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Expression and interaction of the CBLs and CIPKs from immature seeds of kidney bean (*Phaseolus vulgaris* L.)

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ABSTRACT

Protein phosphorylation plays a key regulatory role in a variety of cellular processes. To better understand the function of protein phosphorylation in seed maturation, a PCR-based cloning method was employed and five cDNA clones (*pvcipk1–5*) for protein kinases were isolated from a cDNA library prepared from immature seeds of kidney bean (*Phaseolus vulgaris* L.). The deduced amino acid sequences showed that the five protein kinases (PvCIPK1–5) are members of the sucrose non-fermenting 1-related protein kinase type 3 (SnRK3) family, which interacts with calcineurin B-like proteins (CBLs). Two cDNA clones (*pvcbl1* and *2*) for CBLs were further isolated from the cDNA library. The predicted primary sequences of the proteins (PvCBL1 and 2) displayed significant identity (more than 90%) with those of other plant CBLs. Semi-quantitative RT-PCR analysis showed that the isolated genes, except *pvcbl1*, are expressed in leaves and early maturing seeds, whereas *pvcbl1* is constitutively expressed during seed development. Yeast two-hybrid assay indicated that among the five PvCIPKs, only PvCIPK1 interacts with both PvCBL1 and PvCBL2. These results suggest that calcium-dependent protein phosphorylation-signaling via CBL–CIPK complexes occurs during seed development.

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1. Introduction

Post-translational protein modification is necessary for cell signaling networks to allow plants to dynamically respond to external stimuli. In all eukaryotic cells, reversible protein phosphorylation plays a crucial role in signal-transduction control by regulating the activities of targeted proteins. The eukaryotic protein kinases comprise a superfamily and have been defined in categories by Hanks and Hunter (1995). Sucrose non-fermenting 1 (SNF1), which was first identified in the *Saccharomyces cerevisiae* mutant (*snf1*), encodes a serine/threonine kinase (Celenza and Carlson, 1986) that is essential for expression of the invertase gene in response to glucose depletion (Celenza and Carlson, 1989). Plants have multiple SNF1 homologs, called SNF1-related protein kinases (SnRKs) that are classified into three subgroups (SnRK1–3) based on sequence similarity and domain structure (Halford and Hardie, 1998). For example, *Arabidopsis* contains 38 SnRKs: three are SnRK1, 10 are SnRK2, and 25 are SnRK3 homologs (Hrabak et al., 2003). Nevertheless, there is limited information on their functional roles. SnRK1 members complement the yeast *snf1* mutant phenotype. The antisense expression of a potato SnRK1 homolog (PKIN1) results in a loss of sugar-inducible expression of the sucrose synthase gene in leaves and tubers (Purcell et al., 1998). Recently, KIN10 and KIN11 in *Arabidopsis* have been reported to control transcriptional networks broadly in addition to the sugar response (Baena-González et al., 2007). In barley aleurone layers, the SnRK2 homolog (PKABA) mediates abscisic acid (ABA)-suppressed gene expression (Gómez-Cadenas et al., 1999), and the SnRK2 homologs in *Arabidopsis* are activated by hyperosmolarity (Boudsocq et al., 2004). The SnRK3 homolog in *Arabidopsis* also has been shown to play a role in osmotic signaling (Liu et al., 2000). SnRK3 members, which are unique to plants, are also represented as calcineurin B-like protein (CBL) interacting protein kinases (CIPKs; Shi et al., 1999; Kim et al., 2000) that are involved in responses to salt stress and in sugar and ABA signaling (Guo et al., 2001; Gong et al., 2002; Imamura et al., 2008).

Calcium-signaling mechanisms mediate a multitude of responses to external biotic and abiotic stimuli, and regulate a variety of cellular and developmental processes (Clapham, 1995; Dolmetsch et al., 1998; Reddy and Reddy, 2004). Plant cells have many calcium sensor proteins including calcium-dependent protein kinase (CDPK), calmodulin (CaM), calmodulin-related proteins (Zielinski, 1998; Luan et al., 2002), and CBLs (Luan et al., 2002). CDPK, which includes a calcium binding as well as a catalytic domain, can respond and transmit calcium signals (Harmon et al.,

Abbreviations: 3-AT, 3-aminotrizole; ABA, abscisic acid; CBL, calcineurin B-like protein; CIPK, CBL interacting protein kinase; DTT, dithiothreitol; PvCBL, kidney bean (*Phaseolus vulgaris* L.) CBL; PvCIPK, kidney bean CIPK; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction; SNF1, sucrose non-fermenting 1; SnRK, SNF1-related kinase.

