The Os-AKT1 Channel Is Critical for K⁺ Uptake in Rice Roots and Is Modulated by the Rice CBL1-CIPK23 Complex

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INTRODUCTION

Potassium (K⁺) is one of the essential nutrient elements for plant growth and development. Plants absorb K⁺ ions from the environment via root cell K⁺ channels and/or transporters. In this study, the Shaker K⁺ channel Os-AKT1 was characterized for its function in K⁺ uptake in rice (Oryza sativa) roots, and its regulation by Os-CBL1 (Calcineurin B-Like protein1) and Os-CIPK23 (CBL-Interacting Protein Kinase23) was investigated. As an inward K⁺ channel, Os-AKT1 could carry out K⁺ uptake and rescue the low-K⁺-sensitive phenotype of Arabidopsis thaliana akt1 mutant plants. Rice Os-akt1 mutant plants showed decreased K⁺ uptake and displayed an obvious low-K⁺-sensitive phenotype. Disruption of Os-AKT1 significantly reduced the K⁺ content, which resulted in inhibition of plant growth and development. Similar to the AKT1 regulation in Arabidopsis, Os-CBL1 and Os-CIPK23 were identified as the upstream regulators of Os-AKT1 in rice. The Os-CBL1-Os-CIPK23 complex could enhance Os-AKT1-mediated K⁺ uptake. A phenotype test confirmed that Os-CIPK23 RNAi lines exhibited similar K⁺-deficient symptoms as the Os-akt1 mutant under low K⁺ conditions. These findings demonstrate that Os-AKT1-mediated K⁺ uptake in rice roots is modulated by the Os-CBL1-Os-CIPK23 complex.

In plant roots, K⁺ absorption from soils is mainly mediated by K⁺ channels and transporters whose transcription may be induced and activities may be enhanced in response to K⁺-deficient stress (Wang and Wu, 2013). AKT1 (ARABIDOPSIS K⁺ TRANSPORTER1) has been identified as an inward-rectifying K⁺ channel in Arabidopsis thaliana and plays crucial roles in K⁺ uptake from soil into root cells (Hirsch et al., 1998; Ivashikina et al., 2001; Lagarde et al., 1996; Spalding et al., 1999). Loss of function of At-AKT1 leads to a reduction of K⁺ uptake and makes plants hypersensitive to low-K⁺ stress (Hirsch et al., 1998; Spalding et al., 1999; Xu et al., 2006). AKT1 activity is positively regulated by CBL1/9-CIPK23 protein complexes in Arabidopsis (Xu et al., 2006). The calcineurin B-like protein CBL1 and/or CBL9 interacts with protein kinase CIPK23 at the plasma membrane (PM), where CIPK23 phosphorylates AKT1 and activates AKT1-mediated K⁺ uptake (Xu et al., 2006). Several AKT1 orthologs have been identified in other plant species, such as Os-AKT1 in rice (Oryza sativa; Fuchs et al., 2005), Zm-ZMK1 in maize (Zea mays; Philippart et al., 1999), Ta-AKT1 in wheat (Triticum aestivum; Buschmann et al., 2000), Hv-AKT1 in barley (Hordeum vulgare; Boscari et al., 2009), Si-LKT1 in tomato (Solanum lycopersicum; Hartje et al., 2000), and St-SKT1 in potato (Solanum tuberosum; Zimmermann et al., 1998). Similar regulatory mechanisms, with CBL-CIPK complex-modulating K⁺ channels, were also reported in barley (Boscari et al., 2009) and grape (Vitis vinifera) plants (Cuéllar et al., 2010, 2013).

Rice is one of the most important crops and staple foods in the world, and rice grain yield and quality are significantly dependent on the K⁺ supply in the soils (Wanasuria et al., 1981; Dobermann et al., 1996). However, the K⁺ absorption mechanism in rice roots is still unclear. Several K⁺ channels and transporters have been cloned and considered as candidate components to mediate K⁺ uptake in rice roots, including Os-AKT1 (Golldack et al., 2003; Fuchs et al., 2005), Os-HAK1 (Bañuelos et al., 2002), and others.
Os-AKT1 shares high similarity with other Shaker K+ channels from plant species, such as At-AKT1, SI-LKT1, St-SKT1, Zm-ZMK1, Ta-AKT1, and Hv-AKT1 (58, 60, 60, 73, 76, and 75% identities, respectively) (Supplemental Figure 1). Phylogenetic analysis classified the K+ channels from monocots and dicots separately (Supplemental Figure 2 and Supplemental Data Set 1). The Os-AKT1 P-loop domain contains a typical TxxTxGYG motif, a hallmark of K+-selective channels (Doyle et al., 1998), suggesting that Os-AKT1 is likely to exhibit high ion selectivity for K+. The high degree of similarity of these Shaker K+ channels indicates that they likely have similar physiological functions in the different plant species.

**Protein Structure of Os-AKT1**

Os-AKT1 shares high similarity with other Shaker K+ channels from plant species, such as At-AKT1, SI-LKT1, St-SKT1, Zm-ZMK1, Ta-AKT1, and Hv-AKT1 (58, 60, 60, 73, 76, and 75% identities, respectively) (Supplemental Figure 1). Phylogenetic analysis classified the K+ channels from monocots and dicots separately (Supplemental Figure 2 and Supplemental Data Set 1). The Os-AKT1 P-loop domain contains a typical TxxTxGYG motif, a hallmark of K+-selective channels (Doyle et al., 1998), suggesting that Os-AKT1 is likely to exhibit high ion selectivity for K+. The high degree of similarity of these Shaker K+ channels indicates that they likely have similar physiological functions in the different plant species.

**Subcellular Localization and Expression Pattern of Os-AKT1**

To test the subcellular localization of Os-AKT1, the fusion gene of Os-AKT1-EGFP was constructed and transformed into tobacco (*Nicotiana benthamiana*) leaves. CBL1n-OPF was used as the PM marker (Batistić et al., 2008). When Os-AKT1-EGFP was coexpressed with CBL1n-OPF in tobacco leaves, the green and red fluorescence showed remarkable overlap in tobacco leaves (Figure 1A), which indicates a PM localization for Os-AKT1 in plant cells.

To determine the expression profiles of Os-AKT1 in rice, transgenic rice plants carrying a GUS gene under control of an Os-AKT1 promoter fragment (1010 bp; *O. sativa* ssp *japonica* cv Nipponbare) were generated. The β-glucuronidase (GUS) activity assays showed that the Os-AKT1 promoter drives strong expression in roots (Figures 1B and 1C) and slight expression in shoots (Figure 1B). In root tissues, GUS activity was observed in all cell types (Figures 1C and 1D). The expression of Os-AKT1 in the epidermis and root hairs suggests a physiological role for Os-AKT1 in root K+ uptake from soil (Figures 1C and 1D). Furthermore, GUS activity was also detected in cortex, endodermis, and vascular bundles (Figure 1D), which indicates Os-AKT1 may also participate in K+ translocation in roots. In shoot tissues, Os-AKT1 promoter activity was mainly found in epidermis and vascular bundles (Figures 1E and 1F).

