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Original Paper

Isolation and characterization of a cDNA coding cowpea (*Vigna unguiculata* (L.) Walp.) calcineurin B-like protein-interacting protein kinase, *VuCIPK1*

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Abstract We isolated a calcineurin B-like protein (CBL)-interacting protein kinase (EC 2.7.11.1) homolog (*VuCIPK1*) from cowpea (*Vigna unguiculata* (L.) Walp.) with significant similarity to *AtCIPK3* (86% amino acid identity), which is involved in K^+ transport. *VuCIPK1* mRNA was detected in the whole plant by RT-PCR. Any significant change in *VuCIPK1* mRNA levels was not detected when cowpea was subjected to various environmental stresses. Immunoblot assay with an anti-VuCIPK1 specific antibody and an anti-CBL antibody showed that immunologically CIPK- and CBL-related polypeptides were preferentially associated with membrane fractions of cowpea. The anti-VuCIPK1 antibody cross-reacted to two different CIPK-related polypeptides with relative molecular masses of 51 kDa in root and 55 kDa in mature leaf. Immunoprecipitation assay by anti-VuCIPK1 antibody using cowpea leaf extracts showed that endogenous VuCIPK1 was autophosphorylated on the threonine residues in response to salt stress. The recombinant VuCIPK1 phosphorylated casein in the presence of Mn^{2+}. These observations suggest that membrane-associated VuCIPK1 is involved osmotic stress-activated kinase regulated by its phosphorylation status.

Key words: Calcium, CBL, CIPK, protein phosphorylation, salt stress, SnRK.

Plants use complex mechanisms such as hormonal signaling, regulation of ion transport, and synthesis of osmoprotectants to survive under various environmental stresses (Shinozaki and Yamaguchi-Shinozaki 2000). To breed stress-tolerant crops, it is important to clarify stress signaling mechanisms. It is well known that signal cascades composed of protein kinases and protein phosphatases have essential roles in stress tolerance and adaptation of eukaryotes (Brewster et al. 1993; MAPK Group 1999). In *in-gel* kinase assays, several protein kinases with relative molecular masses of 35 to 50 kDa appeared to be stimulated in response to salt stress, drought, or abscisic acid (ABA) in higher plants (Li and Assmann 1996; Mori and Muto 1997; Hoyos and Zhang 2000) and green algae (Yuasa and Muto 1996). Recent studies of osmotic stress/ABA-activated protein kinases in plants have revealed that SNF1-related protein kinases (SnRKs) play vital roles in osmotic stress signaling in higher plants (Boudsocq and Laurière 2005). *Vicia faba* SnRK2 ABA-activated and Ca^{2+}-independent protein kinase (AAPK) in guard cells (Li et al. 2000) and *Arabidopsis thaliana* SRK2E/SnRK2.6 (Yoshida et al. 2002) appear to be activated by ABA, while *A. thaliana* root tip specific SnRK2, SRK2C/SnRK2.8 (Umezawa et al. 2004) and a tomato fruit specific SnRK2 homolog, SlSnRK2C (Yuasa et al. 2007), are activated by NaCl.

An increase in the cytosolic calcium concentration is a primary response to various environmental stimuli, such as salinity, osmotic stress, low temperature, and ABA (Knight and Knight 2001). In plant cells, calcium signals

Abbreviations: AAPK, ABA-activated and Ca^{2+}-independent protein kinase; ABA, abscisic acid; BSA, bovine serum albumin; CBL, calcineurin B-like protein; CIPK, CBL-interacting protein kinase; EST, expressed sequence tag; GST, glutathione S-transferase; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; MBP, maltose-binding protein; ORF, open reading frame; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; 5′-RACE, rapid amplification of 5′ cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecylsulfate; SnRK&SRK, SNF1-related kinase; TBS, Tris-buffered saline

The nucleotide sequence reported in this paper has been submitted to DDBJ under accession number AB303675 (*VuCIPK1*)

This article can be found at http://www.jspcmb.jp/

appear to have pivotal roles in the regulation of enzymatic activity and gene expression with the actions of many calcium-binding proteins such as calmodulins and calcium-dependent protein kinases. Recent studies have demonstrated that the novel calcium sensor "calcineurin B-like protein" (CBL) has EF-hand motifs, involved in calcium binding, and associates with novel serine/threonine kinases "CBL-interacting protein kinases" (CIPKs) (Halfter et al. 2000; Albrecht et al. 2001). Genetic studies of salt overly sensitive (*sos*) mutants (*sos1*, *sos2*, *sos3*) of *A. thaliana* have revealed that novel families of calcium sensors (SOS3/CBL4) and calcium-regulated protein kinases (SOS2/AtCIPK24) are involved in the acquisition of tolerance under salt stress and the activation of sodium transport (SOS1, Na^+/H^+ exchanger) (Zhu 2000; Qiu et al. 2002). CIPK contains a SNF1-related kinase (SnRK) catalytic domain at the N-terminus and a highly conserved CBL-interacting module with a NAF/FISL domain in the COOH regulatory domain (Albrecht et al. 2001; Guo et al. 2001). On the basis of these structural similarities, the CIPKs have been classified in the SnRK3 subgroup of plant SNF1-related kinases (Hrabak et al. 2003). Biochemical and genetic studies using *Arabidopsis* mutants have established that CBL–CIPK complex system is involved in stress signaling, ion homeostasis, and sodium excretion under salinity and drought stress (Albrecht et al. 2003; Xu et al. 2006).

Cowpea (*Vigna unguiculata* (L.) Walp.) is an extremely drought-tolerant legume, which is one of major crops intercropped with millets in the semi-arid Sahel region of Africa (Zegada-Lizarazu et al. 2006). Under drought stress, stress-inducible genes, such as ABA synthesis enzymes or late embryonic abundant proteins, are rapidly induced in roots and leaves of cowpea to protect the cells from severe dehydration (Iuchi et al. 1996). To understand the molecular mechanisms of intracellular signal cascades involved in the acquisition of drought tolerance in cowpea, we focused on a cowpea CIPK homolog that is a potential candidate for breeding to improve stress tolerance of crops in water-deficient fields.

We isolated a full-length cDNA of the CIPK homolog *VuCIPK1* from *V. unguiculata*. We analyzed its expression levels in cowpea, substrate specificity of recombinant VuCIPK1, and autophosphorylation of endogenous VuCIPK1-related proteins in response to NaCl stress.

Materials and methods

Plant materials and stress treatments

Cowpea (*Vigna unguiculata* (L.) Walp.) seeds were incubated at 25°C under continuous light (80 W m^{-2}) in 9-cm-diameter pots containing expanded vermiculite and fertilized with 1/1000-diluted Hyponex (Hyponex Japan, Osaka, Japan). Two-week-old plants were subjected to three kinds of stresses: drought stress (stopping watering for 24 h and then re-watering) salt stress (application of 200 mM NaCl to pots for 24 h and spraying of the same solution on the leaves) or cold stress (placing in refrigerator at 4°C for 24 h). After treatment, plant tissues were frozen in liquid N_2.

Isolation of a full-length cDNA encoding cowpea CIPK

Two expressed sequence tags (ESTs; RD068337 and RD068338) were identified as partial cDNA sequences of a CIPK homolog in *V. unguiculata* by BLAST search of the NCBI/BLAST database. Total RNA of 2-week-old cowpea was isolated by the SDS/phenol/LiCl method. Single-stranded cDNA was synthesized from RNA by oligo dT primer (5′-GT-GCTCGAGTGCGGCCGCAAGCTTTTTTTTTTTTTTTT-3′) and M-MLV reverse transcriptase (TaKaRa, Tokyo, Japan). The partial cDNA fragment coding ESTs (RD068337 and RD068338) of the CIPK homolog was amplified by PCR with ExTaq DNA polymerase (TaKaRa) and a set of primers (VuCIPK partial-5, 5′-CTTTGACAAAATTGTAAACCATGG-ACG-3′; VuCIPK-3-SmaI-SpeI, 5′-ACTAGTCCCGGGTCA-ATTTGTTTCTCTCATTTGCAT-3′) specific to the ESTs. pCR-partial VuCIPK was constructed with the amplified fragments of the CIPK homolog in a pCR TOPO TA cloning kit (Invitrogen). To clone the full-length cDNA, we amplified the 5′-extension sequence of the partial CIPK with 5′-RACE assay using a set of nested primers (VuCIPK-5-nest1 primer, 5′-ACGTGCTTCATTTTCACTCATACGTCC-3′; VuCIPK-5-nest2 primer 5′-AAGCTGTTGGAAATATCTACGTGCTTC-3′; VuCIPK-3-nest1 primer, 5′-GCTGTTGATTATTGCCA-CAGCAGG-3′; VuCIPK-3-nest2 primer, 5′-AGCAGGGGT-GTCTACCACAGAGATCT-3′) and a VuCIPK-phosphorylated primer, 5′-pTCCAAAGTCGGAAAC-3′. DNA sequences of the 5′-RACE fragments were analyzed. VuCIPKFL-EcoRIXbaI-5 primer (5′-GTTGAATTCTCTAGAATGAGTC-AGCCTAAGATCAAGCGC-3′) was designed according to a 5′-end sequence of the *VuCIPK* open reading frame (ORF) confirmed by 5′-RACE. A DNA fragment coding ORF of the full-length *VuCIPK* cDNA was amplified by PCR with a set of primers (VuCIPKFL-EcoRIXbaI-5 primer and VuCIPK-3-SmaI-SpeI, 5′-ACTAGTCCCGGGTCAATTTGTTTCTCTCA-TTTGCAT-3′). cDNA fragments in pCR TOPO plasmids were sequenced by an ABI Prism 310 DNA sequencer with a Big Dye Terminator Cycle Sequencing Kit ver. 1.1 (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The deduced amino acid sequence of *VuCIPK1* (accession no. AB303675, this study) was aligned manually with the sequences of a soybean CIPK homolog (*GmCIPK*; TC207101 in the DFCI *Glycine max* Gene Index, http://compbio.dfci.harvard.edu/tgi/), *AtCIPK3* (At2g26980) of *Arabidopsis thaliana*, and *OsCIPK3* (accession no. AP003818) of *Oryza sativa*. The sequences used for phylogenic analysis were as follows: *AtCIPK1* (At3g17510), *AtCIPK2* (At5g07070), *AtCIPK4* (At4g14580), *AtCIPK5* (At5g10930), *AtCIPK6* (At4g30960), *AtCIPK7* (At3g23000), *AtCIPK8* (At4g24400), *AtCIPK9* (At1g01140), *AtCIPK10* (At5g58380), *AtCIPK11* (At2g30360), *AtCIPK12* (At4g18700), *AtCIPK13*

(At2g34180), *AtCIPK14* (At5g01820), *AtCIPK15* (At5g01810), *AtCIPK16* (At2g25090), *AtCIPK17* (At1g48260), *AtCIPK18* (At1g29230), *AtCIPK19* (At5g45810), *AtCIPK20* (At5g45820), *AtCIPK21* (At5g57630), *AtCIPK22* (At2g38490), *AtCIPK23* (At1g30270), *AtCIPK24*/*SOS2* (At5g35410), *AtCIPK25* (At5g25110), *AtSnRK2C* (At1g78290), and *Akin10* (At3g01090) of *A. thaliana*; a CIPK homolog (accession no. AJ717348) of *Solanum lycopersicum*, *PsCIPK* (accession no. AY191840) of *Pisum sativum*, and *SNF1* (accession no. M13971) of *Saccharomyces cerevisiae*. The systematic designation of *A. thaliana* genes is given by the Arabidopsis Genome Initiative (http://mips.gsf.de/proj/thal/db/index.html). The amino acid sequences were aligned by using the CLUSTALW program (http://align.genome.jp/), and the corresponding tree was built.