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2000; Sanders et al., 2002). In contrast, CaMs, CaM-related proteins, and CBL proteins do not have enzymatic activity of their own. Binding of calcium changes their affinity and results in activation or deactivation of their target proteins (Roberts and Harmon, 1992; Snedden and Fromm, 1998).

CBL proteins possess four EF-hand motifs to capture calcium and the interacting region with CIPKs. The resulting CBL–CIPK complex forms a signaling network for specific and synergistic stimulus response coupling (Albrecht et al., 2001; Luan et al., 2002; Zhu, 2003; Batistic and Kudla, 2004). For example, reverse genetic analysis of the AtCIPK1 protein kinase established that complex formation with either AtCBL1 or AtCBL9 controls ABA-dependent and -independent stress responses in *Arabidopsis* (D'Angelo et al., 2006). Furthermore, AtCBL1 interacts with a subset of only six CIPKs (Kolukisaoglu et al., 2004). The multiple formations of CBL–CIPK complexes might provide a novel mechanism to integrate and specifically decode signaling in plants. With the exceptions of *Arabidopsis* and rice, the CBL–CIPK pathways have not been established in higher plants. In particular, the regulation and function of CBL–CIPK complexes in seed maturation has not been determined.

In this study, we describe the cDNA cloning, expression analysis, and interaction assay of five novel CIPKs (PvCIPK1–5) and two CBLs (PvCBL1 and 2) from kidney bean (*Phaseolus vulgaris* L.) to better understand protein phosphorylation-signaling in developing seeds.

2. Results and discussion

2.1. Isolation of cDNA clones encoding kidney bean CIPKs

To isolate the protein kinase cDNA clones of kidney bean seeds, two degenerate primers were designed according to the highly conserved sequences of the kinase regions (VI and IX, Fig. 1A) (Wilks, 1991; Hotta et al., 1998) and these were used for PCR amplification of the partial fragments. The nucleotide sequence of the amplified PCR product (about 200 bp) showed a significant similarity to other reported protein kinases. Therefore, to obtain a full-length cDNA clone, the amplified fragment was used as a probe to screen a cDNA library of mid-developing kidney bean seeds. Multiple rounds of screening resulted in isolation of nine positive clones. The digestion of the insert fragment by several restriction enzymes suggested that five of the nine clones were independent. Sequence analysis showed that these five clones (*pvcipk1–5*) encoded SNF1-related protein kinases (SnRKs) and contained open reading frames of 1302, 1302, 1332, 1341 and 1317 bp. The polypeptides (PvCIPK1–5) encoded by the *pvcipk1–5* corresponded to 433, 433, 443, 446, and 438 amino acid residues with predicted molecular masses of 48.2, 48.8, 50.5, 49.9, and 49.4 kDa, respectively. The five deduced sequences displayed significant similarity to other plant SnRK3 members (Fig. 1B). When the primary sequences of the PvCIPKs were analyzed for subcellular location with the WoLF PSORT network program (Horton et al., 2007), all of the proteins were predicted to be cytosolic. The primary sequence alignment of the PvCIPKs is shown in Fig. 1A. The N-terminal domains of PvCIPKs showed significant sequence similarity (78–96%) with each other, and all had 11 of the conserved motifs including an activation loop in the protein kinase catalytic domain (Fig. 1A; Stone and Walker, 1995). Additionally, the catalytic Asp residue (Hanks and Hunter, 1995; Nayak et al., 2006) and the phosphorylated Thr residue (Guo et al., 2001; Gong et al., 2002) were completely conserved in all of the PvCIPKs, suggesting that these protein kinases had enzymatic activities and that their activities were regulated via a phosphorylation-dephosphorylation system. The C-terminal regions of the PvCIPKs displayed lower similarity (52–91%) than the N-terminal regions, but contained