The GUS activity assays showed that the transcription of Os-AKT1 was not affected after K+ deficiency treatment (Supplemental Figure 3A). Meanwhile, the quantitative real-time PCR results also confirmed that the low-K+ treatment did not induce Os-AKT1 expression in both roots and shoots (Supplemental Figures 3B and 3C).

**K+ Transport Activity of Os-AKT1 in Yeast**

The K+ transport activity of Os-AKT1 was tested in auxotrophic yeast mutant strain R5421 (trk1Δ, trk2Δ) and its wild-type strain R757 (Gaber et al., 1988; Nakamura et al., 1997). R5421 was defective in K+ uptake and could not grow under low-K+ conditions. Os-AKT1 was transformed into R5421, and AKT1 from *Arabidopsis* was also introduced into R5421 as a positive control. The yeast growth assays were performed on AP medium (Rodríguez-Navarro and Ramos, 1984) containing different concentrations of K+. Under high K+ conditions (50 mM), all tested yeast strains showed similar growth (Figure 2A). Along...
with the decline of K+ concentration, the growth of R5421 was significantly depressed (Figure 2A), and both rice and Arabidopsis AKT1 could rescue the growth defect of R5421 mutant (Figure 2A), suggesting that the Os-AKT1 channel has a similar function in K+ uptake as the Arabidopsis AKT1 channel. Both channels could mediate K+ uptake under low K+ concentrations (Km AKT1 = 106 μM, Km Os-AKT1 = 114 μM) (Figure 2B).

Functional Characterization of Os-AKT1 in Arabidopsis

AKT1 is an important K+ channel and plays crucial roles in K+ uptake in Arabidopsis roots. The Arabidopsis akt1 mutant plants exhibit low-K+-sensitive phenotypes and a significant decrease in K+ content after growth on low-K+ medium (Hirsch et al., 1998; Spalding et al., 1999; Xu et al., 2006). To examine the possible function of Os-AKT1, the coding sequence of Os-AKT1 was cloned and transformed into Arabidopsis akt1 mutant (ecotype Columbia [Col]) and two transgenic lines were obtained. The phenotype tests showed that the low-K+-sensitive phenotype of akt1 mutant was rescued in these two transgenic lines (akt1/Os-AKT1), which displayed a similar phenotype as wild-type (Col) plants (Figures 2C and 2D). The K+ contents in these two transgenic lines were also rescued (Figure 2E). Moreover, the root K+ content in these two lines was even higher than that in wild-type plants under low-K+ conditions (Figure 2E). These results demonstrate that Os-AKT1 has similar function in K+ uptake as AKT1 in Arabidopsis.

To measure the Os-AKT1-mediated K+ currents in Arabidopsis root cells, patch-clamp whole-cell recording was conducted using root cell protoplasts. Inward K+ currents were observed in root cell protoplasts of wild-type plants, but not in those of the akt1 mutant (Figure 2F), suggesting that the inward K+ currents in wild-type root cells were contributed by AKT1 channel (Hirsch et al., 1998; Reintanz et al., 2002; Xu et al., 2006). However, the akt1/Os-AKT1 transgenic line showed obvious inward K+ currents (Figure 2F), suggesting that Os-AKT1 could mediate K+ uptake in Arabidopsis root cells. It should be noted that the Os-AKT1-mediated K+ currents were different from the currents conducted by Arabidopsis AKT1 channel (Figures 2F and 2G). As shown in Figure 2H, the half-activation voltage of Os-AKT1 (V1/2 = −173 ± 5 mV) was more negative than that of At-AKT1 (V1/2 = −133 ± 4 mV), suggesting that Os-AKT1 requires a more negative membrane potential to be activated.

Phenotype Analysis of Rice Os-akt1 Mutant

The T-DNA insertion mutant line of Os-akt1 from RiceGE (Rice Functional Genomic Express Database, http://signal.salk.edu/cgi-bin/RiceGE) was used to validate the physiological function of Os-akt1 in rice. In this Os-akt1 mutant, the T-DNA fragment is inserted into the 5′-untranslated region of Os-akt1, 303 bp upstream of the start codon (Figure 3A). The insertion leads to the knockdown of Os-akt1 in the mutant plants compared with wild-type (O. sativa ssp japonica cv Dongjin) plants (Figure 3B). The results of DNA gel blot assays indicated that there is only one copy of the T-DNA fragment inserting into the mutant plants (Figure 3C).

Using hydroponic culture, the phenotype of Os-akt1 mutant and wild-type (Dongjin) plants was tested. The 14-d-old rice seedlings were transferred into normal (1 mM K+) and low K+ (100 μM K+) medium. After growth for 7 d, the Os-akt1 mutant seedlings showed overall growth inhibition compared with wild-type plants in both normal and low K+ conditions (Figures 3D, 3F, and 3G). In addition, the Os-akt1 mutant displayed the brown spots on old leaves, which was a typical K+-deficient symptom of rice (Figure 3E). This symptom in Os-akt1 mutant became more remarkable under low K+ conditions (Figure 3E). The K+ content of the Os-akt1 mutant was significantly reduced in both root and shoot compared with wild-type plants (Figure 3H). Since Os-AKT1 is an inward K+ channel, this K+ deficiency in Os-akt1 might be due to the defect of Os-AKT1-mediated K+ uptake. The results of K+ depletion experiments (Drew et al., 1984) showed that the K+ uptake in Os-akt1 was obviously slower than that in the wild type (Figure 3I). These results demonstrated that the loss of function of Os-AKT1 led to the reduction of K+ in Os-akt1 mutants, which caused growth inhibition and K+-deficient symptoms in mutant plants. The function of Os-AKT1 was further confirmed using two complementation lines for the Os-akt1 mutant. As shown in Figure 4, the expression of Os-AKT1 in these two transgenic lines was recovered and the growth inhibition and K+-deficient symptoms were relieved.

The growth of Os-akt1 mutant plants was inhibited throughout development (Figure 5). The heading and grain-filling stages were delayed in Os-akt1 (Figures 5A and 5B). In addition, the lesion of Os-AKT1 also impaired grain yield. The grain number, seed set percentage, and 100-grain weight of the main panicle were all significantly reduced in Os-akt1 (Figures 5C to 5G).

Electrophysiological Analysis of Rice Root Cell Protoplasts

To analyze the Os-AKT1-mediated K+ currents in rice, we tested the whole-cell inward K+ currents in root cell protoplasts isolated from Os-akt1 mutant and wild-type seedlings using patch-clamping methods. In wild-type plants, ~37% of the recorded root cell protoplasts showed inward K+ currents, but this proportion was significantly reduced in the Os-akt1 mutant (Table 1). Furthermore, the magnitude of inward K+ currents recorded in Os-akt1 mutant root cells was much smaller than that in wild-type cells, at only 14% of the currents recorded from wild-type plant cells at ~180 mV (Figure 6, Table 1). In the Os-akt1 complementation line, the magnitude of inward K+ currents was completely recovered (Supplemental Figure 4) even though the inward K+ currents could be recorded only in some of the root cell protoplasts (34%). Although the K+ currents in Os-akt1 were reduced, the voltage dependence of the K+ currents was not altered (Figure 6C, Table 1), indicating that the K+ currents in both Os-akt1 and wild-type root cells were derived from Os-AKT1. We conclude that lesion of Os-AKT1 decreased the inward K+ currents in root cells, which led to the reduction of K+ uptake in Os-akt1 mutant roots.

