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The NAF domain defines a novel protein–protein interaction module conserved in Ca^{2+}-regulated kinases

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The *Arabidopsis* calcineurin B-like calcium sensor proteins (AtCBLs) interact with a group of serine-threonine protein kinases (AtCIPKs) in a calcium-dependent manner. Here we identify a 24 amino acid domain (NAF domain) unique to these kinases as being required and sufficient for interaction with all known AtCBLs. Mutation of conserved residues either abolished or significantly diminished the affinity of AtCIPK1 for AtCBL2. Comprehensive two-hybrid screens with various AtCBLs identified 15 CIPKs as potential targets of CBL proteins. Database analyses revealed additional kinases from *Arabidopsis* and other plant species harbouring the NAF interaction module. Several of these kinases have been implicated in various signalling pathways mediating responses to stress, hormones and environmental cues. Full-length CIPKs show preferential interaction with distinct CBLs in yeast and *in vitro* assays. Our findings suggest differential interaction affinity as one of the mechanisms generating the temporal and spatial specificity of calcium signals within plant cells and that different combinations of CBL–CIPK proteins contribute to the complex network that connects various extracellular signals to defined cellular responses.

Keywords: AtCBL/AtCIPK/calcium sensor proteins/NAF domain/serine-threonine kinases

Introduction

Calcium signalling mechanisms mediate a multitude of responses to external stimuli of biotic and abiotic origin, and regulate a variety of cellular and developmental processes (Clapham, 1995; Dolmetsch *et al*., 1998). In plants, a pivotal function of calcium signals has been established in signalling and adaptive reactions to stress factors, light, phytohormones and pathogens, as well as processes such as root hair elongation, pollen tube growth, guard cell regulation or Nod factor recognition (Malho *et al*., 1998; McAinsh and Hetherington, 1998; Trewavas and Malho, 1998; Sanders *et al*., 1999). Calcium, therefore, simultaneously represents an integrative signal and an important convergence point of many disparate signalling pathways (Clapham, 1995; Bootman *et al*., 1997; Sanders *et al*., 1999). Specificity of signal transduction and co-ordination of numerous cellular processes occurring in parallel are achieved by a tight regulation of the spatial and temporal occurrence of calcium transients and the intensity of their amplitudes (Dolmetsch, 1998; Li *et al*., 1998; Malho *et al*., 1998; McAinsh and Hetherington, 1998). Although this complex network of calcium transients may partially account for the specificity of cellular responses triggered by individual stimuli, an additional level of regulation in calcium signalling is achieved via calcium-binding proteins, such as calmodulin, calcineurin B and frequenin (Snedden and Fromm, 1998; Zielinski, 1998; Hemenway and Heitman, 1999). Such proteins sense and forward local changes in calcium concentration to their specific target proteins. Although calcium-binding proteins usually do not have an enzymatic activity on their own, binding of calcium ions results in an increased affinity for and subsequent enzymatic activation or deactivation of interacting proteins (Roberts and Harmon, 1992; Snedden and Fromm, 1998). Phosphorylation cascades regulated by protein kinases and phosphatases are typical targets of calcium-binding proteins and represent primary downstream transduction routes interpreting calcium signals (Hunter, 1995; Soderling, 1999). Alternatively, calcium transients can also be perceived and transmitted directly by calcium-dependent kinases and phosphatases (Leung *et al*., 1994; Soderling, 1999; Harmon *et al*., 2000). In plants, various calcium/calmodulin-dependent kinases and phosphatases have been identified, and a function for these proteins in processes such as hormone signalling, guard cell regulation, stress and pathogen response has been established (Leung *et al*., 1994; Sanders *et al*., 1999; Harmon *et al*., 2000).

Although considerable progress has been made in elucidating physiological processes involving calcium signalling in plants, and several of the participating protein components have been identified, the mechanisms of generating the temporal and spatial specificity of these signals are only beginning to be understood.

