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Mechanistic Analysis of AKT1 Regulation by the CBL–CIPK–PP2CA Interactions

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ABSTRACT ***Arabidopsis*** **K^+ transporter 1 (AKT1) participates in K^+ uptake in roots, especially under low-K conditions. We recently identified a $Ca2^+$ signaling pathway consisting of multiple calcineurin B-like calcium sensors (CBLs) and multiple target kinases (CBL-interacting protein kinases or CIPKs) that phosphorylate and activate AKT1, whereas a specific PP2C-type phosphatase inactivates CIPK-dependent AKT1 activity. In this study, we analyzed the interactions between PP2Cs and the CBL–CIPK pathway and found previously unsuspected mechanisms underlying the CBL–CIPK–PP2C signaling processes. The interaction between the CIPKs and PP2Cs involves the kinase domain of the CIPK component, in addition to the protein phosphatase interacting motif (PPI) in the regulatory domain. Furthermore, specific CBLs physically interact with and inactivate PP2C phosphatases to recover the CIPK-dependent AKT1 channel activity. These findings provide further insights into the signaling network consisting of CBL–CIPK–PP2C interactions in the activation of the AKT1 channel.**

Key words: Abscisic acid; *Arabidopsis*; environmental stress; K^+ uptake.

INTRODUCTION

As the most abundant inorganic cation in plants, potassium (K^+) plays essential functions in plant growth and development (Armengaud et al., 2010; Leigh and Jones, 1984; Kim et al., 2010; Very and Sentenac, 2003; Wang and Wu, 2010). The *Shaker*-type K^+ channels may play a key role in K^+ uptake from the soil and translocation of K^+ throughout the plant (Very and Sentenac, 2003; Ward et al., 2009; Wang and Wu, 2010). These channels typically consist of six transmembrane domains including an ion selectivity filter P loop with a C-terminus containing putative cyclic nucleotide-binding and KHA domains (Very and Sentenac, 2003; Ward et al., 2009). *Arabidopsis* K^+ transporter 1 (AKT1), one of *Shaker*-type family members, is highly expressed in the root epidermal cells (including root hairs) and functionally participates in K^+ uptake into the roots under low K^+ stress (Lagarde et al., 1996; Hirsch et al., 1998; Xu et al., 2006; Wang and Wu, 2010). AKT1 is inactive in the *Xenopus* oocyte expression system, but is activated by kinase CIPK23 and the specific calcineurin B-like (CBL) Ca^{2+} sensors CBL1 and CBL9 (Li et al., 2006; Xu et al., 2006; Lee et al., 2007; Luan, 2009). Electrophysiological studies showed that disruption of both CBL1 and CBL9 or CIPK23 gene in *Arabidopsis* reduced AKT1 activity in root hair cells (Li et al., 2006; Xu et al., 2006). Indeed, *cbl1cbl9* double mutants and *cipk23* mutants displayed defects similar to *akt1* mutants in the low-K response (Xu et al., 2006; Cheong et al., 2007). In addition to CIPK23, CIPK6, and CIPK16 both activate AKT1 in a CBL-dependent manner (Lee et al., 2007; Luan, 2009).

The *Arabidopsis* genome contains a number of genes for CBL and CIPK proteins. At least 10 CBL and 25 CIPK genes have been confirmed by cDNA cloning and sequencing (Luan et al., 2002; Kolukisaoglu et al., 2004; Pandey et al., 2008). The CIPK proteins are a group of serine–threonine protein kinases, and are most similar to sucrose non-fermenting 1 (SNF1) protein kinase from yeast in the kinase domain. Therefore, this group has also been categorized as SNF1 related kinase 3 (SnRK3) (Hrabak et al., 2003). The kinase domain in CIPKs is separated by a junction domain from the less-conserved C-terminal regulatory domain. Within the C-terminal regulatory domain, a conserved NAF motif (or FISL motif) mediates the binding of CBL proteins and functions as an auto-inhibitory domain (Kim et al., 2000; Albrecht et al., 2001; Guo et al., 2001). In

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contrast, SnRK1 and SnRK2 groups lack specific regulatory domain or auto-inhibitory domain (Hrabak et al., 2003). The binding of CBLs to the NAF/FISL motif expels the auto-inhibitory domain from the kinase domain so that the kinase gets access to the putative substrates (Albrecht et al., 2001; Guo et al., 2001; Kolukisaoglu et al., 2004). Each CBL interacts with a subset of CIPKs and each CIPK interacts with one or more CBLs. Some CBL members share common CIPK targets and some CIPKs share common CBL regulators. The interactions among various members in the CBL and CIPK family are thus both specific and overlapping, forming a complex Ca^{2+} sensitive CBL–CIPK network to function specifically and redundantly in cellular regulation (Luan et al., 2002; Batistic and Kudla, 2004; Luan, 2009; Luan et al., 2009).

In the case of AKT1 regulation by the CBL–CIPK pairs, CIPKs physically interact with the ankyrin repeats in the C-terminal domain of AKT1 (Lee et al., 2007; Luan, 2009) and phosphorylates AKT1 (Li et al., 2006; Xu et al., 2006; Lee et al., 2007; Luan, 2009). In searching for protein phosphatases that inhibit the activity of AKT1, an A-type protein phosphatase 2C (PP2C) member AIP1 was identified to counteract with the CBL1–CIPK23 complex (Lee et al., 2007). However, little is known about the regulatory mechanism underlying CIPK–PP2C action that enables the AKT1 channel to be switched on and off. The present study therefore seeks to investigate how PP2C regulates the CBL–CIPK network to control AKT1 channel activity. Our results show that PP2CA specifically interacts with CIPK6 and directly interacts with the CIPK6 kinase domain to inactivate activation of AKT1. In addition, several CBLs were found to interact with and inhibit the activity of PP2CA, thereby enhancing AKT1 activation induced by CIPK6. These findings provide new insights into the regulation of AKT1 activity by the CBL–CIPK–PP2CA network.

RESULTS

Physical Interaction between A-Type PP2Cs and CIPKs Determines AKT1 Activity

Our previous studies showed that an A-type PP2C member, AIP1 (At1g07430), together with CIPK23, a SnRK3 member, forms a protein phosphorylation/dephosphorylation pair to regulate AKT1 activity (Lee et al., 2007). More recent studies have shown that multiple PP2Cs physically associate with multiple members in the SnRK2 family and regulate their activities (Geiger et al., 2009; Lee et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). Taking together, these studies indicate a common protein kinase–phosphatase interaction network formed between SnRK2- and SnRK3-type kinases and A-type PP2Cs.