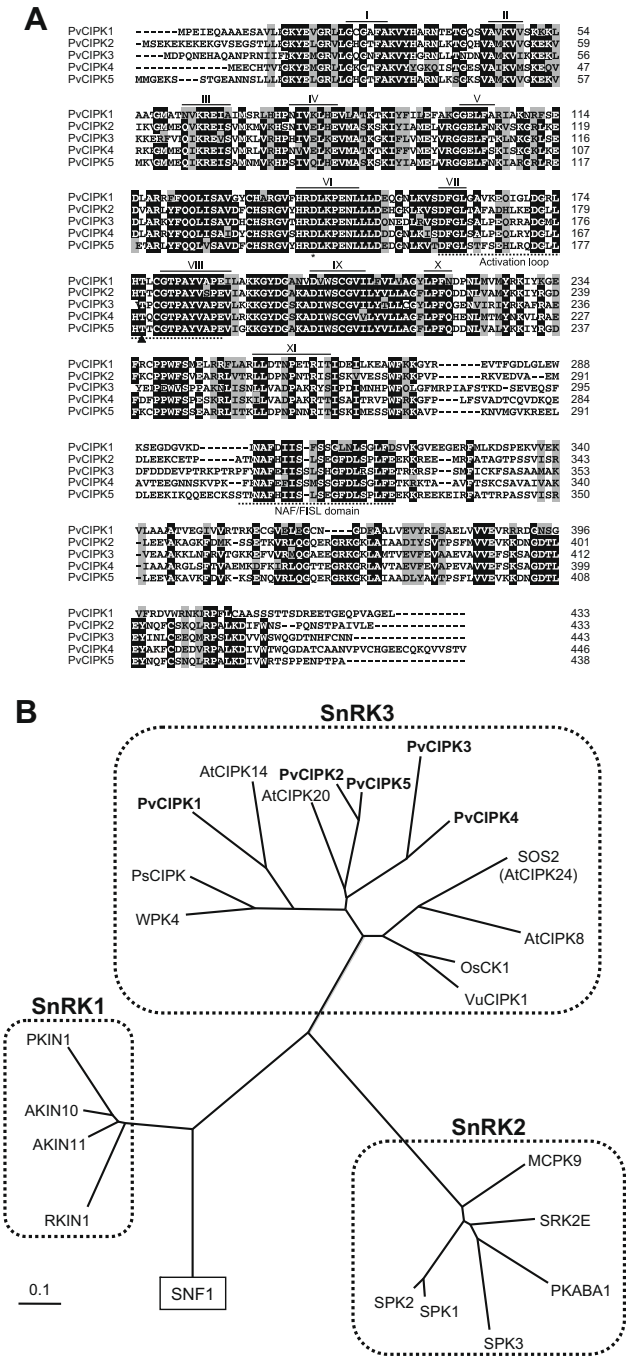

Fig. 1. Primary sequence alignments among isolated PvCIPKs and phylogenetic relationship among plant SnRKs. (A) Multiple sequence alignments were determined using the ClustalW program. Residues showing more than 80% sequence identity are indicated by white letters on a black background. The characters with a gray background are similar residues. The conserved subdomains in the protein kinase family are shown by overbars. The activation loop and NAF/FISL motif are denoted by dotted underlines. The asterisk indicates the catalytic base, and the closed triangle shows the phosphorylated residue in the activation loop. (B) All of the PvCIPKs were classified into the SnRK3 family. The scale indicates the branch length. The relationship was constructed by the TreeView program from a treefile produced in the ClustalW program. The SnRK member sequences were obtained from the GenBank/EMBL/DDBJ database: SNF1 from *Saccharomyces cerevisiae*, M13971; PvCIPK1–5 from *P. vulgaris*, AB378090–AB378094; AKIN10, AKIN11, AtCIPK8, AtCIPK14, AtCIPK20, SOS2 (AtCIPK24) and SRK2E from *Arabidopsis thaliana*, M93023, NM_202645, NM_118573, AF295669, NM_123950, AF395081, and NM_001125639; PKIN1 from *Solanum tuberosum*, X95996; RKIN1 from *Secale cereale*, M74113; MCPK9 from *Mesembryanthemum crystallinum*, Z26846; PKABA1 and WPK4 from *Triticum aestivum*, M94726 and AB011670; SPK1, SPK2 and SPK3 from *Glycine max*, L01453, L19360 and L19360; OsCK1 from *Oryza sativa*, AY256847; PsCIPK from *Pisum sativum*, AY191840; VuCIPK1 from *Vigna unguiculata*, AB303675.

the NAF/FISL motif (Shi et al., 1999; Albrecht et al., 2001) that interacts with calcineurin B-like proteins (CBLs).

2.2. Isolation of cDNA clones encoding kidney bean CBLs

Because the isolated PvCIPKs included an NAF/FISL motif in their C-terminal regulatory domains, the enzymatic activity and substrate specificity of these protein kinases were predicted to be modulated by CBL proteins. To isolate kidney bean CBL cDNA clones, the two specific primers were designed according to the highly conserved regions among several plant CBL proteins and these were used for preparation of the screening probe of a cDNA library. As with the isolation of the *pvcipk* cDNA clones, five positive clones were obtained after multiple rounds of screening. Restriction map and sequencing analysis of the inserted fragments showed that two of the five clones encoded different CBLs and that the other clones were derived from either the same gene or were false positives. The two cDNA clones (*pvcbl1* and *pvcbl2*) contained ORFs of 681 bp and encoded polypeptides (PvCBL1 and PvCBL2) consisting of 226 amino acid residues with predicted molecular masses of 26.1 and 25.8 kDa, respectively. The WoLF PSORT program predicted that the PvCBL1 and PvCBL2, as well as PvCIPKs, were localized in the cytosol. The primary sequences of the PvCBL1 and PvCBL2 were aligned with several plant CBL proteins (Fig. 2). Except for the N-terminal 30 residues, all of the plant CBL-protein sequences were almost completely identical. Several residues (asterisks in Fig. 2) involved in the interaction with a CIPK protein are also completely conserved among plant CBLs (Akaboshi et al., 2008). Like other plant CBLs, PvCBL1 and PvCBL2 contained four EF-hand motifs, denoted EF1–EF4 (Fig. 2), indicating that calcium ion is associated with each EF-hand. Several members of the *Arabidopsis* CBLs have a myristoylation motif (Towler et al., 1988; Kim et al., 2000). Myristoylation permits protein-membrane attachments and, thereby, membrane localization of several protein families, including CBLs and CDPKs (Martín and Busconi, 2000; Batistic et al., 2008). However, there were no myristoylation