Identification of Os-AKT1 Regulators

The previous reports have revealed that the activation of AKT1 channel in Arabidopsis root cells requires protein kinase CIPK23 and calcium sensor CBL1/9 (Li et al., 2006; Xu et al., 2006). Since
Figure 2. Functional Characterization of Os-AKT1 in Yeast and Arabidopsis.

(A) Os-AKT1 and AKT1 complement the K⁺ uptake-deficient yeast mutant R5421 on AP medium containing different K⁺ concentrations. The yeast strain R757 was used as a positive control. Three independent experiments were performed.

(B) The K⁺ uptake kinetic analysis of Os-AKT1 and AKT1 in yeast. The data points are shown as means ± SE (n = 3).

(C) Phenotype comparison of wild-type Arabidopsis (Col), akt1 mutant, and two transgenic lines (akt1/Os-AKT1-1 and akt1/Os-AKT1-2) grown on MS and LK (100 μM K⁺) medium for 7 d.

(D) Real-time PCR verification of Os-AKT1 and AKT1 expression in different plant materials.

(E) Comparison of K⁺ content in different plant materials. The K⁺ content of roots and shoots was determined after the plants grown on MS and LK medium for 7 d. Data are shown as means ± SE (n = 3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance.

(F) Patch-clamp whole-cell recordings of inward K⁺ currents in Arabidopsis root cell protoplasts. The plant materials are indicated above each recording. The voltage protocols, as well as time and current scale bars for the recordings, are shown inside the figure.

(G) The I-V (current-voltage) relationship of the steady state whole-cell inward K⁺ currents in root cell protoplasts. The data are derived from the recordings as shown in (F) and presented as means ± SE (Col, n = 35; akt1, n = 6; akt1/Os-AKT1-2, n = 28).

(H) The voltage dependence of inward K⁺ currents in root cell protoplasts isolated from different plants. The solid lines represented the best fits according to the Boltzmann function: G/Gₘₕₕ = 1/(1+exp((V_m - V_1/2)/S)). G (conductance) was calculated as G=I/(V_m - E_K), where I is the steady state current at voltage V_m. V_1/2 is the membrane potential at which the conductance is half-maximal and S is a slope factor. The data are derived from the recordings as shown in (F) and presented as means ± SE (Col, n = 35; akt1, n = 6; akt1/Os-AKT1-2, n = 28).
Figure 3. Phenotype of Rice Os-akt1 Mutant.

(A) The structure of the Os-AKT1 gene. The black boxes indicate exons and the lines represent introns. The T-DNA insertion site in the Os-akt1 mutant is shown using an arrow.

(B) Real-time PCR verification of Os-AKT1 expression in Dongjin and Os-akt1 mutant. The data are presented as means ± SE (n = 3).

(C) DNA gel blot analysis of T-DNA insertion in Os-akt1. Lanes 1, 4, and 7 were Dongjin (negative control); lanes 2, 5, and 8 were Os-akt1 mutant; and lanes 3, 6, and 9 were the plasmid containing the HPT gene (positive control). Genomic DNA was isolated from rice leaves and digested using BamHI (left), SacI (middle), or HindIII (right). A DNA fragment of HPT included in the T-DNA insertion fragment was used as probe.

(D) and (E) Phenotype comparison between Dongjin and Os-akt1 mutant. The photographs of whole seedlings (D) and first leaves (E) were taken after the rice seedlings were grown in hydroponic solution for 7 d. Bars in (D) = 15 cm; bars in (E) = 2 cm.

(F) to (H) Seedling length (F), dry weight (G), and K⁺ content (H) of Dongjin and Os-akt1 mutant after growth in hydroponic solution for 7 d. Data are shown as means ± SE (n = 3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance of differences between genotypes.

(I) Comparison of K⁺ uptake ability between Dongjin and Os-akt1 using the K⁺ depletion method. Data are shown as means ± SE (n = 3).
Os-AKT1 and Arabidopsis AKT1 displayed the similar K⁺ transport activity (Figures 2A and 2B) and Os-AKT1 could also mediate inward K⁺ currents in Arabidopsis root cells (Figures 2F and 2G), we further hypothesized that Os-AKT1 may be also regulated by Os-CIPKs and Os-CBLs in rice.

Based on sequence alignment, we cloned the Os-CIPK23 and Os-CBL1 genes from rice that showed highest similarities with Arabidopsis CIPK23 and CBL1, respectively. Then, we assayed the protein interaction among Os-AKT1, Os-CIPK23, and Os-CBL1. Yeast two-hybrid assays showed that Os-CIPK23 could interact with Os-CBL1, as well as the cytosolic region of Os-AKT1 (Os-AKT1-C, from 334 to 935 amino acids) (Figure 7A). This interaction was further validated by bimolecular fluorescence complementation (BiFC) assays in tobacco leaves. The yellow fluorescent protein (YFP) fluorescence was detected at the PM in tobacco epidermis in which Os-CIPK23-YN and Os-CBL1-YC were coexpressed, indicating interaction between Os-CIPK23 and Os-CBL1 (Figure 7B). We did not detect interaction fluorescence in leaves coexpressing Os-AKT1-YC and Os-CIPK23-YN (Figure 7B). However, when Os-CBL1 was coexpressed with Os-AKT1-YC and Os-CIPK23-YN, the YFP fluorescence could be observed at the PM in epidermis (Figure 7B). These results suggested that the interaction of Os-CIPK23 and Os-AKT1 in plant cells requires the presence of Os-CBL1. Os-CBL1 may recruit Os-CIPK23 to the PM, so that Os-CIPK23 can interact with PM-located Os-AKT1.

Figure 4. Phenotype of Rice Os-akt1 Complementation Plants.

(A) Phenotype comparison of different plant materials. The photographs of whole seedlings and first leaves were taken after the rice seedlings were grown in hydroponic solution for 7 d. The seedlings transformed with empty vector pBI121 were used as controls. COM1 and COM2 represent the two complementation lines of Os-akt1 mutant. Bars = 5 cm.

(B) Real-time PCR verification of Os-akt1 expression in different plant materials. The data are presented as means ± se (n = 3).

(C) and (D) Dry weight (C) and K⁺ content (D) of different plant materials after the rice seedlings were grown in hydroponic solution for 7 d. Data are shown as means ± se (n = 3). Student’s t test (*P < 0.05 and **P < 0.01) was used to analyze statistical significance of differences from the wild type.
Activation of Os-AKT1 by Os-CBL1 and Os-CIPK23

Based on the protein interaction results as shown in Figures 7A and 7B, we hypothesized that the activity of Os-AKT1 may be regulated by Os-CIPK23 and Os-CBL1. Thus, we tested for Os-CBL1-Os-CIPK23 regulation of Os-AKT1 in Xenopus laevis oocytes using a similar strategy as described in a previous report (Xu et al., 2006). In X. laevis oocytes, neither Arabidopsis AKT1 nor Os-AKT1 alone could form functional K+ channel. However, Arabidopsis AKT1 could be activated by both Arabidopsis CBL1-CIPK23 complex and rice CBL1-CIPK23 complex. By contrast, neither rice CBL1-CIPK23 nor Arabidopsis CBL1-CIPK23 could activate Os-AKT1 in oocytes (Supplemental Figure 5), suggesting differences between these two channels. Os-AKT1 may require some other regulators besides rice CBL-CIPK complex.