RT-PCR analysis

RT-PCR was performed with total RNA prepared from the leaves and other organs of cowpea by M-MLV reverse transcriptase and ExTaq DNA polymerase, according to the manufacturer's manuals (TaKaRa), with gene-specific primers designed to target the 3′ region of the *VuCIPK1* cDNA and *Vigna* actin (accession no. AF143208). The sequences used for RT-PCR were follows: VuCIPK1 forward (VuCIPK-3-nest2 primer) and reverse (VuCIPK1-RT-PCR-3′, 5′-ATCGAGCTCAATTTGTTTCTCTCATTTGCA-3′). cDNA was synthesized in 20 μl of reaction mixture containing 1 μg of total RNA, 2.5 μM oligo-dT15-18-mer, 500 μM each dNTP, and 5 units of M-MLV-reverse transcriptase. Each 20 μl of PCR reaction mixture contained 200 μM each dNTP, 400 nM each 5′- and 3′-primers, 1 unit of ExTaq DNA polymerase, and 1 μl of cDNA solution. The thermal cycle reaction was performed as follows: 94°C for 2 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; 72°C for 5 min; and 4°C for 99 min. The amplified PCR products were analyzed by electrophoresis in 1.5% agarose gels with ethidium bromide staining solution. The identity of these PCR products was confirmed by DNA sequencing.

Construction of plasmids

To produce recombinant VuCIPK1 fused to glutathione S-transferase (GST) in *Escherichia coli*, we constructed pGEX-VuCIPK1. An *Eco*RI–*Xho*I fragment of full-length *VuCIPK1* cDNA in pCR-full-length VuCIPK1 was inserted into *Eco*RI–*Sal*I sites of pGEX (**GE** Healthcare Bio-Sciences, Piscataway, NJ, USA).

To raise a specific antibody against VuCIPK1 in a rabbit, pCR-partial VuCIPK1 was digested with *BamH*I and *Xho*I, and then the resultant DNA fragment was inserted into *BamH*I–*Sal*I sites of pQE9 (Qiagen Inc., Valencia, CA, USA) to construct pQE-partial VuCIPK1 for production of His×6-tagged partial VuCIPK1 polypeptide (99–441 aa).

Expression and purification of GST-fused proteins

GST-VuCIPK1 was expressed in *E. coli* BL21DE3 (Stratagene, La Jolla, CA, USA) containing pGEX-VuCIPK1 by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM when the OD_{600} of *E. coli* culture reached 0.5. After harvesting of *E. coli* cells (250 ml 2×YT medium) by centrifugation at 10,000×*g* for 15 min, the *E. coli* pellet was resuspended in lysis buffer, containing 25 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and 0.1% β-mercaptoethanol, and then the cells were disrupted by sonication. GST-VuCIPK1 was purified from extracts of *E. coli* according to the manufacturer's (GE Healthcare Bioscience) instruction manual. After centrifugation of the mixture at 10,000×*g* for 15 min at 4°C, the resultant supernatant was applied to a column (1 cm×2 cm) of glutathione sepharose CL4B beads, and the column was washed first with 20 ml of washing buffer containing 25 mM Tris-Cl [pH 7.4], 1 M NaCl, 0.5 mM EDTA, 0.1% Triton X-100, and 0.1% β-mercaptoethanol, and then with maltose elution buffer (2 ml), which is washing buffer supplemented with 10 mM glutathione. The elutant was dialyzed overnight at 4°C in 1×TBS (25 mM Tri-HCl [pH 7.4], 150 mM NaCl) containing 0.5 mM EDTA, 0.1% Triton X-100, 0.1% β-mercaptoethanol, and 30% glycerol and then stored at −40°C.

Raising specific antiserum

His×6-tagged partial VuCIPK1 (aa 99–441) (His×6-partial VuCIPK1) polypeptide was produced in BL21DE3 *E. coli* cells. The His×6-partial VuCIPK1 polypeptide was purified from extracts of *E. coli* cells containing pQE-VuCIPK1 with a Ni-NTA His-Bind Resin (Qiagen) column according to the manufacturer's instruction manual. Antiserum was raised against His×6-VuCIPK1 (0.5 mg protein) in a rabbit. We confirmed by immunoblot analysis that the rabbit anti-VuCIPK1 serum cross-reacted with endogenous polypeptides with relative molecular masses of 51 and 55 kDa in cowpea (as shown in Figure 3A).

An anti-tomato CBL serum was raised against His×6-tagged tomato CBL recombinant protein expressed by pQE9-tomato homolog of *CBL4/SOS3* according to a nucleotide sequence data of contig18692 in tomato EST database, MiBASE (http://www.kazusa.or.jp/jsol/microtom/indexj.html) (Takahashi T, Yuasa T, Iwaya-Inoue M, et al., in preparation).

In vitro protein kinase assay

The reaction mixture contained 25 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$ (or $MnCl_2$), 2 mM EGTA, 0.1% Triton X-100, 10 mM Na_2-β-glycerophosphate, 2 mM Na_3-orthovanadate, 0.1% β-mercaptoethanol, 1 μg of the purified GST-VuCIPK1 and 1 mM ATP in a total volume of 50 μl with myelin basic protein, histone, or casein (Sigma) at 0.2 mg ml^{-1} final concentration as substrate. The reactions were performed at 30°C for 180 min and were then terminated by the addition of SDS-PAGE sample buffer. After electrophoresis, acrylamide gels were stained with Pro-Q Diamond phosphoprotein gel stain (Molecular Probes) according to the manufacturer's instruction manual, and then phosphorylated polypeptides in gel were visualized with a FluorChem imaging analyzer (AlphaInnotech) with a 354 nm excitation wavelength and 595 nm emission.