As both A-type PP2C and CIPK families are composed of multiple members, we expected that more CIPK–PP2C pairs may form and regulate the AKT1 activity than previously reported (Lee et al., 2007). To test this idea, we used yeast two-hybrid assay to screen the potential interactions between nine PP2Cs and each of the three CIPKs (CIPK6, CIPK16, and CIPK23) that were shown to activate AKT1 activity (Lee et al., 2007). Among the nine A-type PP2C members tested, AIP1 (At1g07430) and its homologous member At2g29380 (AIP1H) interacted physically with CIPK23 (Figure 1). AIP1 and AIP1H are most similar to each other among the nine A-type PP2C family members. Although CIPK6 and CIPK16 have the low overall protein sequence similarity to CIPK23, both also interacted with AIP1 and AIP1H (Figure 1). These results suggest that these three CIPKs could share the same regulatory mechanisms in the signaling pathway for AKT1 activation. Furthermore, CIPK6 interacted with two more PP2C members (PP2CA and AHG1) (Figure 1). PP2CA and AHG1 share high protein sequence identity and belong to the same clade, including AIP1 and AIP1H.

The above yeast two-hybrid assays show that CIPK6, CIPK16, and CIPK23 physically interact with the specific A-type PP2Cs, probably forming phosphorylation/dephosphorylation pairs to regulate AKT1 activity. In the case of SnRK2–PP2C interaction, the kinase activity is directly inhibited by the PP2Cs (Geiger et al., 2009; Lee et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). It is possible that PP2Cs also directly inhibit CIPK activity, thereby reducing the activity of the AKT1 channel (Lee et al., 2007). To test this idea, we co-expressed AKT1, CBL1–CIPK1 complex, CBL1–CIPK6 complex, and various PP2Cs in the oocytes and measured AKT1 activity. CIPK1 does not physically interact with AKT1 reported in our previous study (Li et al., 2006). The CBL1–CIPK6 complex activated AKT1 activity (Figure 2A), but CBL1–CIPK1 complex did not, consistent with our previous study (Lee et al., 2007). This specific AKT1 activation induced by CBL1–CIPK6 complex was not significantly changed by ABI-1 (Figure 2A), a component that physically interacts with CIPK20/PKS18 (Ohta et al., 2003), but not

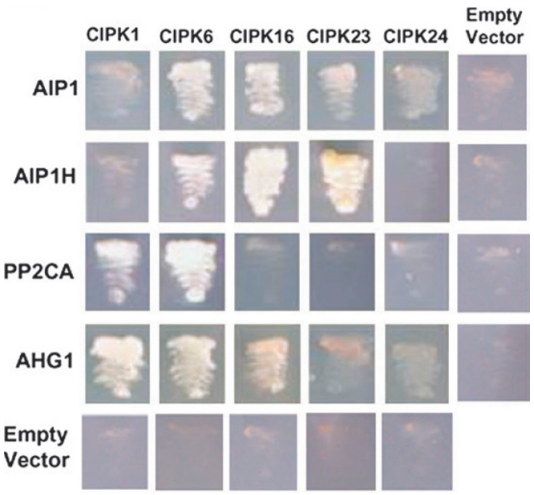

Figure 1. Yeast Two-Hybrid Assay of Interactions between CIPKs and PP2Cs.

Yeast strain PJ69-4A was transformed with CIPKs cloned into pGBT9 and PP2Cs cloned into pGADGH. Growth on the selection medium (SC-LTH including 2 mM 3′-AT) was used as indicator for interaction.

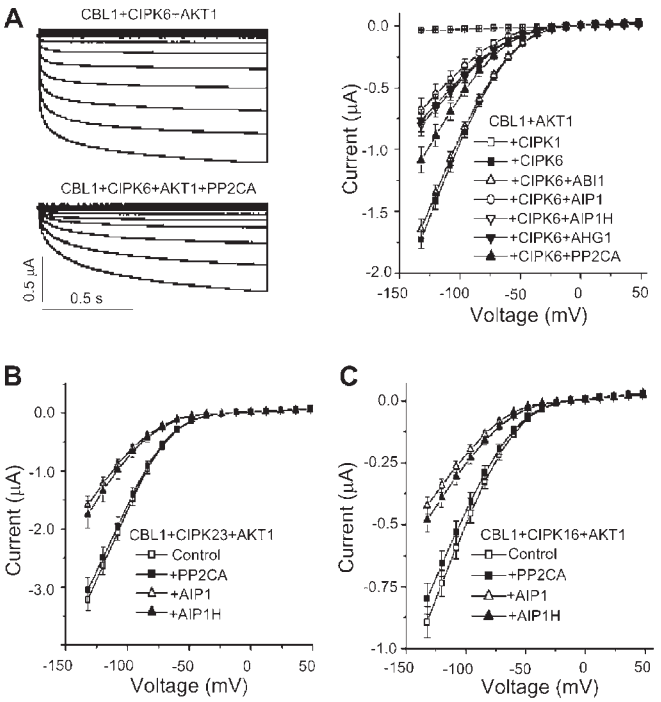

Figure 2. Multiple A-Type PP2Cs Inhibited AKT1 Activation by CBL1–CIPK6 (A), CBL1–CIPK23 (B), or CBL1–CIPK16 (C) in the *Xenopus* oocytes.

(A) AIP1, AIP1H, AHG1, and PP2CA, but not ABI-1, inhibited AKT1 activity induced by CBL1–CIPK6. (Left) Current traces recorded from the oocytes expressing cRNA of AKT1+CBL1+CIPK6 (upper), and AKT1+CBL1+CIPK6+PP2CA (below). Currents were recorded from the voltage steps of 48 to –132 mV with 12-mV decrements. The dotted lines represent zero level. (Right) The voltage–current relationship is shown for AKT1+CBL1+CIPK1, AKT1+CBL1+CIPK6, AKT1+CBL1+CIPK6+ABI1, AKT1+CBL1+CIPK6+AIP1, AKT1+CBL1+CIPK6+AIP1H, AKT1+CBL1+CIPK6+AHG1, and AKT1+CBL1+CIPK6+PP2CA.

(B) The voltage–current relationship is shown for AKT1+CBL1+CIPK23 (Control), AKT1+CBL1+CIPK23+PP2CA, AKT1+CBL1+CIPK23+AIP1, and AKT1+CBL1+CIPK23+AIP1H. AIP1 and AIP1H2, but not PP2CA, inhibited AKT1 activity induced by CBL1–CIPK23.

(C) The voltage–current relationship is shown for AKT1+CBL1+CIPK16 (Control), AKT1+CBL1+CIPK16+PP2CA, AKT1+CBL1+CIPK16+AIP1, and AKT1+CBL1+CIPK16+AIP1H. AIP1 and AIP1H inhibited AKT1 activity induced by CBL1–CIPK16. The pooled current values were at 1.8 s of each voltage-clamp episode and presented as means ± SE, with $n > 10$ in each case.

with CIPK6. However, PP2Cs interacting physically with CIPK6, such as AIP1, AIP1H, PP2CA, and AHG1 (Figure 1) all reduced the magnitude of AKT1 activity by CBL1–CIPK6 (Figure 2A). Among these PP2Cs, PP2CA has the lowest inhibitory potency. We also tested the effects of PP2CA, the component that does not interact with CIPK23 (Figure 1), on AKT1 activity activated by CBL1–CIPK23 and found that this channel activity was not changed by the presence of PP2CA (Figure 2B), providing the further evidence that inhibitory function of PP2CA on AKT1 activity results from the physical interaction with CIPK6. In addition to CIPK6, we tested whether AIP1 and AIP1H, the components interacting with CIPK23 and CIPK16, inhibited AKT1 activation by these two CIPKs. We found that both PP2Cs significantly reduced the magnitude of AKT1 activation by CBL1–CIPK23 (Figure 2B) or by CBL1–CIPK16 (Figure 2C). PP2CA did not significantly change AKT1 activation (Figure 2B and 2C) and we showed PP2CA did not have physical interaction with CIPK23 and CIPK16 (Figure 1). These results suggest that physical interaction with CIPKs is required for PP2Cs to inhibit AKT1 activity induced by CIPK–CBL complex.

PP2Cs Directly Interact with CIPKs Kinase Domain to Inhibit Kinase-Mediated AKT1 Activation

It has been shown that the C-terminal region of CIPKs contains two regulatory domains, one (called NAF/FISL) for interacting with CBLs and the other (PPI domain) for interacting with protein phosphatases such as PP2Cs (Batistic and Kudla, 2004; Gong et al., 2004; Luan, 2009). Now that we have identified the function of PP2C interaction with CIPKs as being inhibitory to the kinase activity, we expect that PPI motif is to provide a docking domain for PP2Cs leading to inhibition of the kinase and that the deletion of this domain should eliminate this inhibition. To test this hypothesis, we made several truncated forms of CIPK6 and tested their interactions with PP2CA in the yeast two-hybrid system. As shown in Figure 3A, the full length of CIPK6 and the truncated forms retaining PPI domain but without *C*-terminal part interacted with PP2CA, consistent with the idea that PPI serves as a docking site for PP2Cs. However, the truncated forms of CIPK6 containing kinase domain only without PPI domain also interacted with PP2CA. These results show that CIPK6 can interact with PP2CA through either PPI domain or the kinase domain.

CIPKs-activating AKT1 activity results from AKT1 protein phosphorylation (Li et al., 2006; Lee et al., 2007). We co-expressed AKT1 with the truncated form of CIPK6 with kinase domain only (CIPK6Kin), the truncated form of CIPK1 with kinase domain only (CIPK1Kin), CIPK6, and CBL1–CIPK6 complex in the oocytes to study the contribution of the kinase domain to AKT1 activation. As shown in Figure 3B, CIPK1Kin did not significantly change AKT1 activity. However, CIPK6Kin significantly activated AKT1 activity compared to CIPK6, although the magnitude of AKT1 activity was lower than CBL1–CIPK6 complex. To assess the contribution of physical interaction between PP2CA and CIPK6 kinase domain in the regulation of AKT1 activation, we co-expressed AKT1 with the truncated form of CIPK6 containing only the kinase domain with or without PP2CA and ABI1 in the oocytes. Figure 3C shows that PP2CA inhibited AKT1 activation induced by CIPK6 kinase domain only, but ABI1 did not, implying that the physical interaction between PP2CA and CIPK6 kinase domain is sufficient for decreasing AKT1 activation by CIPK6.

After finding that PP2CA directly associate with the kinase domain in CIPKs, we examined whether PP2CA deactivate AKT1 activation induced by CIPK6 by dephosphorylating CIPKs. To this end, we changed the glycines at the 159th

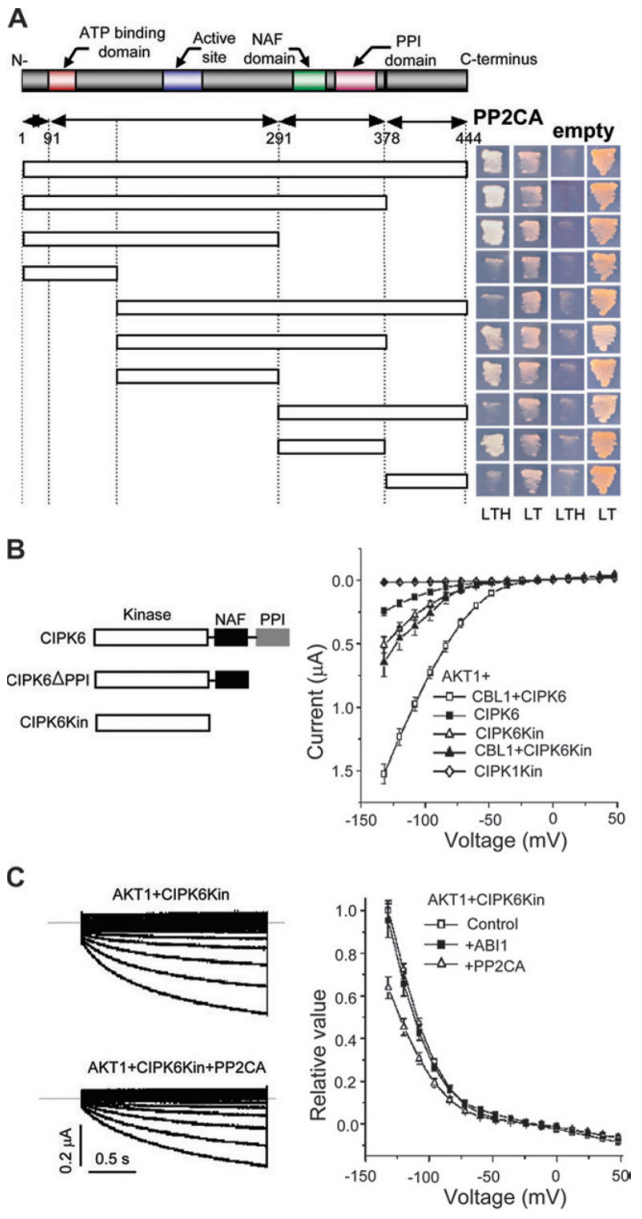

Figure 3. PP2Cs Inhibit AKT1 Activation Induced by CIPK6 Kinase Directly by Interacting with Kinase Domain.