A previous report showed that Os-AKT1 alone could mediate inward K+ currents in HEK293 cells (Fuchs et al., 2005). We further tested the regulation of Os-CIPK23 and Os-CBL1 in HEK293 cells. When Os-CBL1-GFP (green fluorescent protein) and Os-CIPK23 were cotransfected with Os-AKT1 into HEK293 cells, the whole-cell inward K+ currents were remarkably increased compared with the cells transfected with Os-AKT1 alone (Figures 7C and 7D). After the cotransfection with Os-CBL1-GFP and Os-CIPK23, the current density of Os-AKT1-mediated inward K+ currents was increased from $-365 \pm 30$ pA/pF to $-1094 \pm 132$ pA/pF at $-200$ mV (Figure 7D). However, the voltage dependence of Os-AKT1 was not affected ($V_{1/2,Os-AKT1} = -188 \pm 4$ mV, $V_{1/2,Os-CBL1+Os-CIPK23+Os-AKT1} = -184 \pm 2$ mV; Figure 7E).

We also found that Os-CIPK23-GFP alone could not enhance Os-AKT1 activity (Figures 7C and 7D), which indicated that the activation of Os-AKT1 required the presence of both Os-CBL1 and Os-CIPK23. In this experiment, the GFP tag fused to Os-CBL1 or Os-CIPK23 was used as a reporter for transfection efficiency.

In addition, we also searched for any other Os-CIPKs that activate Os-AKT1. We cloned 25 rice CIPK genes and tested for interaction between these Os-CIPK proteins and Os-AKT1 using yeast two-hybrid assays and BiFC experiments. Besides Os-CIPK23, there were two Os-CIPK proteins (Os-CIPK3 and 19) interacting with Os-AKT1 (Supplemental Figure 6). We further tested if these two Os-CIPKs could activate Os-AKT1 in HEK293 cells. As shown in Supplemental Figure 7, only Os-CIPK19 could enhance Os-AKT1 inward K+ currents in the presence of Os-CBL1. However, the activation mediated by Os-CIPK19 was much weaker than that mediated by Os-CIPK23.

Since Os-AKT1 is regulated by CBL proteins, it is possible that Ca2+ signaling might be involved in the regulation of Os-AKT1. We tested for Ca2+-dependent activation of Os-AKT1 in HEK293 cells. The results showed that activation of Os-AKT1 alone was dependent on cytosolic Ca2+ concentration. A high cytosolic Ca2+ concentration could enhance the Os-AKT1 activity (Supplemental Figure 8). After coexpression with rice CBL1-CIPK23, the activation of Os-AKT1 was still dependent on the cytosolic Ca2+ concentration (Supplemental Figure 8). In addition, an Os-CBL1 EF-hand mutation (E172Q in the fourth EF-hand) was used to test the Ca2+-dependent activation of Os-AKT1. In HEK293 cells, the rice CBL1(E172Q)-CIPK23 could no
longer activate Os-AKT1 (Supplemental Figure 8). These data indicated that the regulation of Os-AKT1 activity may be dependent on cytosolic Ca$^{2+}$ signaling, mediated by Os-CBL1.

**Physiological Function Analysis of Os-CIPK23 and Os-CBL1**

To further characterize the functions of Os-CIPK23 and Os-CBL1, Arabidopsis lks1 mutant (a CIPK23 knockout mutant, also named the cipk23 mutant; Xu et al., 2006) and cbl1 cbl9 double mutant plants (Xu et al., 2006) were transformed with Os-CIPK23 and Os-CBL1, respectively. The lks1 and cbl1 cbl9 mutant plants displayed the similar sensitive phenotype as akt1 under low K$^+$ conditions (Xu et al., 2006). The Os-CIPK23 and Os-CBL1 could rescue the sensitive phenotype of lks1 and cbl1 cbl9 in the transgenic lines (lks1/Os-CIPK23 and cbl1 cbl9/Os-CBL1) (Figures 7F and 7G). Furthermore, the K$^+$ contents in these transgenic lines were also resumed (Supplemental Figure 9).

To further characterize the function of Os-CIPK23 in rice, we tested the phenotype of Os-CIPK23 RNA interference (RNAi) lines (Yang et al., 2008; O. sativa ssp japonica cv Nipponbare). Similar to Os-akt1 mutant, the two Os-CIPK23 RNAi lines both displayed the K$^+$ deficiency symptoms, growth inhibition, and leaf brown spots, especially under low K$^+$ conditions (Figures 8A and 8B). The expression of Os-CIPK23 in these two RNAi lines was detected using real-time PCR assays (Figure 8C). The shoot length and dry weight was reduced in the RNAi lines (Figures 8D and 8E). The decline of K$^+$ content in the RNAi lines under low K$^+$ conditions (Figure 8F) was consistent with the K$^+$ deficiency symptoms in Figures 8A and 8B. The K$^+$ depletion experiments indicated that the K$^+$ deficiency in Os-CIPK23 RNAi lines may result from the reduction of K$^+$ uptake capacity (Figure 8G). These data demonstrated that Os-CIPK23 plays important role in rice K$^+$ uptake.

For the Os-cbl1 mutant, the mutant plants did not show a remarkable low-K$^+$-sensitive phenotype compared with wild-type seedlings even though the K$^+$ content in Os-cbl1 mutant roots was reduced (Supplemental Figure 10). The K$^+$ uptake capacity of the Os-cbl1 mutant was slightly reduced (Supplemental Figure 10F), which suggested Os-CBL1 may be partially involved in K$^+$ uptake in rice roots.

**DISCUSSION**

**The Role of Os-AKT1 in Rice K$^+$ Uptake**

In *Arabidopsis*, AKT1 mediates root K$^+$ uptake over a wide range of external K$^+$ concentrations (Lagarde et al., 1996; Hirsch et al., 1998). Here, we report that Os-AKT1, an AKT1 homolog, conducts the K$^+$ uptake in rice roots. The GUS activity analysis indicated that Os-AKT1 is abundantly expressed in rice roots (Figure 1B), especially in root hairs and epidermis (Figures 1C and 1D), which is the precondition that Os-AKT1 mediates the K$^+$ uptake from the soils. The K$^+$ uptake test in both yeast and Arabidopsis (Figures 2A and 2C) indicated that Os-AKT1 could mediate the K$^+$ uptake similar to the Arabidopsis AKT1 channel (Figure 2B).

The Os-akt1 mutant plants showed a typical low-K$^+$-sensitive phenotype compared with the wild-type plants (Figures 3D and 3E). The K$^+$ content in Os-akt1 mutant was significantly reduced (Figure 3H), suggesting that the low-K$^+$-sensitive phenotype of Os-akt1 may be due to the reduction of K$^+$ uptake. This is consistent with the results from patch-clamping experiments and K$^+$ depletion assays, in which the inward K$^+$ fluxes and K$^+$ uptake rate in Os-akt1 mutant root cells were significantly

**Table 1. Electrophysiological Analysis of Rice Root Cell Protoplasts in Patch-Clamp Whole-Cell Recording Experiments**

<table>
<thead>
<tr>
<th>Rice Seedlings</th>
<th>No. of Recorded Cells</th>
<th>No. of Cells with Currents</th>
<th>Current Density at $-180$ mV (pA/pF)</th>
<th>Normalized $G_{\text{max}}$ (nS/pF)</th>
<th>$V_{1/2}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dongjin</td>
<td>144</td>
<td>53 (36.8%)</td>
<td>$-206 \pm 12$</td>
<td>$2.44 \pm 0.86$</td>
<td>$-162 \pm 6$</td>
</tr>
<tr>
<td>Os-akt1</td>
<td>136</td>
<td>28 (20.6%)</td>
<td>$-29 \pm 3$</td>
<td>$0.25 \pm 0.23$</td>
<td>$-167 \pm 5$</td>
</tr>
</tbody>
</table>

Data are shown as means ± SE.

![Figure 6. Electrophysiological Analysis of Inward K$^+$ Currents in Rice Root Cells.](image)
Figure 7. Os-CBL1 and Os-CIPK23 Enhance Os-AKT1 Activity.
reduced compared with those of wild-type rice (Figures 3I and 6A). Since K⁺ is a key nutrient factor that influences plant growth, development, and crop yield, it is plausible to propose that the reduction of K⁺ uptake in the Os-akt1 mutant may result in the inhibition of plant growth and the decline of grain yield. We indeed observed growth inhibition and development delay in Os-akt1 mutant plants (Figures 5A and 5B), and the grain yield was also impaired in the mutant plants (Figures 5C to 5G).

**Characteristic Comparison of Rice and Arabidopsis AKT1**

The yeast complementation experiments showed that rice and Arabidopsis AKT1 not only rescued the growth defect of R5421 under low K⁺ conditions (<250 μM K⁺), but also enhanced the R5421 growth under higher K⁺ conditions (1 and 5 mM K⁺) compared with the wild-type strain R757 (Figure 2A). In addition, the electrophysiological experiments in HEK293 cells also indicated that Os-AKT1 could mediate inward K⁺ currents even though the external K⁺ concentrations varied from 10 μM to 1 mM (Supplemental Figure 11). This suggests that similar to Arabidopsis AKT1, Os-AKT1 could also mediate K⁺ uptake over a wide range of external K⁺ concentrations.

Os-AKT1 could rescue the low-K⁺-sensitive phenotype of the Arabidopsis akt1 mutant (Figure 2C) because the inward K⁺ currents were recovered by Os-AKT1 in akt1/Os-AKT1 root cells (Figure 2F). However, Os-AKT1-mediated K⁺ currents were obviously different from the currents in Arabidopsis wild-type plants (Figures 2F and 2G), suggesting that there are different properties for these two channels. The activation curve analysis derived from patch-clamp recordings showed that the half-activation voltage of Os-AKT1 (V1/2 = −173 ± 5 mV) was much more negative than that of AKT1 (V1/2 = −133 ± 4 mV) (Figure 2H), which suggests that the activation threshold of Os-AKT1 is much more negative than AKT1. In addition, we determined the pH sensitivity of these two channels. Along with the increment of extracellular pH from pH 5.0 to 6.5, the V1/2 of AKT1 channel (in Col plants) and Os-AKT1 channel (in akt1/Os-AKT1 transgenic plants) were both slightly shifted in the negative direction (Supplemental Figure 12). However, the channel activity and V1/2 of AKT1 were more sensitive to extracellular pH compared with Os-AKT1 (Supplemental Figure 12).

The previous reports showed that, in X. laevis oocytes, outward K⁺ currents (K⁺ leakage) were recorded in AKT1-expressed oocytes under low external K⁺ concentrations, when the test voltage was between the K⁺ reversal potential and the AKT1 activation threshold (Duby et al., 2008; Geiger et al., 2009). However, At-KC1 channel subunit could interact with AKT1 and restrain AKT1-mediated K⁺ leakage under low external K⁺ concentrations by shifting the voltage dependence of AKT1 in the negative direction (Duby et al., 2008; Geiger et al., 2009; Wang et al., 2010). In Arabidopsis, KC1 is a general modulator of inward Shaker K⁺ channels that negatively shifts the channel activation threshold and limits the K⁺ leakage under low K⁺ conditions (Jeanguin et al., 2011). A homolog of At-KC1 was not found in the rice genome (Fuchs et al., 2005). It is therefore probable that Os-AKT1 is not regulated by a KC1-like channel in rice. However, it was observed that the half activation voltage of Os-AKT1 was more negative in akt1/Os-AKT1 Arabidopsis root cells (V1/2 = −173 ± 5 mV) than in rice root cells (V1/2 = −162 ± 6 mV). We speculate that At-KC1 may also regulate Os-AKT1 in akt1/Os-AKT1 Arabidopsis lines and negatively shift the activation potential of Os-AKT1. The interaction between Os-AKT1 and At-KC1 was also tested, and At-KC1 indeed interacted with Os-AKT1 at the PM (Supplemental Figure 13).

Despite the absence of a KC1-like channel in rice, it seems that Os-AKT1 alone could restrain the K⁺ leakage under low K⁺ conditions. In HEK293 cells, outward K⁺ currents (K⁺ leakage) were not observed in cells transfected with Os-AKT1, even when the external K⁺ concentration was reduced from 1 mM to 10 μM (Supplemental Figure 11). The results of patch-clamping experiments from root cells showed that the voltage dependence of Os-AKT1 (V1/2 = −162 ± 6 mV in rice root cell protoplasts; Figure 6C) was much more negative than that of AKT1 (V1/2 = −133 ± 4 mV in Arabidopsis root cell protoplasts; Figure 2H). That may be the reason why Os-AKT1 does not require a KC1-like channel and can restrain the K⁺ leakage alone under low K⁺ conditions.

The patch-clamping experiments indicated that the Os-AKT1-mediated inward K⁺ currents could be recorded only in a portion of the rice root cell protoplasts (Table 1), which is not consistent with the ubiquitous expression of Os-AKT1 in rice root cells (Figures 1C and 1D). This might suggest that there is differential regulation of Os-AKT1 in individual root cell types, so that the Os-AKT1 activity is fine-tuned in the specific cell types.

The previous data indicated that AKT1 may not rely on the transcriptional regulation. In Arabidopsis, K⁺ deprivation did not...
affect the expression of AKT1 (Kim et al., 1998; Maathuis et al., 2003; Pilot et al., 2003; Ahn et al., 2004; Hampton et al., 2004; Gierth et al., 2005). Arabidopsis AKT1-overexpressing lines do not show enhanced low-K⁺-tolerance phenotypes nor significantly increased inward K⁺ currents in root cells (Xu et al., 2006). These results suggest that the regulation of Arabidopsis AKT1 may occur at posttranslational level (Wang et al., 2010). As for Os-AKT1, the real-time PCR results and GUS assays also confirmed that the expression of Os-AKT1 in rice roots was not changed under K⁺ deficiency conditions (Supplemental Figure 3), indicating that Os-AKT1 may be also regulated at posttranslational level.