Recently, two studies reported the identification of novel calcium sensor proteins from plants (Liu and Zhu, 1998; Kudla *et al*., 1999). These proteins are most similar to the regulatory B subunit of calcineurin (CNB) and neuronal calcium sensors (NCS) from animals. Like other calcium-binding proteins, these plant CBLs (calcineurin B-like proteins) contain typical EF-hand motifs providing the structural basis for calcium binding. Expression of AtCBL1 is strongly induced by drought, cold and wounding stress, suggesting a function for this calcium sensor protein in the respective signalling cascades. AtCBL4/SOS3 (salt overlay sensitive) was identified by positional cloning of the *Arabidopsis SOS3* locus. Loss-of-function mutants of this gene render plants hypersensitive to sodium and affect the cellular sodium/potassium homeostasis. Taken together, these findings suggested a function

for this unique family of calcium sensor proteins in plant signal transduction processes in response to stress stimuli.

A family of novel serine-threonine kinases was identified as cellular targets of the AtCBL1 calcium sensor protein by using two-hybrid screens (Shi *et al*., 1999). Sequence analysis of these CIPKs (CBL-interacting protein kinases) revealed a two-domain structure. The N-terminal part of these proteins comprises a conserved domain typical of serine-threonine kinases and most related to SNF-like and AMP-dependent kinases from various organisms (Hardie *et al*., 1998). A novel C-terminal domain was found to be unique to this subgroup of kinases and showed a considerable degree of conservation only with other plant SNF-like kinases. A 150 amino acid region in this domain proved to be required and sufficient for interaction with AtCBLs (Shi *et al*., 1999). Moreover, interaction of AtCBL1 and AtCIPK1 required micromolar concentrations of calcium, suggesting that calcium-dependent complex formation between AtCBL1 and AtCIPK1 provides a regulatory mechanism in deciphering cellular calcium signals. Subsequent positional cloning of the *Arabidopsis SOS2* gene, another mutated locus rendering plants hypersensitive to salt stress, revealed that the *SOS2*-encoded protein also belongs to this specific family of protein kinases (Liu *et al*., 2000). SOS2 interacted most strongly and preferentially with the AtCBL4/SOS3 calcium sensor protein in yeast two-hybrid assays (Halfter *et al*., 2000). These elegant genetic approaches therefore led to the identification of a plant salt stress-specific signalling cascade involving the calcium sensor protein AtCBL4/SOS3 and the CBL-interacting kinase SOS2.

In this study, we have identified a novel protein interaction domain (NAF domain) permitting interaction of AtCBL calcium sensors with their target kinases. Mutational analysis revealed amino acid residues critical for the observed interaction. Extensive two-hybrid screens with several CBLs led to the isolation of 15 potentially interacting kinases. Additionally, the NAF domain defines a group of heterologous kinases implicated in different signalling processes, including light, hormone and stress responses from numerous plant species, as targets of CBL calcium sensor proteins. Comparative protein interaction assays revealed preferential interaction of several CBL–CIPK combinations. This differential affinity might add to the mechanisms generating a temporal and spatial specificity of calcium-triggered processes in plant cells. Together with other factors, such as expression pattern, subcellular localization and differential calcium dependence of protein–protein interaction and kinase activity, the multitude of possible CBL–CIPK combinations may provide a novel mechanism to integrate and specifically decode the calcium signalling system in plant cells.

Results

AtCBL2 interacts with a 24 amino acid C-terminal domain of AtCIPK1

A C-terminal domain covering amino acid positions 276–398 of AtCIPK1 has been shown to be required and sufficient for interaction with AtCBL1–4 (Shi *et al*., 1999). Yeast two-hybrid assays were applied to map and narrow down further the peptide motif responsible for AtCBL–AtCIPK1 interaction. A series of deletion constructs (KinD1–KinD9) was generated by cloning AtCIPK1 fragments into the pGAD.GH activation domain vector. The constructs were then transformed into yeast strains harbouring the pGBT.AtCBL2 plasmid. To avoid potential interference of a possible myristylation and subsequent membrane localization of AtCBL1 in the two-hybrid assays, AtCBL2 instead of AtCBL1 was chosen for these analyses. Interactions were assayed by growth on selective medium due to the activation of the nutritional marker gene *HIS3*. Activation of the second reporter gene *lacZ* was monitored by measuring the β-galactosidase activity (Figure 1A). In control experiments, neither AtCBL2 nor the kinase fragments activated reporter gene expression when co-transformed with an empty vector, while a peptide harbouring amino acids 312–336 (KinD8) was sufficient to mediate interaction with AtCBL2 (Figure 1B). A fragment covering amino acid residues 319–369 (KinD6) did not yield detectable reporter gene activation, indicating that the seven N-terminal amino acids of KinD8 represent a motif indispensable for AtCBL2–AtCIPK1 interaction. In addition, a construct harbouring the complete coding region of AtCIPK1 except amino acids 312–336 (KinD9) did not interact with AtCBL2.

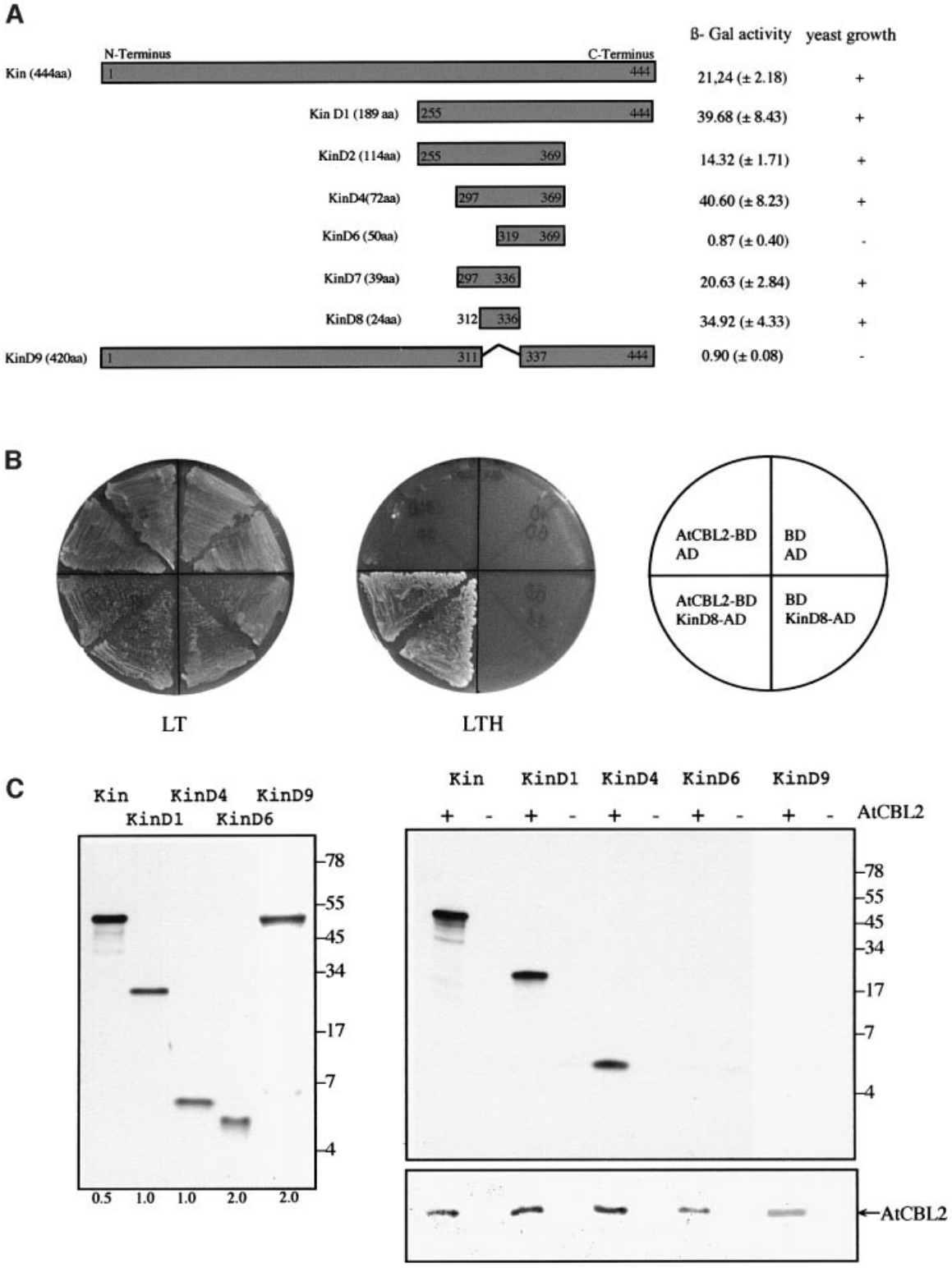
To corroborate these results, we investigated the AtCBL2–AtCIPK1 interaction *in vitro*. Full-length AtCIPK1 (Kin) as well as KinD1, KinD4, KinD6 and KinD9 were expressed and ^{35}S-labelled *in vitro* (Figure 1C, left panel). Subsequently, the different polypeptides were incubated with or without recombinant AtCBL2-His$_6$ protein. Co-affinity purification was performed on Ni-NTA beads, and kinase polypeptides were visualized by autoradiography. As shown in Figure 1C (right panel), full-length AtCIPK1 (Kin), KinD1 and KinD4, but not

Fig. 1. A 24 amino acid motif in AtCIPK1 is sufficient for interaction with AtCBL2. (**A**) The complete coding region (Kin) and different deletion constructs of AtCIPK1 (KinD1–9) were cloned into the pGAD.GH vector and introduced into yeast reporter strains containing the pGBD.AtCBL2 plasmid. Yeast growth (+ or –) was monitored on selective media (SC –Leu, –Trp, –His; supplemented with 25 mM 3-AT) and β-galactosidase activity was estimated as described in Materials and methods. The amino acid positions covered by each construct are indicated in the bars. The length of each peptide is depicted separately in parentheses. (**B**) Representative two-hybrid assay with the KinD8 construct. The arrangement of the yeast strains containing the different plasmids is indicated in the circle on the right. AD and BD refer to the Gal4 activation domain and binding domain plasmids, respectively. (**C**) *In vitro* interaction of AtCBL2 with a set of representative AtCIPK1 polypeptides. Left panel: autoradiograph of *in vitro* transcribed/translated Kin polypeptides. The indicated polypeptides were produced in reticulocyte lysate in the presence of [^{35}S]methionine. A 0.5–2.0 µl aliquot of the translation reactions was separated by SDS–PAGE to analyse the *in vitro* expression of the polypeptides by autoradiography. Right panel: ^{35}S-labelled Kin polypeptides (see left panel) and recombinant AtCBL2-His$_6$ protein were co-incubated for 2 h on ice (+ AtCBL2). Co-purification was carried out on Ni-NTA–Sepharose and bound protein complexes were eluted with an EDTA-containing buffer. As a control, the assay was also performed without addition of recombinant protein (– AtCBL2). Eluted samples were separated on two separate SDS–polyacrylamide gels. One gel was autoradiographed (upper part) and the other was analysed by western blotting using an antiserum produced against AtCBL1 to verify AtCBL2-His$_6$ purification (lower part). Molecular weight standards in kilodaltons are indicated on the right.

KinD6 and KinD9, interacted with AtCBL2-His_6 *in vitro*, thus confirming our yeast two-hybrid results.

Identification of the amino acid residues critical for AtCBL2–AtCIPK1 interaction

To elucidate further the amino acids essential for the protein–protein interaction, we mutated individual residues within the identified minimum peptide motif. Protein interactions were investigated initially by using the yeast two-hybrid system. As depicted in Figure 2A, conversion of Ala319 into the imino acid proline completely abolished binding of AtCBL2. Since this alanine is centred in a highly conserved NAF motif (Figure 4), we refer to the 24 amino acid peptide as the 'NAF domain'. In addition, mutation of the aliphatic amino acid Leu334 to a charged amino acid (aspartic acid) also prevented complex



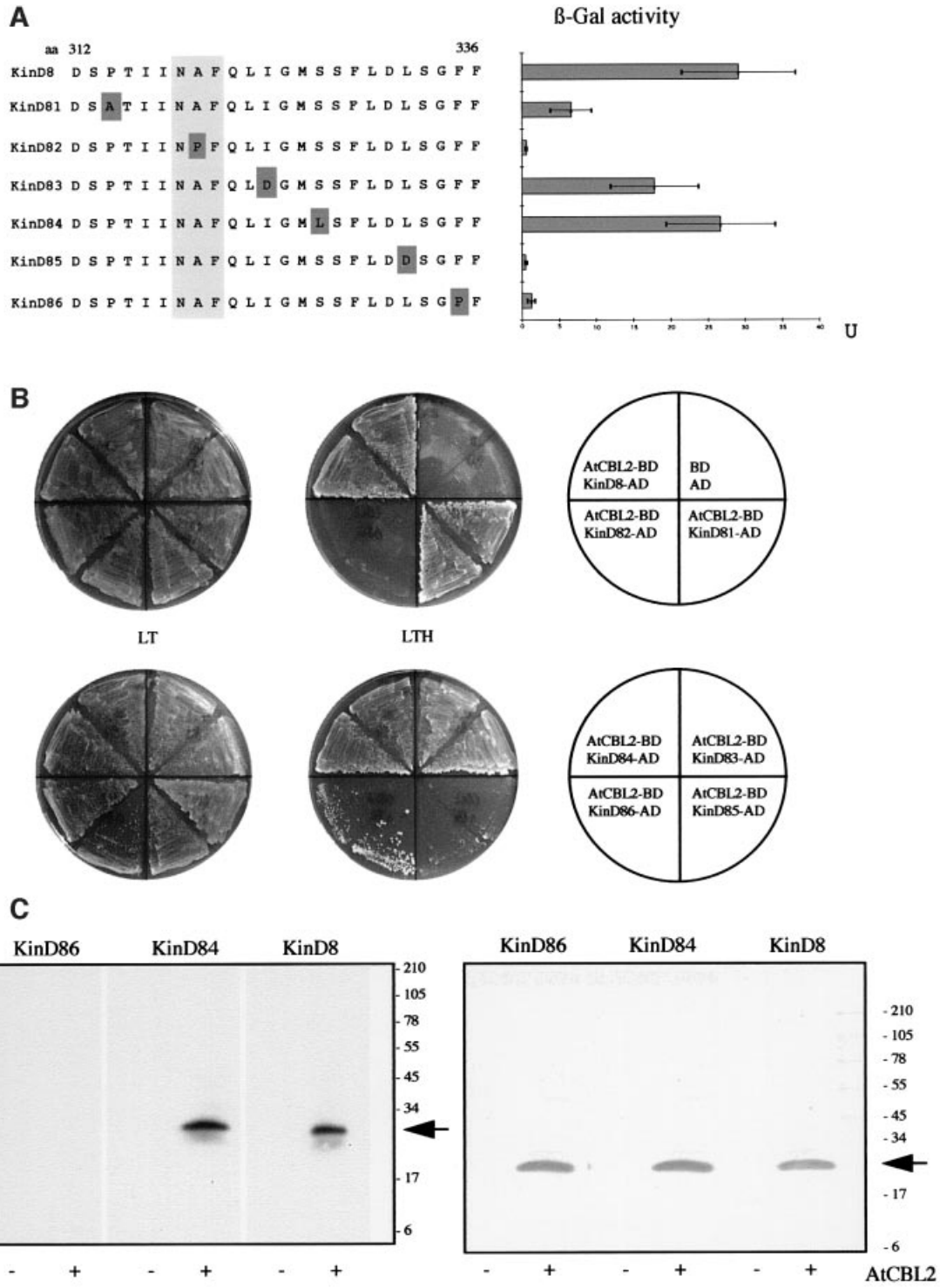


Fig. 2. Effect of mutations in the NAF domain of AtCIPK1 on binding to AtCBL2. (**A**) Mutational analysis of the NAF domain. The wild-type sequence of the NAF domain of AtCIPK1 is presented in the uppermost line (KinD8) with amino acid positions indicated on top. Amino acid residues substituted by mutagenesis are dark shaded. The conserved NAF motif is marked by a light grey background. The β-galactosidase activity was measured as described in Materials and methods. The measured activity is indicated in the graph on the right. (**B**) The yeast strain SMY3 harbouring the pGBD.AtCBL2 plasmid was transformed with vectors harbouring either the 24 residues of the wild-type NAF domain or the indicated mutated versions fused to the Gal4 activation domain. As controls, yeast cells were transformed with either vectors pGBD.BS and pGAD.GH or a combination of these vectors with the corresponding vectors expressing AtCBL2 or the NAF domain of AtCIPK1. The yeast cells were grown for 3 days at 30°C on SC medium lacking Leu and Trp (LT) or SC medium without Leu, