses to abiotic stress in *DREB1* overexpressor plants (Lee et al., 2004; Ito et al., 2006). Although the exact mechanism underlying the connection between the up-regulation of *OsCPK4* expression and the down-regulation of *OsDREB1A*, *OsDREB1B*, (and *OsLEA21*) expression is unclear, the dramatic effect of *OsCPK4* on the expression of these genes in leaves of rice plants reveals such a connection. On the other hand, *OsCPK4* overexpressor plants showed an up-regulation of *OsWRKY76* in their leaves. Very recently, it was reported that the overexpression of *OsWRKY76* improves tolerance to cold stress in rice plants, a protective effect that appears to be mediated by protection against membrane damage from oxidative stress (Yokotani et al., 2013). The results presented here for *OsCPK4* rice plants are reminiscent of those reported by Yokotani et al. (2013) for *OsWRKY76* overexpressor plants. Furthermore, examination of the *OsWRKY76*-regulated genes (Yokotani et al., 2013) revealed the down-regulation of *OsDREB1B* in leaves of *OsWRKY76* overexpressor plants (as is also the case in *OsCPK4* overexpressor plants). These observations might be indicative of a functional connection between *OsCPK4* and *OsWRKY76* in conferring stress tolerance in rice.

Also intriguing is the finding that there is no overlap between *OsCPK4*-regulated genes in roots and leaves. Based on the results presented here, it is tempting to speculate that different *OsCPK4*-mediated protective mechanisms might operate in roots and leaves of rice plants in relation to abiotic stress tolerance. In favor of this hypothesis, Minh-Thu et al. (2013) recently reported that although leaf and root tissues shared some common gene expression during drought stress, the two tissues appeared to act differently in response to dehydration. Other studies in maize (*Zea mays*) indicated that under salt stress conditions, maize leaves and roots employed distinct mechanisms to cope with salt stress (Qing et al., 2009). Therefore, tolerance mechanisms to abiotic stress may be differentially regulated based on plant organs. Another possibility is that OsCPK4 might act on different protein substrates that might, in turn, activate different responses (i.e. phosphorylation cascades) in one or another tissue. Further studies are needed to decipher whether OsCPK4 exerts its protective effect in abiotic stress tolerance by modulating specific mechanisms in either root or leaf tissues.

The results presented here demonstrate that OsCPK4 localizes to the plasma membrane. Clearly, the plasma membrane contains proteins that are essential for receiving signals from the environment and transducing them for the activation of downstream responses. As mentioned previously, OsCPK4 harbors potential myristoylation and palmitoylation sites at its N-terminal end. The N-terminal myristoylation appears to mediate the membrane localization of distinct CPKs, whereas other

CPKs require both myristoylation and palmitoylation for their membrane association (Martín and Busconi, 2000; Benetka et al., 2008; Coca and San Segundo, 2010; Mehlmer et al., 2010; Lu and Hrabak, 2013). In other studies, it was shown that the variable domain of a plant CPK not only dictates its proper subcellular localization but also confers substrate specificity (Asai et al., 2013). If demonstrated, the myristoylation and/or palmitoylation of OsCPK4 would explain that OsCPK4 is localized exclusively at the plasma membrane.

It is also well recognized that the level of Ca^{2+} rapidly increases in plant cells in response to abiotic stress (Reddy et al., 2011). Ca^{2+} homeostasis is achieved by the combined action of channels, pumps, and antiporters. Very recently, Choi et al. (2014) proposed the existence of a plant-wide signaling system based on the rapid, long-distance transmission of Ca^{2+} waves. This Ca^{2+}-dependent signaling system elicits molecular responses in distant parts of the plant upon the perception of localized salt stress (e.g. the application of salt stress to the root tip triggers systemic molecular responses in shoot tissue). The peculiar structural features of CPKs make these proteins ideally suited to sense the stress-induced alterations in cytoplasmic Ca^{2+} levels by binding Ca^{2+} to its intrinsic calmodulin-like domain for the activation of downstream phosphorylation processes. Then, in addition to its transcriptional activation, a direct regulation by Ca^{2+} of the OsCPK4 enzyme activity might induce changes in the phosphorylation status of a variety of protein targets in response to salt stress. In this regard, alterations in the phosphoproteome of rice plants in response to salt stress are documented, some of these events occurring at the plasma membrane (Chitteti and Peng, 2007). Multiple phosphorylation of plasma membrane aquaporins in response to salt stress has also been described in Arabidopsis (Prak et al., 2008). Furthermore, the guard cell anion channel *SLOW ANION CHANNEL-ASSOCIATED1* is phosphorylated by AtCPK21 and AtCPK23 (Geiger et al., 2010), and another vacuolar channel, TWO-PORE K^+ CHANNEL1, is phosphorylated by AtCPK3 (and other CDPKs) in response to salt stress (Latz et al., 2013). The important role of Ca^{2+}-dependent phosphorylation processes during the salt stress response in plants is also illustrated by the SOS pathway in which SOS3 (a Ca^{2+} sensor) activates the protein kinase SOS2, which then regulates the plasma membrane Na^+/H^+ antiporter SOS1 (Quintero et al., 2002). These findings raise the question of whether OsCPK4 could play a role at the plasma membrane, for example by regulating the function of ion channels or transporters through phosphorylation. Indeed, the observation that *OsCPK4* overexpression has a low impact in the rice transcriptome favors that OsCPK4 might act at the posttranslational level for the regulation of target proteins, perhaps in a tissue-specific manner, during the adaptation of rice plants to abiotic stress conditions.

In Arabidopsis, certain CPKs are known to be involved in abiotic stress tolerance and ABA signaling (Sheen, 1996; Zhu et al., 2007; Boudsocq and Sheen, 2010; Xu et al., 2010; Franz et al., 2011). In rice, however, the function of most CPK genes in relation to abiotic stress is less characterized. Overexpression of *OsCPK12*, *OsCPK13* (encoding OsCDPK7), and *OsCPK21* was reported to confer salt tolerance (Saijo et al., 2000; Asano et al., 2011, 2012), whereas *OsCPK23* overexpression confers cold tolerance (Komatsu et al., 2007). As is the case in *OsCPK4* overexpressor plants, no significant differences in the expression levels of *LEA* and *OsNAC6* genes were observed between plants overexpressing one or another of the above-mentioned *CPK* genes (*OsCPK12*, *OsCPK21*, and *OsCPK13*) under unstressed conditions (Saijo et al., 2000; Asano et al., 2011, 2012). The knowledge gained in this study not only reveals an important regulatory function of *OsCPK4* in abiotic stress tolerance but also provides a foundation for the further investigation of stress-induced signaling pathways in which rice *CPK* genes participate. For instance, it will be of interest to determine whether *OsCPK4* acts in a cooperative manner with one or more other *CPK* genes during the adaptation of rice plants to salt and/or drought conditions. Knowing that rice has been adopted as the model system for cereal genomics (Goff, 1999), a better understanding of abiotic stress mechanisms in rice might also benefit stress tolerance in other monocot cereal crops.

Finally, in this study, we determined the salt tolerance of *OsCPK4* rice plants at the seedling stage. Rice is considered to be a salt-sensitive crop, its tolerance to high salinity varying through the life cycle of the plant (Maas and Hoffman, 1977). Thus, the rice plant is very sensitive to high salinity at the seedling and reproductive stages (Zeng and Shannon, 2000). Further studies will determine whether *OsCPK4* overexpression confers tolerance to salt/drought stress at any other developmental stage (i.e. reproductive stage). Equally, the practical question is whether the benefits observed by the transgenic expression of *OsCPK4* in rice in terms of salt and drought tolerance could be used to engineer tolerant rice plants under field conditions. The results described in this study point to the need to further dissect the pathways in which *OsCPK4* is involved in tolerance to abiotic stresses in rice plants

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Treatments

Rice (*Oryza sativa* 'Nipponbare') plants were grown at 26°C to 28°C with a 16-h-light/8-h-dark cycle. For expression studies of *OsCPK4* in response to salt treatment, 7-d-old rice plants were transferred to nutrient solution containing 100 mM NaCl. To examine the expression pattern of *OsCPK4* in relation to drought stress, 7-d-old rice plants were left to air dry or stressed in a 20% (w/v) PEG 8000 solution for the required period of time. In all cases, roots of at least 10 individual plants were collected and used for total RNA isolation. Control seedlings were treated with water. For treatment with ABA, 2-week-old plants were treated with a solution of 100 μM ABA (100 mM stock solution in ethanol) for 1 h. T-DNA insertional mutant lines for *OsCPK4* were grown as described above and genotyped by PCR (Supplemental Methods S1).

Rice Transformation

The full-length complementary DNA sequence of *OsCPK4* (Os02g03410) was cloned into the pCAMBIA1300 vector under the control of the maize

ubiquitin1 (*ubi*) promoter and the *nopaline synthase* (*nos*) terminator. Transgenic rice (cv Nipponbare) plants were produced by *Agrobacterium tumefaciens*-mediated transformation (strain EHA105). As control, transgenic rice lines expressing the empty vector (pCAMBIA1300) were also produced. Details on construct preparation and rice transformation are presented in Supplemental Methods S1.

qRT-PCR

Total RNA was extracted from plant tissues using the Trizol reagent (Invitrogen) and used for reverse transcription reactions. The first complementary DNA was synthesized from DNase-treated total RNA (1 μg) with Transcriptor Reverse Transcriptase (Roche) and oligo$(dT)_{18}$ following the manufacturer's instructions. qRT-PCR was performed on optical 96-well plates in the Roche Light Cycler 480 instrument using SYBR Green I dye. Primers were designed using Primer Express software (Applied Biosystems; primers are listed in Supplemental Tables S3 and S4). Routinely, three replicate reactions were used for each sample. Data were normalized using the geometric mean of the *CYCLOPHYLIN* (Os02g02890) and *UBIQUITIN* (Os06g46770) genes as internal controls. Three independent biological replicates were analyzed. Controls of the qRT-PCR without adding the reverse transcriptase enzyme were systematically included in our experiments.

Microarray Analysis

Total RNA was isolated from tissues, both roots and leaves, of *OsCPK4* and vector control plants that had been grown hydroponically for 15 d using the Plant Total RNA Extraction kit (Qiagen). Two independent homozygous *OsCPK4* lines (lines 1 and 13) and two vector control lines (lines 3 and 11) were examined. The quality and concentration of the RNA were analyzed using the Agilent 2100 bioanalyzer and NanoDrop ND-1000 (Thermo Scientific). Samples with an RNA integrity number less than 6 were discarded. Three biological samples and three replicates per biological sample were analyzed. Details of microarray construction, probe labeling, and hybridization conditions are presented in Supplemental Methods S1. Bootstrap analysis with Significance Analysis of Microarrays was used to identify differentially expressed genes using a cutoff of 2 (Tusher et al., 2001). Significance Analysis of Microarrays calculates the fold change and significance of differences in expression. The delta value, false significant number, and false discovery rate were 0.749, 8.88, and 4.99, respectively. We considered genes with $P \leq 0.05$. Data sets for microarray analyses (roots and leaves) have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus with accession number GSE52353.

Tolerance to Salt and Drought Stress

For salt stress treatment, plants were grown hydroponically in Yoshida medium for 15 d and transferred to nutrient solution containing 60 mM NaCl or fresh nutrient solution (control plants). Five independent homozygous *OsCPK4* lines, three independent vector control plants, and at least 30 plants per line were assayed. Salt tolerance was evaluated by determining the percentage of surviving plants after 25 d of salt treatment.

For drought stress treatment, plants were grown in soil for 15 d under a normal watering regime. Drought stress was induced by withholding water for 17 d. Plants were then irrigated normally for 9 d. Drought tolerance was evaluated by determining the percentage of surviving plants after the period of recovery. To measure the water loss under dehydration conditions, leaves of wild-type and homozygous transgenic plants at the three-leaf stage were cut and exposed to air under controlled conditions (27°C ± 2°C). The leaves were weighed 4 h after being cut down. Water loss was calculated from the following equation: water loss (%) = (fresh weight – desiccated weight)/fresh weight × 100. The water retention ability was measured according to the following formula: water retention ability (%) = (desiccated weight – dry weight)/(fresh weight – dry weight) × 100. Statistical analysis of the data obtained in these experiments was performed using the ANOVA test at a 5% confidence level.

Subcellular Localization

An *OsCPK4-GFP* fusion gene was prepared and used for particle bombardment experiments in onion (*Allium cepa*) epidermal cells as described previously (Murillo et al., 2003; for details, see Supplemental Methods S1). Confocal microscopy was used to visualize GFP fluorescence. To confirm plasma membrane localization, cells were plasmolyzed with 0.75 M mannitol for 15 min.