Preparation of extracts from plant tissues

Frozen tissues (2 g each) were homogenized in an ice-cooled motor in 6 ml of lysis buffer containing 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS, 50 mM Na_2-β-

glycerophosphate [pH 7.6], 10 mM EDTA, 5 mM EGTA, 30 mM NaF, 5 mM Na_3VO_4, 5% glycerol, 0.1% β-mercaptoethanol, 1 mM PMSF, 5 mM *n*-aminocaproic acid, 1 mM benzamidine, 1 mM Na-bisulfite, and 1 μg ml^{-1} leupeptin, with or without 1% Triton X-100). The resultant homogenates were centrifuged at 10,000×*g* for 20 min at 4°C. 100,000×*g* supernatants (soluble fractions) and 100,000×*g* pellets (microsomal fractions) were prepared by centrifugation at 100,000×*g* for 60 min at 4°C (P65A rotor and 55P-7 ultra-centrifugator, Hitachi Inc., Tokyo, Japan) with 10,000×*g* supernatant fraction (without Triton X-100) described above. Protein concentrations in the samples were measured by a Bio Rad protein assay kit (BioRad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

Immunoblot assay by anti-VuCIPK, anti-tomato CBL, anti-phosphoserine and anti-phosphothreonine antibodies

Polypeptides in the gel were electroblotted onto PVDF membrane at 10 V cm^{-1} for 90 min in 25 mM Tris-base containing 0.05% SDS and 20% methanol, and then the PVDF membrane (Millipore Co., Ltd.) was blocked by incubating in 1×TBS containing 3% skim milk (TBS-milk) for 60 min. The PVDF membrane was incubated with anti-VuCIPK1 serum (1/2,000 dilution [v/v]) or anti-tomato-CBL serum (1/2,000 dilution [v/v]) in TBS-milk containing 0.05% Tween 20 at room temperature for 2 h. Subsequently, the PVDF membrane was incubated at room temperature for 60 min in TBS containing horseradish peroxidase (HRP)–conjugated protein G (GE Healthcare Bio-Sciences) (1/10,000 dilution [v/v]), and 0.05% Tween 20. The immunodecorated protein bands were visualized by an ECL Plus kit (GE Healthcare Bio-Sciences) and a FluorChem imaging analyzer. To detect phosphoproteins by immunoblotting, the electroblotted PVDF membrane was blocked by incubating at room temperature for 4 h in 1×TBS containing 1% BSA (TBS-BSA) and then at room temperature for 2 h in TBS-BSA containing either anti-phosphoserine monoclonal mouse antibody (clone PSR-45, Sigma) or anti-phosphothreonine monoclonal antibody (clone PTR-8, Sigma) (1/3000-dilution, [v/v]) and 0.05% Tween 20. After washed in TBS-Tween, the PVDF membrane was incubated for 2 h in TBS-BSA containing HRP-conjugated anti-mouse goat IgG antibody (1/3000-dilution [v/v]) (NIF825, GE Healthcare Bio-Sciences) and 0.05% Tween 20. The immunodecorated protein bands were visualized by an ECL Plus kit and a FluorChem imaging analyzer.

Immunoprecipitation

The soluble fraction obtained from the 20,000×*g* centrifugation (containing 5 mg protein) was mixed with 15 μl rabbit anti-VuCIPK1 serum and 30 μl protein G-Sepharose CL-4B beads (50% [v/v] slurry) (GE Healthcare Bio-Sciences). The suspensions were rotated for 2 h at 4°C. The immune-complex beads were precipitated by centrifugation at 10,000×*g* for 1 min at 4°C, then washed in 1 ml of washing buffer containing 25 mM Tris·HCl (pH 7.4), 1 M NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Triton X-100, and 0.1% β-mercaptoethanol. After six or more washes, the immuno-decorated beads were subjected to SDS-PAGE and immunoblot.

Results

Cloning of cowpea CIPK homolog full length cDNA

By searching for cowpea EST clones in the NCBI gene database, we identified VuRD068337 and VuRD068338, which encode a kinase domain closely related to that of CIPK/SnRK3 family. The full-length cDNA of the cowpea CIPK homolog was obtained by PCR and 5′-RACE using a cowpea cDNA library and a set of primers designed with the two ESTs. The resultant cDNA, named *VuCIPK1* (accession no. AB303675), had an ORF of 1326 bp, encoding a predicted protein of 441 amino acids with a relative molecular weight of 50.4 kDa and a putative pI of 6.4 (Figure 1A). VuCIPK1 is composed of two parts, a highly conserved N-terminal kinase domain similar to that of SNF1/AMPK and a unique COOH-terminal domain containing CBL-interacting region, called "NAF/FISL domain," with more varied amino acid sequences than the kinase domains have (Albrecht et al. 2001; Guo et al. 2001). The predicted protein and the parameters of the *VuCIPK1* cDNA are highly similar to those of a soybean CIPK homolog (GmCIPK; TC207101 in the DFCI *Glycine max* Gene Index, http://compbio.dfci.harvard.edu/tgi/; predicted ORF of 441 aa, 50.5 kDa, putative pI 6.8; 98% amino acid identity), AtCIPK3 of *Arabidopsis* (At2g26980; predicted ORF of 451 aa, 51.8 kDa, putative pI of 7.6; 86% amino acid identity), and OsCIPK3 of rice (accession no. AP003818, predicted ORF of 445 aa, 51.0 kDa, putative pI 6.9; 73% amino acid identity) (Kolukisaoglu et al. 2004). VuCIPK1 belongs to the SnRK3(CIPK)-class kinase subfamily of the plant SNF1-related protein kinase family (Hrabak et al. 2003) as AtCIPK3 and OsCIPK3 also does (Figure 1B).

The expression pattern of *VuCIPK1* mRNA was analyzed by RT-PCR of total RNA prepared from mature leaf, young leaf, stem, and root of 2-week-old cowpea plants. Signals of expression of *VuCIPK1* and actin were detected in all organs by RT-PCR. The difference among organs was marginal (Figure 2A). The expression profiles of *VuCIPK1* in leaf, stem, and root of cowpea resemble with those of *AtCIPK3* in *A. thaliana* (Kim et al. 2003a). *PsCIPK* in pea which belongs to the other major subclass of CIPK family (Figure 1B), is expressed at higher levels in leaf and root than in other tissues (Mahajan et al. 2006).

Next, we analyzed the effects of various environmental stresses on levels of *VuCIPK1* mRNA in leaf. Any significant change of *VuCIPK1* expression was observed by RT-PCR when 2-week-old cowpea plants were subjected to drought, NaCl, or chilling (Figure 2B). After drought stress, rehydration had also a marginal effect. While RT-PCR was carried out with the same cDNA sample in conditions of 28 and 32 cycles on PCR, no

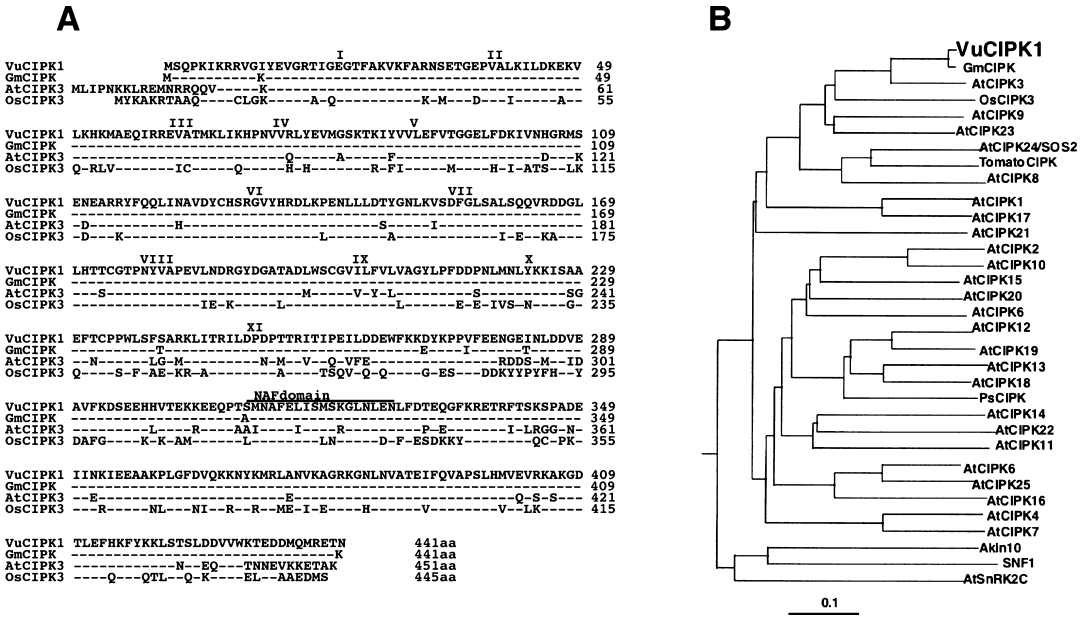

Figure 1. Deduced amino acid sequence of *VuCIPK1* and alignment with related sequences, and phylogenic analysis. (A) Deduced amino acid sequences of *VuCIPK1*, *GmCIPK*, *AtCIPK3*, and *OsCIPK3*. Roman numerals indicate the subdomains of Ser/Thr protein kinases; the rule indicates the NAF/FISL domain, which interacts with CBLs. The dashes in the sequences indicates identical amino acids to those of VuCIPK1. (B) Phylogenic tree of VuCIPK1 and related protein kinases: GmCIPK of *G. max*; PsCIPK of *P. sativum*; AtCIPK1–25, Akin10, and AtSnRK2C of *A. thaliana*; OsCIPK3 of *O. sativa*; tomato CIPK of *S. lycopersicum*; and SNF1 of *S. cerevisiae*. Details of phylogenic analysis are described in Materials and Methods.

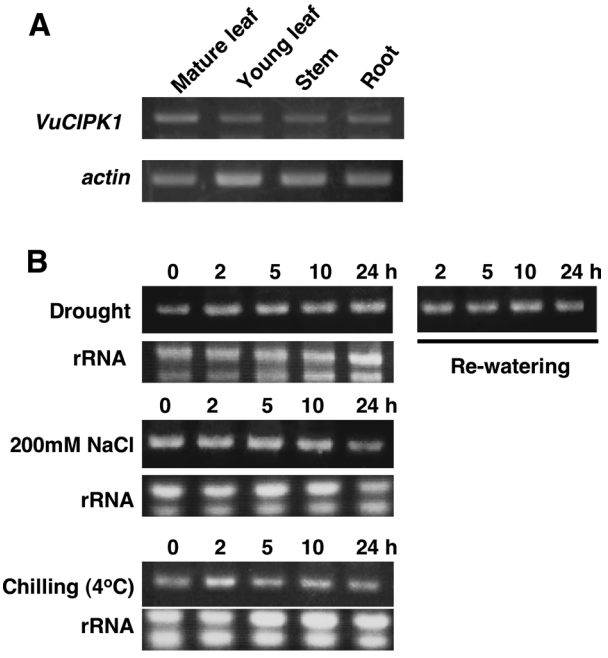

Figure 2. Organ-specific expression of *VuCIPK1* and effects of stress treatments. (A) Expression analysis of *VuCIPK1* and *actin* in mature leaf, young leaf, stem, and root of 2-week-old cowpea. (B) Expression profiles of *VuCIPK1* mRNA in leaves of 2-week-old cowpea in response to drought and re-watering, NaCl, and chilling. Total RNA samples were isolated from tissues harvested at the indicated times.

significant difference of signals of RT-PCR was observed among the control sample and the strees-given samples (data not shown).

Immunoblot of CIPK-related proteins

It was confirmed that the rabbit anti-VuCIPK1 antibody cross-reacted with recombinant CIPKs, maltose binding protein (MBP)- fused VuCIPK1 and MBP-GmCIPK, but not with MBP only (data not shown). The antibody also cross-reacted with ~55-kDa polypeptides in the leaf extracts of soybean, peanut, and tomato but not rice (Figure 3). The observation that the anti-VuCIPK serum cross-reacted with CIPK-related proteins in soybean is consistent with the significantly high similarity of the predicted amino acid sequences between VuCIPK1 and GmCIPK (Figure 1A). The immunoreactive signals in the extracts of peanut and tomato suggest that the antibody can cross-react broadly with CIPK-related proteins in higher plants. In contrast to legumes and tomato, the antibody failed to cross-react with peptides in rice seedlings. It is conceivable that the failure of immunological detection is due to the low expression level of CIPK1-related proteins in rice seedlings or due to the difference of between dicots and monocots.

Localization of CIPK-related proteins and CBL-related proteins in cowpea

To detect the levels of endogenous VuCIPK1 in cowpea, we immunoblotted 10,000×*g* supernatant fractions prepared from several organs of 2-week-old-cowpea plants with rabbit anti-VuCIPK1 serum. Immuno-cross-reacting signals were detected at significant levels in fully expanded leaves at 55 kDa, and in roots at 51 kDa, showing different mobilities in SDS-PAGE (Figure 4A).

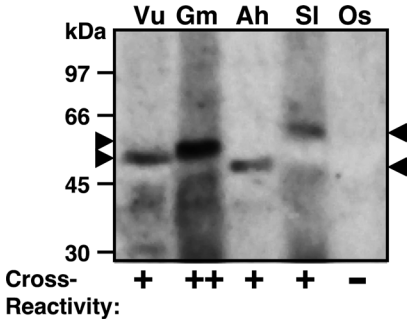

Figure 3. Detection of immunologically CIPK-related proteins in dicot and monocot plants. Anti-VuCIPK1 serum cross-reacted with polypeptides from cowpea (Vu), soybean (Gm), peanut (Ah), and tomato (Sl), but not rice (Os) (arrowheads). The 10,000×*g* soluble fractions were prepared from crude extracts of leaves of each plants in the presence of 1% Triton X-100. Each 50 μg protein per lane was subjected to SDS-PAGE (10% acrylamide gels) and immunoblot by anti-VuCIPK serum.

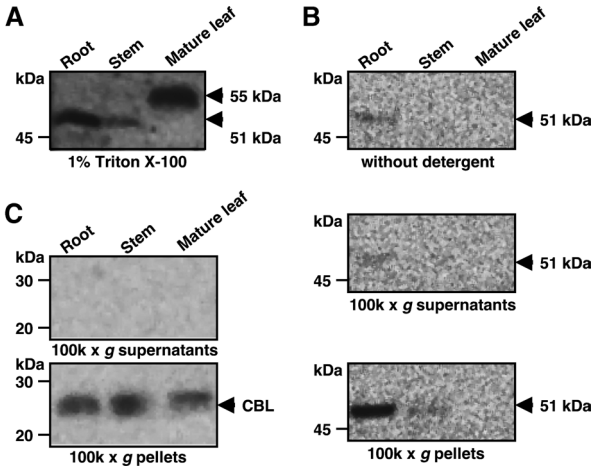

Figure 4. Immunoblot analysis by anti-VuCIPK1 serum and anti-CBL serum. (A) Localization of VuCIPK1 in various organs of cowpea. The 10,000×*g* soluble fractions were prepared from crude extracts of root, stem, and leaves of 2-week-old cowpea in the presenceof 1% Triton X-100. Each 50 μg protein per lane was subjected to SDS-PAGE (10% acrylamide gel) and immunoblot by anti-VuCIPK serum). (B) Immunoblots by anti-VuCIPK serum with 10,000×*g* supernatants (without detergent), soluble fractions (100k×*g* supernatants) and microsomal fractions (100k×*g* pellets). (C) Immunoblot by anti-tomato CBL serum with soluble fractions and microsomal fractions. Fractionation was carried out at 100,000×*g* for 60 min at 4°C after preparation of 10,000×*g* supernatants in the absence of Triton X-100. Each 50 μg protein per lane was subjected to SDS-PAGE (10% or 12.5% acrylamide gels) and immunoblot by anti-VuCIPK serum oranti-tomato CBL serum, respectively.

Weak signals were also detected in the stem at 51 kDa. Interestingly, the immuno-cross-reactive signals were detected when tissues were homogenized in the presence of Triton X-100 (Figure 4A), but signals were relatively weak for root or even a null signal for stem and leaf in the absence of Triton X-100 (Figure 4B, upper). The effect of Triton X-100 suggests that VuCIPK1 protein binds tightly to the 10,000×*g* pellets, and perhaps to subcellular organelles and nuclear membranes.