(A) Yeast two-hybrid interaction analysis of PP2CA with various domains of CIPK6. Each of the domains of CIPK6 was cloned into the pGBT9BS and transformed into Yeast PJ69-4A strain with PP2CA cloned into pGADGH. Growth on the selection medium (SC-LTH including 2 mM 3′-AT) was used as an indicator for interaction (left row). Growth in SC-LT was used as control (right row).

(B) The truncated form of CIPK6 with kinase domain only activates AKT1 activity. (Left) Diagram of, from top to bottom, full length of CIPK6 with kinase domain (white bar), NAF domain (black bar), and PPI domain (gray bar), CIPK6 with kinase and NAF domains (CIPK6ΔPPI), and CIPK6 with kinase domain only (CIPK6Kin). (Right) The voltage–current relationship is shown for AKT1+CBL1–CIPK6, AKT1+CIPK6, AKT1+CIPK6Kin, AKT1+CIPK6Kin+CBL1, and AKT1+CIPK1Kin. The summarized values were at 1.8 s of each voltage-clamp episode.

(C) PP2CA inhibited AKT1 activity induced by CIPK6Kin. (Left) Current traces recorded from the oocytes expressing cRNA of AKT1+CIPK6Kin (upper) and AKT1+CIPK6Kin+PP2CA (below). The dotted lines represent zero level. (Right) The voltage–current relationship is shown for AKT1+CIPK6Kin (Control), AKT1+CIPK6Kin+PP2CA, and AKT1+CIPK6Kin+ABI1. Relative value was calculated as summarized currents generated by various combinations/summarized currents at –132 mV generated by AKT1+CIPK6Kin. Summarized current data are from 10 cells per condition in three separate experiments and presented as means ± SE, with $n > 10$ in each case.

and 165th amino acid positions of the catalytic domain of PP2CA into aspartic acids, previously shown to eliminate the phosphatase activity of PP2Cs (Sheen, 1998; Lee et al., 2009). We measured the physical interaction of this mutant ($PP2CA_{G139D/G145D}$) with full-length CIPK6, CIPK6 truncated form without PPI domain (CIPK6Δ PPI), or CIPK6 truncated form with kinase domain only (CIPK6Kin), in comparison with the wild-type PP2CA in the yeast two-hybrid assay. As shown in Figure 4A, $PP2CA_{G139D/G145D}$, as well as the wild-type PP2CA, interacted with the wild-type CIPK6 and other CIPK6 truncated forms. These results indicate that amino acids required for phosphatase activity are not necessary for PP2CA interaction with CIPK6.

To test whether PP2CA phosphatase activity is essential for inhibition of CIPK6 and thus AKT1 activity, we co-expressed $PP2CA_{G139D/G145D}$ in the oocytes with AKT1, CBL1, and CIPK6. Figure 4B shows that $PP2CA_{G139D/G145D}$ still inhibited AKT1 activity induced by CBL1–CIPK6, although the wild-type PP2CA seems to have a higher potency. However, increasing $PP2CA_{G139D/G145D}$ cRNA concentration enhanced its inhibitory potency. When its concentration is three times that of PP2CA, $PP2CA_{G139D/G145D}$ showed a higher inhibitory function than PP2CA on AKT1 activity induced by CBL1–CIPK6, suggesting that the reduction in CIPK6 kinase activity could be caused by the physical interaction between PP2CA and CIPK6 kinase, thereby reducing the AKT1 channel activity. Furthermore, $PP2CA_{G139D/G145D}$ also inhibited AKT1 activation by the CIPK6 truncated form with only kinase domain (Figure 4C), indicating that PP2CA can inhibit CIPK6 kinase activity through a direct interaction with the kinase domain.

The PPI Domain of CIPK6 Contributes to the Inhibition of CIPK6 Kinase Activity by PP2Cs

The assays described above showed PP2CA interacts with CIPK6 through both PPI and the kinase domain, and the latter is sufficient for decreasing CIPK6 activity by PP2Cs. However, it remains unknown about the function of PPI motif in the regulation of the kinase by PP2Cs. In the yeast two-hybrid assay, PP2CA had stronger interaction with CIPK6 than the other two CIPK6 truncated forms containing no PPI domain (CIPK6Δ PPI and CIPK6Kin) (Figure 4A), suggesting that PPI contributes to PP2CA interaction with CIPK6. To evaluate the contribution of the PPI domain, we compared the inhibitory potency of PP2Cs on the full-length CIPK6 and the truncated form of CIPK6 (with PPI deleted, CIPK6ΔPPI) in the oocytes with AKT1. As shown in Figure 5A,

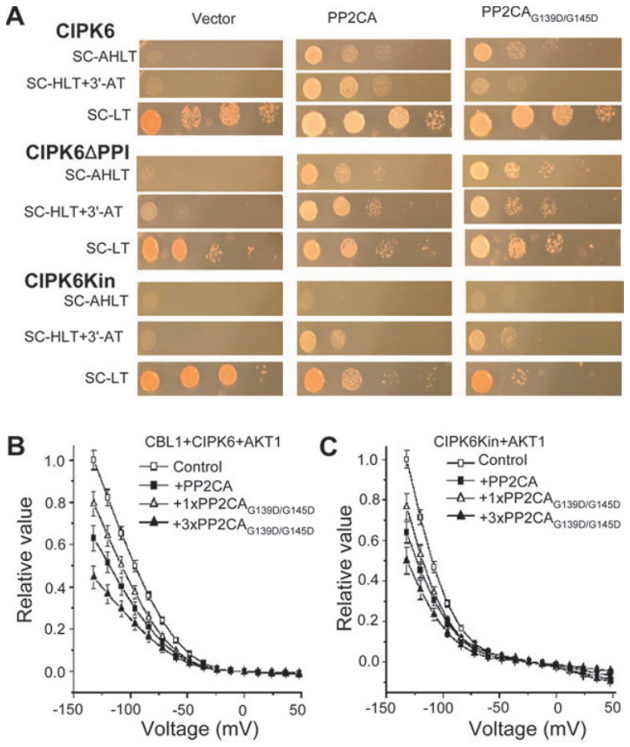

Figure 4. PP2CA Mutant without Phosphatase Activity Inhibits AKT1 Activity Induced by CIPK6.