Preparation of Anti-OsCPK4 N-Terminal Antiserum and Immunoblotting

The N-terminal domain of OsCPK4 (Met-1 to Arg-58) was produced in *Escherichia coli*, and the purified polypeptide was used for antiserum production in rabbits. Protein extracts were prepared from roots of control and *OsCPK4* rice plants, separated by SDS-PAGE or two-dimensional PAGE, and probed with the anti-OsCPK4 antiserum produced in this work (1:1,000 dilution). Blots were then incubated with protein A-peroxidase (Sigma) at a dilution of 1:10,000. Peroxidase activity was made visible by incubating the blot with ECL Western Blotting Substrate (Pierce) for 5 min on an LAS-4000 Image analyzer (Fujifilm). Details on bacterial expression, preparation of the antiserum, and immunoblotting are presented in Supplemental Methods S1.

Lipid Peroxidation, Electrolyte Leakage, and Detection of Na^+

The level of lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction as described by Hodges et al. (1999). Leaf samples (0.05 g) were homogenized in 1 mL of 80% (v/v) ethanol on ice and then centrifuged at 16,000*g* for 20 min at 4°C. The supernatant (0.6 mL) was mixed with 0.6 mL of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) thiobarbituric acid. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000*g* for 10 min, the absorbance of the supernatant was measured at 532 nm. The value for nonspecific absorption at 600 nm was subtracted from the 532-nm reading. The MDA content was calculated using its molar extinction coefficient (155 mM^{-1} cm^{-1}), and the results are expressed as μmol MDA mg^{-1} fresh weight. For the measurement of relative electrolyte leakage, three OsCPK4 and OsCPK2 vector control lines were treated with 150 mM NaCl for 2 h. Leakage ratio values were determined following the method described by Cao et al. (2007). Briefly, the leaf segments from at least three plants of each line were placed in deionized water, and the conductivities of the obtained solutions were determined. Then, the leaf segments in deionized water were boiled for 15 min. After being thoroughly cooled to room temperature, the conductivities of the resulting solutions were determined. For each line, the relative electrolyte leakage after salt treatment was calculated as the ratio of the conductivity before boiling the rice leaves to that after boiling. Each data point represents an average from two independent experiments. Intracellular Na^+ was detected in 5-d-old rice roots, control and salt-treated roots (150 mM NaCl for 16 h), by staining with 10 μM CoroNa-Green dye (Molecular Probes), and images were obtained by confocal laser scanning microscopy using the Olympus FV1000 microscope. Quantification of the fluorescent signals from the cells was performed using the FV10-ASW4.1 software for image analysis. The data were subjected to statistical analysis using ANOVA.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *OsCPK4* (Os02g03410), *OsLEA23* (Os02g44870), *OsLEA26* (Os11g26750), *OsCYP* (Os02g02890), and *OsUbi1* (Os06g46770).

Supplemental Data

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

Supplemental Figure S1. Expression of *OsCPK4* in rice leaves in response to salt stress.

Supplemental Figure S2. Amino acid sequence of OsCPK4 and alignment of the N-terminal variable domain of rice CPKs.

Supplemental Figure S3. Overexpression of *OsCPK4* in transgenic rice.

Supplemental Figure S4. Expression of salt-associated genes in roots of rice plants overexpressing *OsCPK4*.

Supplemental Table S1. The cis-related motifs identified in the 2,000-bp upstream region of the *OsCPK4* promoter.

Supplemental Table S2. Genes misregulated in leaves of plants overexpressing *OsCPK4* relative to vector control plants, identified by microarray analysis.

Supplemental Table S3. List of primers used for *OsCPK4* cloning purposes and mutant analysis.

Supplemental Table S4. List of primers used for expression analysis of rice genes by qRT-PCR.

Supplemental Methods S1. Rice transformation and mutant analysis, microarray analysis, subcellular localization of OsCPK4, and immunoblotting.

ACKNOWLEDGMENTS

We thank Dr. Mauricio Soto for assistance in microarray data analysis, Dr. Montserrat Pagés for critical reading of the article, and Gisela Peñas, Vanessa Alfaro, and Vanessa Vega for technical assistance.

Received October 11, 2013; accepted May 1, 2014; published May 1, 2014.

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