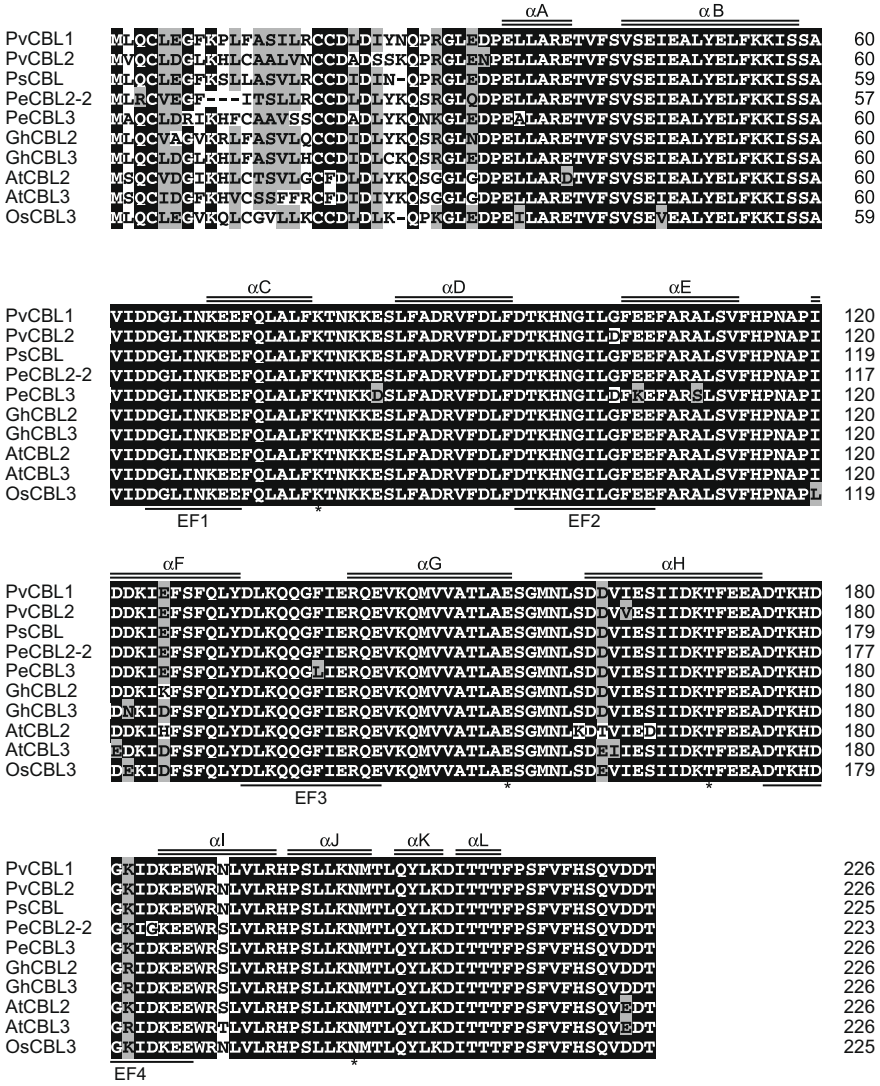

Fig. 2. Primary sequence alignment among several plant CBLs. Residues showing more than 80% sequence identity are indicated by white letters on a black background and those with 80% similarity are indicated by black letters on a gray background. The a-helices determined on the crystal structure of AtCBL2 are shown by double overbars (aA–aL). Four calcium binding domains are underlined. The asterisks show the positions corresponding to residues of AtCBL2, which are involved in hydrogen bonds with AtCIPK14 (Akaboshi et al., 2008). The multiple sequence alignments were determined using the ClustalW program. The sequences of plant CBL members were obtained from the GenBank/EMBL/DDBJ database: PvCBL1 and PvCBL2 from *P. vulgaris*, AB378095 and AB378096; PsCBL from *P. sativum*, AY883569; PeCBL2-2 and PeCBL3 from *Populus euphratica*, DQ907708 and DQ907709; GhCBL2 and GhCBL3 from *Gossypium hirsutum*, EU085039 and EU085040; AtCBL2 and AtCBL3 from *A. thaliana*, NM_124981 and NM_118791; OsCBL3 from *O. sativa*, NM_001057239.