The Regulatory Mechanism of AKT1-Like Channels in Plants

In Arabidopsis, the activity of AKT1 channel is controlled by CBL1/9 and CIPK23 (Li et al., 2006; Xu et al., 2006). Similar regulatory mechanisms involving CBL-CIPK complexes modulating K⁺ channel were reported for several different plant species based on electrophysiological analyses. Two AKT1-like
channels, Hv-AKT1 from barley and Vv-K1.1 from grapevine both could be activated by Arabidopsis CBL1 and CIPK23 in X. laevis oocytes (Boscari et al., 2009; Cuéllar et al., 2010). Furthermore, another K+ channel from grapevine, Vv-K1.2, was characterized as a voltage-gated inward K+ channel. In X. laevis oocytes, Vv-K1.2 alone could not form functional channel. However, after coexpression with Vv-CBL01-Vv-CIPK04 or Vv-CBL02-Vv-CIPK03, Vv-K1.2 was capable of mediating inward K+ currents (Cuéllar et al., 2013). It seems that the regulation of AKT1-like channels via a CBL-CIPK complex might be a universal mechanism in different plant species.

In this study, we tried to identify the candidate Os-CBLs and Os-CIPKs that may regulate Os-AKT1 activity. Based on the sequence alignment between rice and Arabidopsis, we cloned Os-CBL1 and Os-CIPK23 from rice. In HEK293 cells, Os-AKT1 alone could mediate the inward K+ currents (Fuchs et al., 2005), whose activity was further enhanced when coexpressed with rice or Arabidopsis CBL1-CIPK3 (Figures 7C and 7D; Supplemental Figure 14). Os-CBL1 and Os-CIPK23 could rescue the low-K+-sensitive phenotype of Arabidopsis cbl1 cbl9 and lks1 mutants, respectively (Figures 7F and 7G). These results indicated that the function of CBL and CIPK proteins in K+ channel regulation may be conserved in different plant species.

The phenotype test showed that Os-CIPK23 RNAi lines exhibited similar K+-deficient symptoms as the Os-akt1 mutant under low K+ conditions (Figures 3E and 8B). In these two mutant lines, the K+ uptake rates were reduced (Figures 3I and 8G), which led to the decline of K+ content (Figures 3H and 8F) and inhibition of plant growth (Figures 3G and 8E). Therefore, the Os-CIPK23 should be the functional regulator of Os-AKT1 in rice. As for Os-CBL1, the Os-cbl1 mutant did not show an obvious low-K+-sensitive phenotype (Supplemental Figure 10). There are two possible reasons for this. On the one hand, the Os-cbl1 mutant used in this study is a knockdown line (Supplemental Figure 10). The leaky expression of Os-CBL1 might be sufficient to accomplish its physiological function in Os-AKT1 regulation. On the other hand, there might be some other CBLs in rice that are functionally redundant with Os-CBL1. This would be quite similar to the situation in Arabidopsis, in which only the cbl1 cbl9 double mutant showed obvious low-K+-sensitive phenotypes and neither cbl1 nor cbl9 single mutants exhibited the sensitive phenotype (Xu et al., 2006).

In conclusion, our findings demonstrate that the inward K+-channel Os-AKT1 functions in K+ uptake in rice roots, whose activity is regulated by Os-CBL1 and Os-CIPK23. These findings may provide insight to improve rice K+ uptake efficiency and enhance rice tolerance to K+ deficiency stress.

METHODS

Plant Material and Growth Conditions

For rice (Oryza sativa) harvest, the rice seeds were first germinated in Petri plates. One week later, the rice seedlings were transplanted into growing trays with soil mixture (rich soil: vermiculite = 1:1, v/v) and kept in growth chamber with a 12-h-light/12-h-dark (25°C) photoperiod. About 3 weeks later, the plants were transplanted into the field and grown until harvest. The rice seeds were sterilized with 20% NaClO for 1 h, soaked in deionized water at room temperature for 2 d, and pregerminated at room temperature for one night. Then, the seeds were sown on wet filter papers.

Uniformly germinated seeds were selected and transplanted into nutrient solution one week later. The 14-d-old seedlings were transferred into nutrient solution with different K+ concentrations (1 mM and 100 μM). The nutrient solution (pH 5.7) consisted of macronutrients (mg L−1): NH4NO3 114.25, NaH2PO4 2H2O 50.38, K2SO4 39.25, CaCl2 110.75, MgSO4·7H2O 40.5, FeSO4·7H2O 34.75, Na2EDTA 46.53; and micronutrients (mg L−1): MnCl2·4H2O 1.88, (NH4)6Mo7O24·4H2O 0.09, H3BO3 1.17, ZnSO4·7H2O 0.44, CuSO4·5H2O 0.39 (International Rice Research Institute, Philippines). The nutrient solution was renewed every 2 d. For the low K+ (100 μM) hydroponics, the amount of K2SO4 was reduced to achieve the low K+ concentration. Other components in nutrient solution were not changed.

The Arabidopsis thaliana seeds were germinated on Murashige and Skoog (MS) medium containing 0.8% (w/v) agar and 3% sucrose at 22°C under constant illumination. Then, 4-d-old seedlings were transferred to MS and low K+ (LK; 100 μM) medium, which was described previously (Xu et al., 2006). The low-K+ phenotype was observed after 7 d.

Subcellular Localization Analysis

The coding sequence of Os-AKT1 was fused with GFP in the pCAMBIA1300 vector. The plasmids pCAMBIA1300-Os-AKT1-GFP and pGPTVII-CBL1n-OFP (Batisti et al., 2008) were electroporated into Agrobacterium tumefaciens (GV3101) using a Bio-Rad Gene Pulser. The methods for plasmid transformation and expression were described in the previous literature (Rajamäki and Valkonen, 2009).

GUS Assays

The 1010-bp fragment before the ATG codon of Os-AKT1 was cloned from the japonica rice cultivar Nipponbare. This fragment was used as Os-AKT1 promoter in this study and constructed into pCAMBIA1381 vector. The plasmid was transformed into Nipponbare rice via callus transformation, which was mediated by Agrobacterium (EHA105). The T2 heterozygous transgenic rice plants were treated with the GUS staining solution which contained 1% (v/v) N,N-dimethyl formamide, 0.1% X-Gluc, 0.1% Triton X-100, 0.1 M PBS, 0.05 mM KFe(CN)6, and 0.05 mM K4Fe(CN)6·3H2O. Then, the rice plants were immersed in 70% (v/v) ethanol. The GUS-stained roots and leaves were sliced into semithin sections for microscopy observation.

K+ Content Analysis

The 14-d-old rice seedlings were transferred into nutrient solution with different K+ concentrations (1 mM and 100 μM) and treated for 7 d. 4-d-old seedlings were collected, rinsed with deionized water, and dried at 80°C to constant weight in paper bags. The dry weights of samples were measured as dry biomass. Then the dry plant tissues were incinerated in a muffle furnace at 575°C for 9 h. The ashes were dissolved with 0.1N HCl in a 30°C water bath. The solutions were transferred to a 100-mL volumetric flask and diluted 20 times. The nutrient solution was renewed every 2 d. K+ concentrations were determined by Zeeman atomic absorption spectrophotometry (Hitachi Z-2000). The method of K+ content analysis for Arabidopsis plants was described previously (Xu et al., 2006).

Real-Time PCR

The total RNA was extracted from rice seedlings grown under normal conditions using TRIzol reagent (Invitrogen). The 4 μg-DNase-treated total RNA was reverse transcribed into cDNA with random primers. The cDNA was diluted 40 times, and 6 μL diluted cDNA was used as the template in each well for quantitative real-time PCR analysis. The cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems) on the ABI 7500 thermocycler (Applied Biosystems). The
amplification reactions were performed in a total volume of 20 μL, which contained 6 μL cDNA, 2 μL forward and reverse primers (1 μM), 10 μL SYBR Green premix, and 2 μL PCR-grade sterile water. The PCR was programmed as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. An Actin gene was used as an internal standard to normalize the expression data for the tested gene. All the primers used in this experiment are listed in Supplemental Table 1. Three replications were performed for each sample. The fluorescence signal was detected during the annealing step. All experiments were repeated three times.

**Yeast Complementation**

The coding sequences of Os-AKT1 and AKT1 were constructed into p416-GPD vector and transformed into the yeast strain R5421 (trk1Δ trk2Δ), in which the two endogenous K+ transporter genes (TRK1, 2) were deleted. The yeast strain R757 was used as a positive control. Single colonies were picked from different transformed yeast cells and cultured at 30°C overnight in 50 mL YPDA medium, until the OD600 reached 2.5. The yeast cells were collected by centrifugation at 6000g for 1 min and washed in double-distilled water three times. Cells were resuspended in double-distilled water with the OD600 0.8, and 10-fold serial diluted cultures were incubated on AP plates containing different K+ concentrations. These plates were incubated at 30°C for 2 d.

For kinetic analysis of K+ uptake in yeast, yeast colonies expressing Os-AKT1 and AKT1 were cultured at 30°C overnight in 50 mL YPDA medium, until the OD600 reached 2.5. The yeast cells were collected and washed in double-distilled water for three times. Cells were resuspended in double-distilled water with the OD600 3.0. The 30 mL liquid AP medium containing different KCl concentrations was added in each 50-mL flask. Yeast cells (100 μL) were transferred into the AP medium, and flasks were shaken at 30°C. The OD600 values of the yeast cells were recorded every 1.5 h after the OD600 reached 2.0. The slope for each K+ concentration was calculated according to the linear regression of the growth curves during the logarithmic growth phase. The curve in the graph was obtained by applying nonlinear regression analysis using the Michaelis-Menten equation (Horie et al., 2011).

**Yeast Two-Hybrid Assays**

The coding sequences of Os-CBL1 and the cytosolic region of Os-AKT1 (Os-AKT1-C, from N334) were cloned into pGADT7 vector. Os-CIPK23 coding sequence was introduced into pGBK7 vector. The plasmids were transformed into yeast strain AH109 for yeast two-hybrid assays following the lithium acetate method (according to TRANFOR protocol). The positive clones of Os-CBL1/Os-CIPK23 and Os-CIPK23/Os-AKT1-C were selected from SC medium (-Leu-Trp) without 3-aminotriazole. Single colonies were picked from different transformed yeast cells and cultured from SC medium (-Leu-Trp, without 3-aminotriazole) incubated at 28°C after the lithium acetate method (according to TRANFOR protocol). The positive transformed into yeast strain AH109 for yeast two-hybrid assays following the lithium acetate method (according to TRANFOR protocol). The positive clones of Os-CBL1/Os-CIPK23 and Os-CIPK23/Os-AKT1-C were selected from SC medium (-Leu-Trp-His-Ade) without 3-aminotriazole. Four days later, β-galactosidase activity was detected, and photographs were taken subsequently.

**BiFC Assays**

The coding regions of Os-CBL1 and Os-AKT1 were cloned into vector pSPYCE (MR) (Waadt and Kudla, 2008; Waadt et al., 2008). The coding sequence of Os-CIPK23 was cloned into vector pSPYNE (R) 173 (Waadt and Kudla, 2008). The BiFC assays were performed as described previously (Walter et al., 2004; Waadt and Kudla, 2008). The tested construct pairs were expressed in leaves of Nicotiana benthamiana for 5 d before microscopy observation. The YFP fluorescence in the transformed leaves was imaged using a confocal laser scanning microscope (Leica DMI2020).

**Generation of Arabidopsis Transgenic Plants**

Full-length coding sequences of Os-AKT1, Os-CIPK23, and Os-CBL1 were constructed into the overexpression vector pU113D01 (vector pCAMBIA1301 modified with ubiquitin promoter instead of cauliflower mosaic virus 35S promoter, Yu et al., 2007). The three constructs were transformed into akt1, cbl1, and cbl9 Arabidopsis mutant plants, respectively. The Arabidopsis transformation was performed using the floral dip method with Agrobacterium (strain GV3101; Clough and Bent, 1998). The T4 homozygous transgenic plants were used to examine the phenotype under low-K+ conditions. The expression of Os-AKT1 in transgenic plants was detected using real-time PCR. The Os-CIPK23 and Os-CBL1 expression in transgenic plants were detected using RT-PCR.

**Construction of Os-akt1 Complementation Rice Plants**

The CDS of Os-AKT1 was cloned into the vector pBII121 (Chen et al., 2003) with Smal and XbaI. The 35S promoter in pBII121 vector was replaced by the Os-AKT1 promoter (1010 bp) using Scal and Smal. The plasmid was transformed into the Os-akt1 mutant using a gene gun. The empty vector pBII121 was also transformed into the Os-akt1 mutant, as a control.

**Kinetic Analysis of K+ Uptake**

Rice seeds were germinated on half-strength MS medium containing 0.2% (w/v) plant phytagel and 3% sucrose at 28°C under constant illumination. For K+-depletion experiments (Drew et al., 1984), 7-d-old seedlings were pretreated in starvation solution (0.2 mM CaSO4 and 5 mM MES, pH 5.75 adjusted with Tris) at 28°C for 18 h. Each sample included seven seedlings, the fresh weight of which was near 0.8 g. The tests began 5 min after transfer of seedlings into the depletion solution (0.25 mM KNO3, 0.2 mM CaSO4, and 5 mM MES, pH 5.75 adjusted with Tris). The experiments were conducted at 28°C in the light, and all the samples were shaking on a shaking table during the experiments (Xu et al., 2006; modified). The solution samples were collected at different time points as indicated, and the K+ concentrations were measured by Zeeman atomic absorption spectrophotometry (Hitachi Z-2000).

**DNA Gel Blot Analysis**

Genomic DNA was isolated from the leaves of Os-akt1 mutant and Dongjin by the CTAB method. The 20 μg DNA was digested with different restrictive enzymes, separated on 1% agarose (Invitrogen) gel, and transferred onto Amersham Hybond-N+ membrane (GE Healthcare). Prehybridization, hybridization, and bolt washing were performed as recommended by the manufacturer. A segment of the hygromycin resistance gene HPT (846 bp) was used as the probe and was labeled with the digoxin labeling system (DIG DNA Labeling and Detection Kit; Roche). The membrane was exposed to the Kodak XAR-50 x-ray film.

**Protoplast Isolation and Patch-Clamp Whole-Cell Recording**

The rice root cell protoplasts were isolated from 7-d-old primary roots of rice seedlings that were grown on filter paper immersed in half-strength MS with 1% sucrose at 25°C in the dark. The root tips (3 to 5 mm) were cut into small pieces and incubated in enzyme solution at 28°C for 1 h to release root cell protoplasts. The enzyme solution contained 1% (w/v) cellulase (Onozuka R-10), 0.3% (w/v) pectolyase Y-23 (Onozuka), 0.5% (w/v) BSA, 0.5% (w/v) polyvinylpyrrolidone, 1 mM CaCl2, 10 mM MES/Tris (pH 5.6), and α-sorbitol (α = 300 mosmol kg−1). The protoplasts were filtered through 80-μm nylon mesh and washed three times with standard solution, which contained 1 mM CaCl2, 10 mM MES/Tris (pH 5.6), and α-sorbitol (α = 300 mosmol kg−1). The protoplast suspension was kept on
ice at least for 1 h and then used for patch-clamping experiments. Standard whole-cell recording techniques were applied (Hamill et al., 1981). The bath solution contained 100 mM K-glucuronate, 10 mM CaCl$_2$, 10 mM MES/Tris (pH 5.6), and 100 mM sorbitol ($\sigma = 300$ mosmol kg$^{-1}$). The pipette solution contained 150 mM K-glucuronate, 20 mM MgCl$_2$, 100 mM HEPES/Tris (pH 7.4), and 200 mM sorbitol ($\sigma = 300$ mosmol kg$^{-1}$). The patch-clamping recordings were conducted at ~20°C in dim light. Whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments). The methods for Arabidopsis root cell protoplast isolation and patch-clamp whole-cell recording were described previously (Xu et al., 2006).

**Cell Culture, Transfection, and Whole-Cell Recording**

For the patch-clamping recordings from HEK293 cells, we purchased HEK293 cells from ATCC (American Type Culture Collection) and cultured in DMEM (Dulbecco’s modified eagle medium) with 4500 mg L$^{-1}$ glucose (Gibco) and 10% fetal calf serum (Gibco) for 24 h at 37°C, 5% CO$_2$. Then, the HEK293 cells were transfected with different combinations of recombinant plasmids using Lipofectamine 2000 Transfection Reagent (Invitrogen). Os-AKT1 was cloned into pcDNA3.1 vector (Invitrogen). Os-CBL1 was cloned into pEGFP-N1 vector (Clontech). The vector pBudCE4.1 (Invitrogen) was used for the simultaneous expression of both Os-CBL1-GFP and Os-CIPKs. In pBudCE4.1 vector, Os-CBL1-GFP was driven by CMV promoter, and Os-CIPKs were driven by EF-1a promoter. The plasmids pcDNA3.1-Os-AKT1 and pBudCE4.1-Os-CBL1-GFP-Os-CIPKs were cotransfected into HEK293 cells. Then, the transfected cells were treated with Trypsin (Gibco), centrifuged at 160g for 5 min, and kept on ice for patch-clamp recording. The cells with GFP fluorescence were selected for whole-cell recording. The components of the pipette solution were the same as described previously (Fuchs et al., 2005). The bath solution contained 100 mM K-glucuronate, 10 mM MES/Tris (pH 5.6), and 100 mM sorbitol ($\sigma = 300$ mosmol kg$^{-1}$). The patch-clamp recordings were conducted at ~20°C in dim light. Whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments).

**Accession Numbers**

Sequence data from this article can be found in the Rice Genome Annotation Project under the following accession numbers: Os10g41510 for Os-CBL1, Os07g05620 for Os-CIPK23, Os03g20380 for Os-CIPK3, Os05g43840 for Os-CIPK19, and Os01g45990 for Os-AKT1.

**Supplemental Data**

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

**Supplemental Figure 1.** Amino Acid Sequence Alignment of Shaker K$^+$ Channels from Different Plant Species.

**Supplemental Figure 2.** Phylogenetic Analysis of AKT1-Like K$^+$ Channels from Different Plant Species.

**Supplemental Figure 3.** Relative Expression of Os-AKT1 under Low-K$^+$ Stress.

**Supplemental Figure 4.** Electrophysiological Analysis of Inward K$^+$ Currents in Rice Root Cells from Os-akt1 Complementation Line.

**Supplemental Figure 5.** Os-CBLs and Os-CIPKs Could Not Activate Os-AKT1-Mediated Inward K$^+$ Currents in Xenopus Oocytes.

**Supplemental Figure 6.** Interaction Analysis between Os-CIPKs and Os-AKT1.

**Supplemental Figure 7.** Electrophysiological Analysis of Os-AKT1 Regulation by Other Os-CIPKs in HEK293 Cells.

**Supplemental Figure 8.** Electrophysiological Analysis of Os-AKT1 Activation by Cytoplasmic Ca$^{2+}$ in HEK293 Cells.

**Supplemental Figure 9.** Os-CIPK23 and Os-CBL1 Rescue the K$^+$ Content of Arabidopsis lks1 and cbl1 cbl9 Mutants.

**Supplemental Figure 10.** Phenotype of Rice Os-cbl1 Mutant.

**Supplemental Figure 11.** Os-AKT1-Mediated K$^+$ Currents in HEK293 Cells under Different K$^+$ Concentrations.

**Supplemental Figure 12.** The pH Sensitivity of AKT1 and Os-AKT1 Channels in Arabidopsis Root Cell Protoplasts.

**Supplemental Figure 13.** Interaction Analysis between At-KC1 and Os-AKT1.

**Supplemental Figure 14.** Electrophysiological Analysis of Os-AKT1 Regulation by Arabidopsis CBL1 and CIPK23 in HEK293 Cells.

**Supplemental Table 1.** Primer Sequences Used in Real-Time PCR Experiments.

**Supplemental Table 2.** Primer Sequences Used for Cloning.

**Supplemental Data Set 1.** Text File of the Alignment Used for the Phylogenetic Analysis in Supplemental Figure 2.

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**AUTHOR CONTRIBUTIONS**

Y.W. and W.-H.W. designed the research; J.L., Y.L., G.-N.Q., J.L., and Z.-J.X. performed research and analyzed data; J.L., Y.L., and Y.W. wrote the article. Y.W. and W.-H.W. revised the article. Received January 22, 2014; revised July 3, 2014; accepted July 21, 2014; published August 5, 2014.

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The Os-AKT1 Channel Is Critical for K⁺ Uptake in Rice Roots and Is Modulated by the Rice CBL1-CIPK23 Complex
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