(A) Yeast two-hybrid interaction analysis of PP2CA$_{G139D/G145D}$ with the full length of CIPK6, CIPK6 truncated form with PPI domain deletion (CIPK6ΔPPI), or CIPK6 truncated form with kinase domain only (CIPK6Kin). For serial dilution assay, exponentially grown yeast cells were harvested and adjusted to OD_{600} = 0.5 with sterilized double-distilled water and diluted 1/10, 1/100, and 1/1000. Yeast cells, 2 μl, were spotted onto SC-Leucin-Tryptophan media and SC-Adenine-Histidine-Leucine-Tryptophan media.

(B) The relative currents generated by AKT1–CIPK6–CBL1 with or without PP2CA, or PP2CA$_{G139D/G145D}$. PP2CA$_{G139D/G145D}$ cRNA was injected one or three times into PP2CA. Relative value was calculated as summarized currents generated by various combinations/summarized currents at –132 mV generated by AKT1+CIPK6+CBL1.

(C) The relative currents generated by AKT1–CIPK6Kin with or without PP2CA or PP2CA$_{G139D/G145D}$. Relative value was calculated as summarized currents generated by various combinations/summarized currents at –132 mV generated by AKT1+CIPK6Kin. Summarized currents are from >10 cells per condition in three separate experiments.

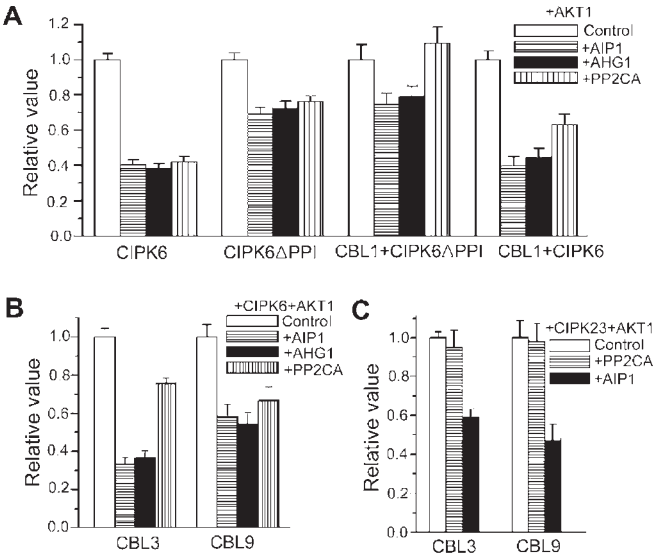

Figure 5. The PPI Domain of CIPK6 Contributes for PP2CA to Inhibit AKT1 Activation Induced by CIPK6.

(A) PP2CA regulates AKT1 activation by CIPK6, CIPK6 mutant without PPI domain (CIPK6ΔPPI), CIPK6+CBL1, or CIPK6ΔPPI+CBL1. The relative value in the group of CIPK6–AKT1, CIPK6ΔPPI–AKT1, CIPK6ΔPPI–AKT1, and CBL1–CIPK6–AKT1 was calculated as summarized currents at –132 mV generated by various combinations/currents at –132 mV generated by CIPK6–AKT1, CIPK6ΔPPI–AKT1, CIPK6ΔPPI–AKT1, and CBL1–CIPK6–AKT1, respectively.

(B) PP2CA regulates AKT1 activation by CIPK6 with CBL3 or CBL9. The relative value in the group of CBL3–CIPK6–AKT1 and CBL9–CIPK6–AKT1 was calculated as summarized currents at –132 mV generated by various combinations/summarized currents at –132 mV generated by CBL3–CIPK6–AKT1 and CBL9–CIPK6–AKT1, respectively.

(C) PP2Cs regulates AKT1 activation by CIPK23 with CBL3 or CBL9. The relative value in the group of CBL3–CIPK23–AKT1 and CBL9–CIPK23–AKT1 was calculated as summarized currents at –132 mV generated by various combinations/currents at –132 mV generated by CBL3–CIPK23–AKT1 and CBL9–CIPK23–AKT1, respectively. Summarized data are from at least seven cells per condition in three separate experiments.

AIP1 and AHG1 suppressed CIPK6- or CBL1–CIPK6-dependent AKT1 activity by 60%. However, these two PP2Cs inhibited AKT1 activity by 30% (Figure 5A) when AKT1 activity was induced by CIPK6ΔPPI or by CBL1–CIPK6Δ PPI. These results indicated that the interaction with PPI domain contributes to but is not required for the PP2Cs to inhibit the kinase activity.

In the analysis of PP2CA inhibition of AKT1 activity in the oocytes, we had an intriguing observation. Although PP2CA, AHG1, and AIP1 inhibited AKT1 activity in a similar manner when AKT1 was induced by CIPK6 or by CIPK6ΔPPI (Figure 5A), PP2CA did not inhibit AKT1 activation by CIPK6ΔPPI plus CBL1, while AHG1 and AIP1 still inhibited this activation by 30%. Furthermore, PP2CA had a less inhibitory potency than AHG1 and AIP1 on AKT1 activity induced by full-length CIPK6–CBL1. The results appear to suggest that the less inhibitory effect of PP2CA results from the presence of CBL1. In our previous study, we showed CIPK6 interacted with CBL2, CBL3, and CBL9, besides CBL1 (Lee et al., 2007). To test the possible role of CBLs in the CBL–CIPK–PP2C network, we co-expressed two other CBLs (CBL3 and CBL9), CIPK6, and AKT1 in the oocytes with various PP2Cs. As shown in Figure 5B, PP2CA, AHG1, and AIP1 had a similar inhibition of AKT1 activity induced by CIPK6–CBL9. However, PP2CA showed a less inhibitory potency than AHG1 and AIP1 on AKT1 activity induced by CIPK6 and CBL3. PP2CA did not inhibit AKT1 activation by CIPK23 and CBL3 or CBL9, while AIP1 inhibited AKT1 activation by CIPK23 and CBL3 or

CBL9 (Figure 5C). This provided further evidence that the special inhibitory function of PP2CA on AKT1 activation by CIPK6–CBLs may depend on CBL species.

CBLs Directly Interact with PP2CA and Inhibit Its Phosphatase Activity

To examine the mechanism of PP2CA regulation by CBLs, we used yeast two-hybrid assay to probe the possible interaction between A-type PP2Cs and CBLs. As shown in (Figure 6A), PP2CA interacted with CBL1, CBL2, CBL3, CBL5, and CBL7, but not CBL4, CBL6, and CBL9. Furthermore, AHG1 interact with two CBLs: CBL5 and CBL7 (Figure 6A). Other PP2Cs tested did not interact with any CBL family member, indicating that interactions between PP2Cs and CBLs are specific. These results show that CBLs directly interact with PP2Cs, in addition to interacting with CIPKs through NAF previously reported (Albrecht et al., 2001; Guo et al., 2001), and suggest that CBLs may regulate phosphorylation/dephosphorylation controlled by CIPKs and PP2Cs through more than one mechanism.

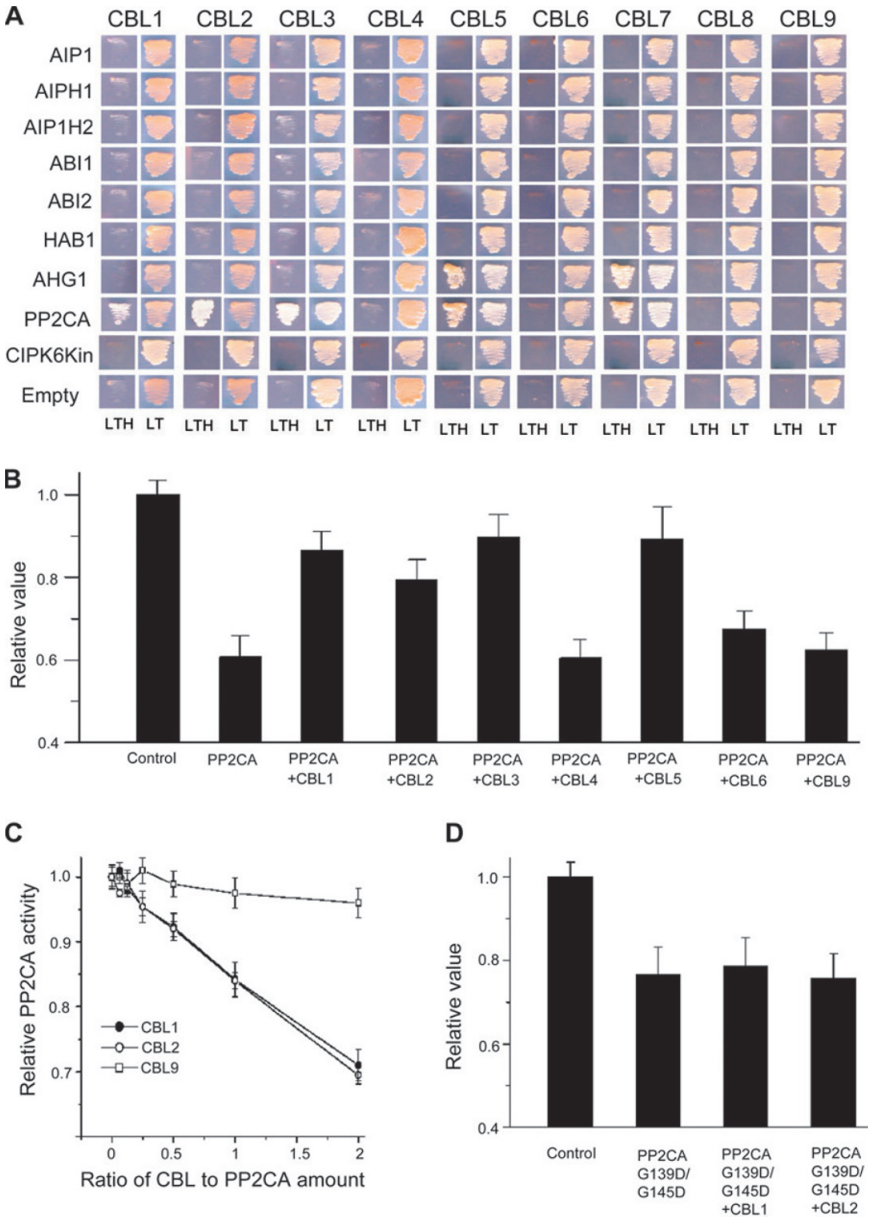

Figure 6. Specific CBLs Recovered AKT1 Activation Induced by CIPK6 Kinase Inhibited by PP2CA.

(A) Yeast two-hybrid assay of interactions between CBLs, PP2CA, and CIPK6-Kin.

(B) CBLs regulate CIPK6Kinase-activating AKT1 activity inhibited by PP2CA. Currents were recorded from the oocytes expressing AKT1+CIPK6Kin (control), AKT1+CIPK6Kin+PP2CA (PP2CA), AKT1+CIPK6Kin+PP2CA+CBL1 (PP2CA+CBL1), AKT1+CIPK6Kin+PP2CA+CBL2 (PP2CA+CBL2), AKT1+CIPK6Kin+PP2CA+CBL3 (PP2CA+CBL3), AKT1+CIPK6Kin+PP2CA+CBL4 (PP2CA+CBL4), AKT1+CIPK6Kin+PP2CA+CBL5 (PP2CA+CBL5), AKT1+CIPK6Kin+PP2CA+CBL6 (PP2CA+CBL6), or AKT1+CIPK6Kin+PP2CA+CBL9 (PP2CA+CBL9). Relative value was calculated as summarized currents at –132 mV generated by various combinations/summarized currents at –132 mV generated by AKT1+CIPK6Kin.

(C) *In vitro* PP2CA phosphatase activity. CBL1 and CBL2 inhibited PP2CA phosphatase activity in a dose-dependent manner, but CBL9 did not.

(D) CBL1 and CBL2 did not change CIPK6-Kin-activating AKT1 activity inhibited by $PP2CA_{G139D/G145D}$. Currents were recorded from the oocytes expressing AKT1+CIPK6Kin (control), AKT1+CIPK6Kin+$PP2CA_{G139D/G145D}$ ($PP2CA_{G139D/G145D}$), AKT1+CIPK6Kin+$PP2CA_{G139D/G145D}$+CBL1 ($PP2CA_{G139D/G145D}$+CBL1), or AKT1+CIPK6Kin+$PP2CA_{G139D/G145D}$+CBL1 ($PP2CA_{G139D/G145D}$+CBL2). Relative value was calculated as summarized currents at –132 mV generated by various combinations/summarized currents at –132 mV generated by AKT1+CIPK6Kin. Summarized data are from at least seven cells per condition in three separate experiments.

Because CBL10 is found to interact with CIPK24/SOS2 through kinase domain (Lin et al., 2009), we tested whether CBLs may interact with CIPK6 kinase domain in yeast two-hybrid assay, and found no interaction (Figure 6A), and CBL1 did not change AKT1 activation induced by CIPK6Kin (Figure 3B). Based on this observation, we co-expressed various CBLs, PP2CA, and AKT1 in the oocytes with CIPK6 truncated form (kinase domain only, CIPK6Kin), to analyze the possible relationship of CBL and PP2Cs. As shown in Figure 6B, CBL4, CBL6, and CBL9, the CBLs that do not interact with PP2CA, had little effect on PP2CA inhibition of AKT1 (activated by CIPK6Kin). However, CBL1, CBL2, CBL3, and CBL5 all recovered PP2CA-inhibited AKT1 activity to a certain degree (Figure 6B).

To distinguish these possibilities, we performed an *in intro* phosphatase assay, and tested the possible effect of CBL1, CBL2, and CBL9 on the phosphatase activity of PP2CA. As shown in Figure 6C, both CBL1 and CBL2 reduced PP2CA activity in a dose-dependent manner, but CBL9 did not. To confirm further that CBLs binding directly deactivates PP2CA phosphate activity, we co-expressed these two CBLs (CBL1 and CBL2) with $PP2CA_{G139D/G145D}$, CIPK6Kin, and AKT1 in the oocytes and found that CBLs did not change the magnitude of CIPK6Kin-activated AKT1 activity inhibited by $PP2CA_{G139D/G145D}$ (Figure 6D). This result indicates that these CBLs could directly interact with and act as a negative regulator of the PP2Cs in signaling pathways.

DISCUSSION

Calcium sensors CBLs interact with their target kinases (CIPKs) to form a complex signaling network regulating many physiological processes in plants (Luan, 2009; Luan et al., 2009). Several CBL–CIPK complexes have been shown to regulate membrane transport in plant cells under salt stress and in low-K response (reviewed in Luan et al., 2009). In these processes, the CBL–CIPK complexes are targeted to the cell membranes where the CIPK kinases phosphorylate the substrate protein and activate the transport activity (Luan et al., 2009). In the negative regulation of this pathway, PP2C-type phosphatases inactivate the membrane transport processes. In the case of AKT1 activation by the CBL–CIPK pairs, AIP1 was identified as a negative regulator (Lee et al., 2007). The present study identified multiple A-type PP2C members as negative regulators in the CBL–CIPK pathway leading to activation of AKT1 channel. The phosphatase could inactivate the AKT1 channel by dephosphorylating the channel protein, or by physical interaction with and inhibition of CIPK kinases as recently described for SnRK2 and PP2C function in the ABA signaling pathway (Geiger et al., 2009; Lee et al., 2009; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). Our results here suggest that PP2CA physically interact with and inactivate CIPK6 kinase activity required for activation of AKT1. Interestingly, CBLs physically interact with and inhibit PP2CA, indicating that CBLs activate CIPK6 kinase activity by at least two mechanisms, one by interacting with the CIPK6 directly and another by inhibiting PP2CA phosphatase activity.

Several CIPKs, including CIPK23, CIPK16, and CIPK6, interact with and are activated by multiple CBLs in the activation of AKT1 (Li et al., 2006; Xu et al., 2006; Lee et al., 2007). Specific interaction of AIP1, a PP2C-type phosphatase, with CIPK23 reversed the AKT1 activation by CIPK23, suggesting that AIP1 serves as a negative regulator in the CIPK23 activation of AKT1 (Lee et al., 2007). The present study identified multiple CIPKs and multiple A-type PP2Cs interacting with one another to form an interaction network that regulates AKT1. For example, CIPK23 interacts with AIP1 and another PP2C referred to as AIP1H (At2g29380), CIPK16 interacts also with AIP1 and AIP1H, CIPK6 interacted with four PP2Cs, AIP1, AIP1H (At2g29380), AHG1, and PP2CA. These four PP2Cs are more similar to each other than to others, such as ABI1, ABI2, HAB1, and HAB2 that interact with both CIPKs (Ohta et al., 2003) and SnRK2 family members involved in response to abscisic acid (ABA) (Fujii et al., 2009; Lee et al., 2009; Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). These results suggest that the specific interactions between PP2Cs and CIPKs form phosphorylation/dephosphorylation pairs to control distinct processes including but not limited to AKT1 channel activity and ABA response.

A motif with the conserved amino acid residues in the CIPK regulatory domain is identified to serve as a docking site for the interaction with PP2Cs, and therefore is designated as the PPI (protein phosphatase-interacting) motif (Ohta et al., 2003). The sequence variations of this motif may determine the interaction specificity of CIPKs with PP2Cs (Ohta et al., 2003; Sanchez-Barrena et al., 2007). In the present study, we also show that PP2CA physically interacts with the PPI motif in CIPK6 (Figure 3). Unexpectedly, we also found that PP2CA physically interacts with the truncated forms of CIPK6 without the PPI motif in the yeast two-hybrid assay (Figure 3A) and inhibits CIPK kinase domain-mediated AKT1 activation (Figure 3B). We conclude, therefore, that CIPK6 has two PP2CA docking sites: the PPI motif and the kinase domain. In our previous study on the interaction between PP2Cs and SnRK2 family, we also found that PP2Cs directly interact with the catalytic domain of these kinase proteins (Lee et al., 2009). As the PPI motif is not a common feature of SnRK protein kinases in *Arabidopsis* (Hrabak et al., 2003), we speculate that the direct physical interaction with the catalytic domain would be a more ubiquitous mechanism for protein phosphatases to regulate SnRK protein kinases.

To keep the phosphorylation activity of CIPKs in the basal state, the kinase domain physically interacts with the autoinhibitory domain (Guo et al., 2001). However, full-length CIPK23, CIPK16, or CIPK6 alone still activates AKT1 channel in the oocyte expression system (Figure 4) (Li et al., 2006; Lee et al., 2007; Fujii et al., 2009), indicating that the regulatory domain can not completely eliminate kinase activity of the catalytic domain. The present results indicate that PP2CA interact with and inhibit CIPK activity and that PP2Cs act as an

additional exogenous suppressor to inhibit CIPK6. The PP2CA mutant without phosphatase activity still interacts with CIPK6 and inhibits AKT1 activation by CIPK6–CBLs or by CIPK6Kin (Figure 6), but the inhibition was less strong compared to that caused by wild-type PP2CA. We suggest, thus, that PP2CA regulates CIPK6 kinase activity through the dual mechanisms: physical interaction and dephosphorylation. The dual regulatory mechanisms are also found in the interaction of PP2Cs with SnRK2 family members (Fujii et al., 2009; Lee et al., 2009; Umezawa et al., 2009). Therefore, although these mechanisms need to be further elucidated in detail, they seem to be common in the interactions of PP2Cs with SnRK protein kinases including those in the SnRK2 and SnRK3 subfamilies.

In addressing PP2CA inhibition of CIPK6 activity, we uncovered a previously unsuspected interaction: CBLs directly interact with PP2Cs and inhibit their phosphatase activity. By doing so, the CBLs relieve inhibition of CIPKs by PP2Cs. Several of our observations support this idea. First, interaction between ABLs and PP2Cs was specific. Only specific CBLs, such as CBL1, CBL2, CBL3, and CBL5, physically interact with PP2CA in the yeast two-hybrid assay (Figure 6A). Second, CBL1 and CBL2 that interacted with PP2CA reduced PP2CA phosphatase activity when proteins were incubated together (Figure 6C). And, third, the inhibition of PP2CA on CIPK6Kin-dependent AKT1 activation was reduced by the CBLs interacting with PP2CA (Figure 6B). These results suggest that, in the interaction between PP2Cs and CIPKs, CBLs protect CIPK kinase activity by directly inhibiting PP2Cs phosphatase activity, in addition to interacting with the regulatory domain to free kinase domain from auto-inhibition.

In summary, the data presented here enable us to develop the model of AKT1 regulation by the interaction of CIPKs and PP2Cs previously proposed (Li et al., 2006; Lee et al., 2007). The NAF/FISL motif locks the catalytic domain in the absence of CBLs (Figure 7A), and specific CBLs bind to NAF/FISL domain and liberate the catalytic domain to phosphorylate AKT1 (Figure 7B). PP2Cs interact with the PPI motif and the kinase domain to inhibit AKT1 activation by CIPK–CBL complex through physical binding and dephosphorylation (Figure 7C). Specific CBLs interact with and reduce PP2Cs phosphatase activity to recover the AKT1 activation by CIPKs (Figure 7D).

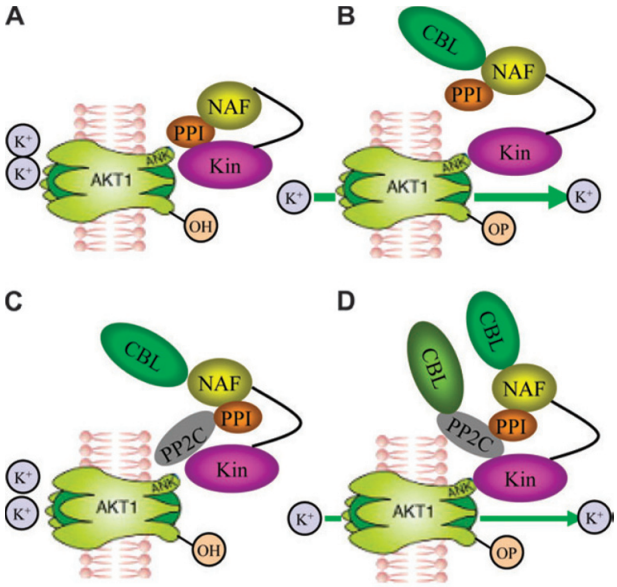

Figure 7. Proposed Model for the Negative Regulation of PP2Cs on AKT1 Activation by CIPK–CBL Complex.

(A) In the absence of CBLs, the kinase domain is enclosed by the NAF/FISL motif and can not interact with the AKT1 ankyrin domain to activate AKT1.

(B) CBLs interact with the NAF/FISL motif to liberate the catalytic domain to phosphorylate AKT1.

(C) PP2Cs interact with the PPI motif and the kinase domain to inhibit AKT1 activation by CIPK–CBLs complex, in which CBLs do not interact with PP2Cs, through physical binding and dephosphorylation.

(D) Specific CBLs interact with PP2Cs and reduce phosphatase activity to recover CIPKs kinase activity.

METHODS

Yeast Two-Hybrid Assay

Each construct was built by cDNA fragments amplified by PCR and cloned into the pGBT9BS and pGADGH vectors. The lithium acetate method was used to introduce BD and AD plasmids into yeast strain PJ69-4A and AH109 (Ito et al., 1983). Yeast two-hybrid assays were performed as before (Li et al., 2006; Lee et al., 2007). Transformants were selected in SC-Leucin-Tryptophan media and transferred on the interaction selection media (SC-Histidine-Leucine-Tryptophan and SC-Adenine-Histidine-Leucine-Tryptophan for PJ69-4A and AH109, respectively) to score growth as an indicator of protein–protein interaction. Numbers show the designed nucleotide number of the primer for making truncated CIPK6 form, while Fr presents the forward primer and Re presents the reverse primer:

CIPK6-1Fr: GAACTAGTGATGGTCGGAGCAAAACCGGTG,
CIPK6-91Fr: GAACTAGTGATGGCGAGTAAATCCAAGATCTAT,
CIPK6-91Re: GAGTCGACGACTTCGTGAAGCTCGACGATGT,
CIPK6-291Fr: GAACTAGTGGCTGCCACAATCACAACCACGG,
CIPK6-291Re: GAGTCGACCACTGGTTCGTTTCTTGATCTCGT,
CIPK6-378Fr: GAACTAGTGGTAAGGATTGAAGGTAAACAGAATG,
CIPK6-378Re: GAGTCGACTCTACTCTCACTCTTCCTCACATCAAA,
CIPK6-444Re: GAGTCGACTCAAGCAGGTGTAGAGGTCCAGAA.

Protein Phosphatase Assay

To produce GST fusion proteins in *E. coli*, CBL1, CBL2, and PP2CA were cloned into pGEX4T-1 vector. All GST fusion constructs were transformed into *E. coli* strain BL21(DE3) cells. Protein expression and purification of GST fusion protein were performed as described earlier (Li et al., 2006). Protein phosphatase assays of PP2CA with CBLs were performed by using

the ProFluor Ser/Thr PPase assay kit (Promega, WI, USA) according to the manufacturer's protocol.

Electrophysiological Procedures

The concentration of the capped cRNA prepared by the mMESSAGE mMACHINE T_7 RNA transcription Kit was determined at A260/A280. The total volume of cRNA injected into freshly isolated *Xenopus* oocytes was 23 nl. The final concentration of CBL, CIPK, and AKT1 was 125 mg L^{-1}, while PP2C was 250 mg L^{-1}. The oocytes, 40–50 h after injection, were used for two-electrode voltage clamp experiments. Electrophysiological recording procedures were carried out as previously described (Lee et al., 2007). Histograms were generated from the pooled currents, recording 1.8 s of each voltage-clamp episode. Data are presented as representative recordings or as mean ± SE of *n* observations with three repetitions, in which *n* is the number of samples. Statistical comparisons were made using either Student's paired or unpaired *t*-tests as appropriate, and differences were considered to be significant at $p < 0.05$.

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