In the next stage, ultra-centrifugation was carried out with the 10,000×*g* supernatant fractions without Triton X-100 to examine whether VuCIPK1 binds to membrane fraction. A significant signal was detected in 100,000×*g* pellet (Figure 4B, lower), but weak or even no signals in 100,000×*g* supernatant (Figure 4B, middle) prepared from root by immunoblot with anti-VuCIPK1 serum. It is interesting that rabbit anti-VuCIPK1 serum cross-reacted with the 55-kDa polypeptide in mature leaf and the 51-kDa polypeptide in root, whereas PCR fragments of *VuCIPK1* in RT-PCR analysis did not differ in size among organs (Figure 2A). An alternative splicing of the 5′-region of *VuCIPK1* mRNA to give different sizes of translational products in its N-terminal regions is possible. In the other case, it is also possible that the rabbit anti-VuCIPK1 serum cross-reacted with various CIPK isoforms that have different mobilities in SDS-PAGE. Accordingly, the presence of the 55- and 51-kDa CIPKs in cowpea suggests that different isoforms of CIPKs are expressed between leaf and root, respectively.

Considering that several CBL isoforms are conjugated with a myristoyl hydrophobic moiety at the N-terminus and recruit CIPKs onto microsomes (Kolukisaoglu et al. 2004), it is reasonable that VuCIPK1 is associated with membrane structures *via* interaction with CBLs. Therefore, localization of CBL-related proteins in cowpea plants was examined using the same sets of soluble and microsomal fractions of cowpea by immunoblot with anti-tomato CBL serum (Figure 4C). Immunoreactive signals were detected in 100,000×*g* pellets (Figure 4C, lower) but not in 100,000×*g* supernatants (Figure 4C, upper). These observation indicated that VuCIPK1 and cowpea CBL-related proteins are preferentially associated with the membrane fractions but not with the soluble fractions .

Protein kinase activity and autophosphorylation of VuCIPK1

We analyzed whether VuCIPK1 phosphorylated exogenous substrates. GST-VuCIPK1 was purified by glutathione sepharose resin from crude extracts of *E. coli* containing pGEX-VuCIPK1. GST-VuCIPK1 was incubated with histone H1, myelin basic protein, or dephosphorylated casein in the presence of Mg^{2+} or Mn^{2+} with ATP. A fluorescence image of the Pro-Q-stained gel (Figure 5A) shows that GST-VuCIPK1 preferentially phosphorylated a polypeptide with a low molecular mass of 17 kDa (arrow) of dephosphorylated casein in the presence of Mn^{2+}. The bands at 17 kDa in the presence of Mg^{2+} showed the same signal level as in the lane with no kinase, indicating that recombinant VuCIPK1 did not phosphorylate the substrate. When histone H1 and myelin basic protein were assayed, all the bands in each lane showed the same level as in the absence of kinase, indicating that the recombinant

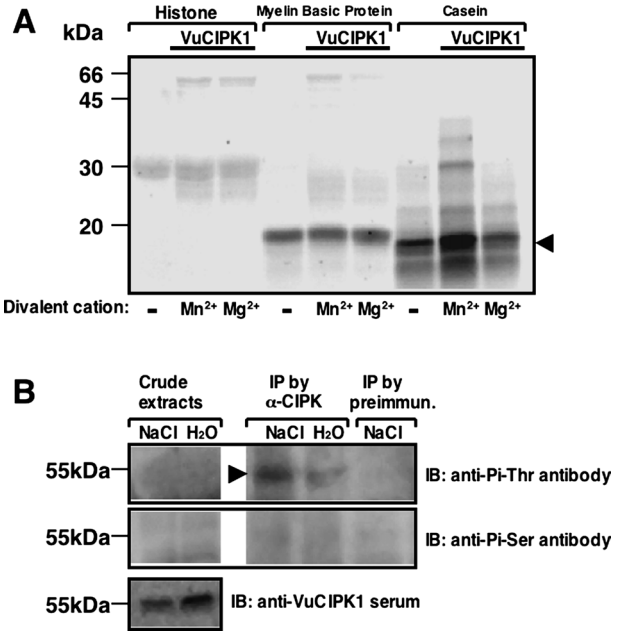

Figure 5. Biochemical properties of VuCIPK1. (A) Substrate specificity of recombinant GST-VuCIPK1. *In vitro* protein kinase assay was carried out with 1 μg recombinant GST-VuCIPK1 and 20 μg substrate proteins (histone H1, dephosphorylated casein, or myelin basic protein) in the presence of divalent cations (Mg^{2+} or Mn^{2+}) or their absence (−). Arrowhead indicates Pro-Q-stained signals of phosphorylated proteins. (B) Detection of autophosphorylation of endogenous VuCIPK1-related polypeptides in cowpea leaf extracts. 20,000×*g* soluble fractions were prepared from cowpea leaves 30 min after treatment with NaCl stress or water. After immunoprecipitation by anti-VuCIPK1 serum, the immunoprecipitates and crude extracts were immunoblotted with anti-phosphothreonine antibody (upper), anti-phosphoserine antibody (middle), or anti-VuCIPK1 serum (lower).

VuCIPK1 did not phosphorylate either (Figure 5A). Autophosphorylation of GST-VuCIPK1 was not observed under these conditions. The presence of free Ca^{2+} and recombinant GmCBL2 in the assay did not stimulate the kinase activity of VuCIPK1 (data not shown). These observations suggest that the recombinant VuCIPK1 showed substrate specificity distinct from that of OsCK1 and PsCIPK. The *in vitro* kinase assay indicated that recombinant OsCK1 phosphorylated myelin basic protein and autophosphorylated its polypeptide in the presence of Mn^{2+}, but not in the presence of Mg^{2+} (Kim et al. 2003a). Recombinant PsCIPK phosphorylated myelin basic protein, PsCBL, and itself in the presence of Mn^{2+} or Mg^{2+} (Mahajan et al. 2006).