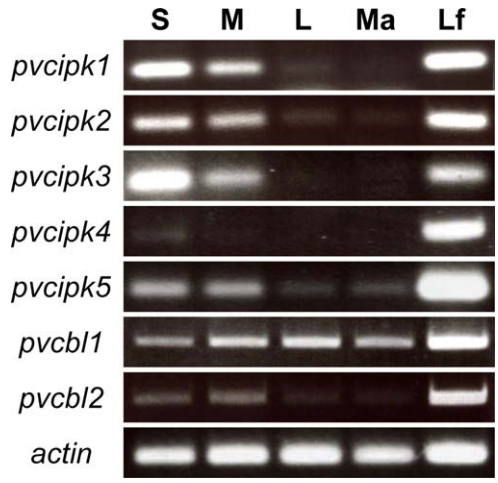

Fig. 3. Expression profiles of *pvcipk* and *pvcbl* in seeds and leaves. The RT products were prepared from the same amount of total RNA of small- (S), mid- (M), and large-size (L) developing seeds, mature seeds (Ma) and leaves (Lf), and used as templates. The products were separated on a 1.0% agarose gel. An actin fragment was amplified and used as a control (bottom panel).

motifs found in the N-terminal regions of the cloned PvCBL1 and PvCBL2. This suggests that PvCBL1 and PvCBL2 are partly responsible for a calcium-dependent signal-transduction pathway through interactions with the NAF/FISL motifs of PvCIPKs in cytosol of the kidney bean cells.

2.3. Expression profiles of pvcipk and pvcbl

Because we found no *pvcipk* and *pvcbl* expression signal by RNA gel blot analysis under the same conditions as described in previous reports (Hamada et al., 2001; Isono et al., 2003), the expression profiles were estimated by semi-quantitative RT-PCR analysis. The specific primer sets were designed to distinguish *pvcipk* or *pvcbl*, and were used to amplify the transcripts with the first strand cDNA mixtures from developing and mature seeds and leaves (Fig. 3). The expression levels varied by gene, but the expression profiles for the *pvcipk* and *pvcbl*, except for the *pvcbl1*, showed similar patterns. That is, these transcripts accumulated markedly in leaves and early developing seeds. This suggests that the gene expression was regulated by a similar process, and that the PvCIPKs and PvCBLs encoded by these genes might interact to display kinase activities. In contrast, the *pvcbl1* transcript accumulated constitutively during seed development, indicating that *pvcbl1* expression was regulated by different promoter factors, and that PvCBL1 in the late stage of seed development might interact with unknown PvCIPK(s) other than PvCIPK1–5. It has been reported that the transcriptional activation of *WPK4* (one of wheat SnRK3 members) was negatively regulated by sucrose; that is, the levels of *WPK4* transcript in wheat seedlings increased in the absence of and decreased in the presence of sucrose in the medium (Ikeda et al., 1999). Our preliminary experiment suggests that sucrose content soars dramatically in the middle stage of seed development (data not shown). This elevation may cause decreased levels of *pvcipk* transcripts in the late stage, as in the case of *WPK4*. We are currently conducting detailed expression analysis of *pvcipk* and *pvcbl* and their relationship to sugar induction.

2.4. Interaction of PvCIPKs and PvCBLs

We examined the interaction between five PvCIPKs and two PvCBLs using the yeast two-hybrid assay. The yeast cells co-transformed with pDEST32/PvCIPK1–5 and pDEST22/PvCBL1 or pDEST22/PvCBL2 were spotted on the selection medium containing 3-AT, an inhibitor of the His3 product. The cells co-expressed PvCIPK1 and PvCBL1 or PvCBL2 proliferated on the selection medium containing 100 mM of 3-AT, whereas other cells did not grow on the same medium, indicating that PvCIPK2–5 had no affinity for PvCBL1 and PvCBL2 (Fig. 4). Based on sequence similarity, PvCIPK1–PvCBL1 corresponds to the PsCIPK–PsCBL from pea leaves whose transcript levels are coordinately increased in response to various abiotic and biotic stresses (Mahajan et al., 2006). In the presence of 150 mM 3-AT, only the cells with PvCIPK1 and PvCBL1 grew on the plate. These results show that PvCIPK1 can interact with PvCBL1 and PvCBL2 with different affinities. Compared to the growth with positive control cells, PvCIPK1 showed a stronger interaction with PvCBL1 than with PvCBL2. Interestingly, except for the N-terminal region, only two residues (Gly103 and Ile164 of PvCBL1 corresponding to Asp103 and Val164 of PvCBL2) were different in the primary sequences between PvCBL1 and PvCBL2 (Fig. 2). The recent structural analyses of the complexes formed between the regulatory domains of *Arabidopsis* CIPKs and the calcium sensor CBLs provide direct evidence for plant CBL structure. Furthermore, these analyses provide critical insights into the conformational change of CBL that occurs through binding of calcium

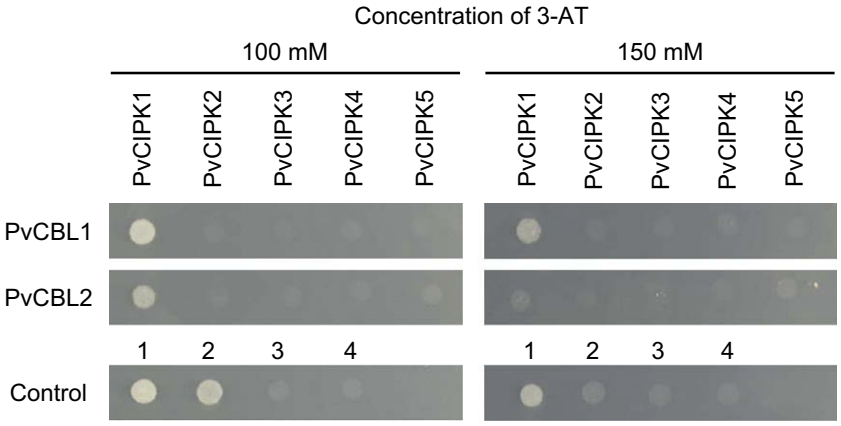

Fig. 4. Yeast two-hybrid assay between PvCIPKs and PvCBLs. The *pvcipk* and *pvcbl* cDNAs were inserted into the pDEST32 bait vector and pDEST22 prey vector to yield pDEST32/PvCIPK1–5 and pDEST22/PvCBL1–2, respectively. Each plasmid combination was introduced into MaV203 yeast cells and their growth was monitored on SC-Leu-Trp-His medium containing 3-AT (100 or 150 mM). The numbers above the control panel indicate the following plasmid combinations: 1, pEXP32/Krev1 and pEXP22/RalGDS-wt (for strong interaction); 2, pEXP32/Krev1 and pEXP22/RalGDS-m1 (for weak interaction); 3, pEXP32/Krev1 and pEXP22/RalGDS-m2 (for no interaction); 4, pDEST32 and pDEST22 (for vector control).

and the interaction of CIPK with CBL via the NAF/FISL region (Sánchez-Barrena et al., 2007; Akaboshi et al., 2008). For example, one of the *Arabidopsis* CBL proteins, AtCBL2, is composed of 12 continuous a-helices (aA–aL, Fig. 2) folded into two globular domains, N-lobe and C-lobe, which are formed by EF-1 and EF-2, and EF-3 and EF-4, respectively (Akaboshi et al., 2008). The N-terminal region of the CBLs is located out of the globular domains and upstream of the first a-helix A (aA), suggesting that the amino acid substitutions in the N-terminal region have almost no contribution to the interaction strength with their CIPK partners. Apart from the N-terminal regions, the two different residues between PvCBL1 and PvCBL2 exist on the EF-2 and a-helix H (aH), respectively (Fig. 2). Calcium binding to the EF-2 of AtCBL2 results in a large conformational change (Akaboshi et al., 2008), suggesting that the structural change resulting from the difference in the 103rd residues may affect partner interaction. The NAF/FISL region of the CIPKs is inserted into a hydrophobic crevice within the CBLs and is accompanied by a large displacement of the helices. The aH in the AtCBL2–AtCIPK14 complex is displaced considerably compared to the CBL free form. Moreover, the Thr171 in the aH of AtCBL2, which is conserved in PvCBL1 and PvCBL2, forms a hydrogen bond with the NAF/FISL motif Ala312 residue of AtCIPK14 (Akaboshi et al., 2008). These results suggest that the difference in the 164th residues of the aH might also influence the interaction between CBL1 and CIPK. Site-directed mutagenesis is required to better understand the differences in affinity between PvCIPK1 and PvCBL1 or PvCBL2.

3. Conclusions

The cDNA clones encoding five CIPKs (PvCIPK1–5) and two CBLs (PvCBL1 and 2) were isolated from developing seeds of kidney bean (*P. vulgaris* L.). Yeast two-hybrid analysis showed that the PvCIPK1 interacts with both PvCBL1 and PvCBL2, suggesting that calcium-dependent protein phosphorylation-signaling via the PvCIPK1-PvCBL1 and -PvCBL2 complexes occurs in kidney bean seed development. Identification of the targets of the PvCIPK1-PvCBL1 and -PvCBL2 complexes is required for an understanding of protein phosphorylation-signaling in seed development.

4. Experimental

4.1. Plant materials

Kidney bean (*P. vulgaris* L. cv. toramame) plants were grown on an experimental field at the Hokkaido University, Sapporo, Japan. The seeds were isolated at different developmental stages and separated as small- (4–8 mm), mid- (10–12 mm), or large-size (14–16 mm) developing seeds, or mature seeds. We also collected leaves for RNA preparation. All plant materials were stored in liquid nitrogen until use. The intact expanded leaves were harvested early morning.

4.2. Reverse transcription polymerase chain reaction (RT-PCR) and 3′ rapid amplification of cDNA ends (3′-RACE)

Total RNA was extracted from developing and mature seeds and leaves by the standard phenol/SDS method (Ausubel et al., 1992) as previously described (Hamada et al., 2001). RT-PCR and 3′-RACE were performed with an RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio, Kyoto, Japan). The first strand cDNA mixtures, which were prepared from the total RNA with the oligo-dT adaptor primer and avian myeloblast virus reverse transcriptase, were used for PCR as templates.

Table 1
Oligonucleotide primers used in this study.

Primers	Sequence (5′–3′)	Comments
PvPK-SEN	CAICGIGAYCTNAARCTNGARAA	For preparation of the probe to screen protein kinase cDNA clones
PvPK-ANT	GTIACICCRCANGACCANACRTC	For preparation of the probe to screen protein kinase cDNA clones
CBL-SEN	GATGGACTGATTAACAAGGA	For preparation of the probe to screen CBL cDNA clones
CBLANT	GAATGGAATACAAAGCTTGG	For preparation of the probe to screen CBL cDNA clones
λgt10-forward	GCTGGGTAGTCCCCACCTTT	For isolation and sequencing of the inserted cDNA fragments in phage DNAs
λgt10-reverse	CTTATGAGTATTTCTTCCAGGGTA	For isolation and sequencing of the inserted cDNA fragments in phage DNAs
PvCIPK1-RTsen	TGAATTATGATTCGGCCTCA	For RT-PCR of *pvcipk1*
PvCIPK1-RTant	CTACTGTAATATAAGCATGAC	For RT-PCR of *pvcipk1*
PvCIPK2-RTsen	CTTCAAGTGTCCACCTTGG	For RT-PCR of *pvcipk2*
PvCIPK2-RTant	CGTCTCACACTTCTCTTCC	For RT-PCR of *pvcipk2*
PvCIPK3-RTsen	ATCTCCTCGTTGCTGATCC	For RT-PCR of *pvcipk3*
PvCIPK3-RTant	GTTTCCTCGTTGGAACCTC	For RT-PCR of *pvcipk3*
PvCIPK4-RTsen	TGTGACAGATAGAAACTGTG	For RT-PCR of *pvcipk4*
PvCIPK4-RTant	TATTCCGAAAGTAACAGCAG	For RT-PCR of *pvcipk4*
PvCIPK5-RTsen	TGGTTTTCATCCGAAGCACG	For RT-PCR of *pvcipk*
PvCIPK5-RTant	ATTCATCGTGCTGGACTTGC	For RT-PCR of *pvcipk5*
Actin-RTsen	GGACGAGGCTCAATCGAAGA	For RT-PCR of actin gene
Actin-RTant	ACTGACACCGTCTCCGGAGT	For RT-PCR of actin gene
PvCIPK1-TOPOsen	CACCATGCCGGAAATTGAACAGGCG	For cloning of *pvcipk1* into entry vector
PvCIPK1-TOPOant	TCATAATTCACCGGCAACCGGCTGC	For cloning of *pvcipk1* into entry vector
PvCIPK2-TOPOsen	CACCATGAGTGAGAAAGAGAAAGAG	For cloning of *pvcipk2* into entry vector
PvCIPK2-TOPOant	TCATTCAAGCACTATGGCAGGTGTG	For cloning of *pvcipk2* into entry vector
PvCIPK3-TOPOsen	CACCATGGATCCGCAGAACGAACAC	For cloning of *pvcipk3* into entry vector
PvCIPK3-TOPOant	CTAATTGTTGCAGAAGTGATTGGTA	For cloning of *pvcipk3* into entry vector
PvCIPK4-TOPOsen	CACCATGGAAGAGTGTCACACGGTG	For cloning of *pvcipk4* into entry vector
PvCIPK4-TOPOant	TTATACAGTGCTAACGACCTGCTTT	For cloning of *pvcipk4* into entry vector
PvCIPK5-TOPOsen	CACCATGATGGGTGAGAAAAGCAGC	For cloning of *pvcipk5* into entry vector
PvCIPK5-TOPOant	TCAAGCAGGTGTAGGATTCTCAGGA	For cloning of *pvcipk5* into entry vector
PvCBL1-TOPOsen	CACCATGTTGCAGTGCTTAGAGGGA	For cloning of *pvcbl1* into entry vector and for RT-PCR of *pvcbl1*
PvCBL1-TOPOant	TCAAGTATCGTCTACTTGTGAATGG	For cloning of *pvcbl1* into entry vector and for RT-PCR of *pvcbl1*
PvCBL2-TOPOsen	CACCATGGTTCAGTGCCTAGACGGA	For cloning of *pvcbl1* into entry vector and for RT-PCR of *pvcbl2*
PvCBL2-TOPOant	TCAAGTATCATCAACTTGTGAGTGA	For cloning of *pvcbl1* into entry vector and for RT-PCR of *pvcbl2*

I, inosine; Y, T/C; N, A/T/G/C; R, A/G.

4.3. Isolation of cDNA clones and their sequence analysis

To isolate cDNA clones encoding CIPKs and CBLs, we screened a cDNA phage library generated from the RNA of mid-size developing seeds (Hamada et al., 2001). To amplify partial cDNA fragments for the protein kinases, the first strand cDNA mixtures prepared from the mid-size developing seeds were subjected to RT-PCR with the two degenerated primers (PvPK-SEN and PvPK-ANT, Table 1). Similarly, to amplify the cDNA fragments of the CBL proteins, the same procedure was followed using the two specific primers (CBL-SEN and CBL-ANT, Table 1). The amplified fragments were subcloned into pT7Blue T-vector (Novagen, Madison, WI, USA), sequenced on both strands, and used as probes for screening. Each probe was prepared from cloned PCR fragments labeled with a digoxigenin (DIG) DNA-labeling kit (Roche Diagnostics K.K., Tokyo, Japan), and screened as previously described (Hamada et al., 2001). The cDNA inserts of the positive clones were subcloned into pBluescript II SK(+) (Stratagene, CA, USA) after amplification by PCR using the primer set (lgt10-forword and lgt10-reverse, Table 1). The plasmids carrying the protein kinase cDNAs (*pvcipk1–5*) and CBL cDNAs (*pvcbl1* and *2*) were named pBS-PvCIPK1–pBS-PvCIPK5 and pBS-PvCBL1–pBS-PvCBL2, respectively. Nucleotides were sequenced by the dideoxynucleotide chain-termination method using an automated DNA sequencer (ABI PRISM 310; Applied Biosystems Japan Ltd., Tokyo, Japan). The complete nucleotide sequences of the open reading frames of PvCIPKs and PvCBLs have been submitted to the DDBJ/EMBL/GenBank DNA database under Accession Nos. AB378090–AB378094 and Nos. AB378095–AB378096, respectively.

4.4. Semi-quantitative RT-PCR analysis

The first strand cDNA mixtures were used for semi-quantitative PCR to analyze the transcript levels from developing seeds and leaves. Using the specific primer sets (Table 1), PCR amplification was performed with initial denaturation at 98 °C for 30 s, followed by 27 cycles of incubation at 95 °C for 10 s, 55 °C for 5 s and 72 °C for 30 s, with a final extension at 72 °C for 1 min (Table 1). The expression level for actin gene was used as a quantitative control. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized under UV light with ethidium bromide.

4.5. Yeast two-hybrid assay

To examine whether there was a direct *in vivo* interaction between the CIPKs and CBLs, we employed a yeast two-hybrid system (ProQuest™ Two-Hybrid System, Invitrogen Japan K.K., Tokyo, Japan). The pDEST32 and pDEST22 vectors that carry the GAL4 DNA-binding domain and the GAL4 DNA-activation domain, respectively, were used to construct the bait and prey plasmids. The coding regions of *pvpick1–5* and *pvcbl1* and *2* were amplified with each primer set (Table 1) and inserted in the pENTR/D-TOPO vector. The inserted *pvpick1–5* fragments were recombined in the pDEST32 vector using LR Clonase II (Invitrogen) to yield the bait plasmids (pDEST32/PvCIPK1–5). Likewise, the inserted *pvcbl1* and *2* were recombined in the pDEST22 vector to generate the prey plasmids (pDEST22/PvCBL1 and 2). Each combination of the bait and prey plasmids was introduced into the MaV203yeast strain (Dohmen et al., 1991). Transformants were plated on synthetic complete (SC) medium without tryptophan and leucine. Interaction assays were performed on plates containing the same medium supplemented with 100 or 150 mM of 3-aminotriazole (3-AT).

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