Next, we examined the phosphorylation status of endogenous VuCIPK1-related polypeptides by using rabbit anti-VuCIPK1 serum to immunoprecipitate the soluble fraction prepared from cowpea leaves treated by salt stress. Thirty minutes after treatment with water or 200 mM NaCl, the 10,000×*g* soluble fraction of cowpea leaves was prepared in lysis buffer containing 1% Triton X-100. The soluble fractions were subjected to immunoprecipitation assay with rabbit anti-VuCIPK1 serum (or preimmune serum) and protein A sepharose beads. After washing with high salt buffer, the resultant immune complexes on beads were subjected to SDS-PAGE and immunoblotting by anti-phosphothreonine monoclonal antibody or anti-phosphoserine monoclonal antibody. Immunoreactive signals by anti-phosphothreonine monoclonal antibody were detected at 55 kDa in immunoprecipitates with rabbit anti-VuCIPK1 serum, but not with preimmune serum. The signal immunodetected by anti-threonine antibody in immunoprecipitate derived from NaCl-treated leaf was higher than that from water-treated leaf (Figure 5B), suggesting that the endogenous VuCIPK1 and/or immuno-related cowpea CIPKs is autophosphorylated at its threonine residues in response to NaCl stress. No signal at 55 kDa was detected by immunoblotting with anti-phosphoserine monoclonal antibody. When cowpea plants were subjected to chilling (4°C, 1 h) or treatment of abscisic acid (50 μM, 1 h), there was no or marginal changes of immuno-reactive signals by anti-phosphothreonine antibody nor anti-phosphoserine antibody in immunoprecipitation assay (data not shown).

Discussion

Examination of the phylogenic tree of *Arabidopsis* CIPK and other SnRKs showed that the CIPK subfamily kinases can be subdivided into two major subclasses (Figure 1B), leaving SnRK1s (SNF1 and Akin10; Celenza and Carlson 1984; Le Guen et al. 1992) and AtSnRK2C as outgroups. VuCIPK1 is placed in the subclass including AtCIPK3, which is involved in signaling of ABA and cold stress (Kim et al. 2003b), AtCIPK24/SOS2, which is involved in Na^+ excretion (Qiu et al. 2000), and AtCIPK23, which is involved in regulation of K^+ channels (Xu et al. 2006). AtCIPK1 belonging to the same sub-class of CIPK family as VuCIPK1 (Figure 1B) is involved in drought resistance (Batistic and Kulda 2004), while CIPK14 belonging to another subclass of CIPK family is involved in sugar response (Kim et al. 2003a). In contrast to the expression profiles of *VuCIPK1* mRNA (Figure 2), transcripts of CIPK molecules in other plants are highly induced by various stress treatments: *AtCIPK3* is induced by drought, NaCl, ABA, and chilling stress in *Arabidopsis* (Kim et al. 2003b); *OsCK1* (accession no. AAP82174), with high sequence similarity to *AtCIPK3* and *OsCK1*, by chilling stress, light, NaCl, and high $CaCl_2$ in rice (Kim et al. 2003a); and *PsCIPK* by chilling stress, NaCl, and high $CaCl_2$, but not by drought or ABA, in *Pisum sativum* (Mahajan et al. 2006).

In the present study, VuCIPK1 appeared to have high similarity of amino acid sequence to AtCIPK3

and OsCK1. However, biochemical properties and immunochemical data indicate that VuCIPK1 has unique features: (1) VuCIPK1 prefers to phosphorylate casein rather than histone or myelin basic protein. (2) Triton X-100 is necessary for solubilization of VuCIPK1-related proteins from homogenates of cowpea tissues. (3) VuCIPK1 and CBL-related proteins are tightly associated to membrane fractions. (4) VuCIPK1 is autophosphorylated in response to salt stress. These observations suggest that VuCIPK1, co-localized onto membrane structures and/or specific intracellular organella, is regulated post-translationaly by phosphorylation at its threonine residues. The NaCl-induced autophosphorylation of threonine residues in endogenous cowpea CIPK(s) (Figure 5B) is consistent with the observation that PsCIPK is autophosphorylated at its threonine residues (Mahajan et al. 2006). It is conceivable that VuCIPK1 is regulated mainly by phosphorylation of threonine residues in the activation loop, like other SnRK3 (Guo et al. 2001). However, this does not mean to exclude a possibility that environmental stresses regulates VuCIPK1 by changing transcription levels in specific tissues involving transport and/or up-take of minerals. It is necessary for clarifying details of localization and expression of VuCIPK1 to perform real-time PCR and *in situ* hybridization.

We detected autophosphorylation of endogenous VuCIPK under salt stress (Figure 5B) but nothing of the recombinant VuCIPK1 expressed in *E. coli* in *in vitro* kinase assay (Figure 5A), it is conceivable that the stress-stimulated full activation of endogenous VuCIPK requires plant specific chaperones for reffolding of VuCIPK1 and/or involving in association of VuCIPK1 onto membrane in addition of CBL and calcium ion. The significant tolerance of cowpea against extremely drought stress may be accounted for stress signaling cascades utilizing cowpea-specific sets of CIPKs and CBLs which regulate ion transport and water permieability acrossing plasma membrane and/or vacuoles in root and leaf cells. It remains to be clarified whether VuCIPK1 is a stress-activated kinase involved in the expression of stress-inducible genes, or whether it is involved in the direct regulation of ion transporters by phosphorylation under salinity, drought, chilling, and ABA.

Increasing evidence suggests that CBL–CIPK complexes phosphorylate and activate various ion transporters, such as Na^+/H^+ exchangers and K^+ channels (Zhu 2003; Xu et al. 2006), while SnRK2 family members appear to be involved in phosphorylation and activation of various stress-responsive transcription factors (Kobayashi et al. 2005; Boudsocq and Laurière 2005; Furihata et al. 2006). Recently, it has been reported that drought stress altered expression profiles of aquaporines and phosphorylation status of membrane proteins in root of cowpea (Yamauchi et al. 2008). VuCIPK1 is one of the candidate kinases involving regulation of such membrane when cowpea is subjected to environmental stresses. Therefore, identification of the endogenous substrate of VuCIPK1 in cowpea is important for unveiling its physiological functions under drought or other stresses.

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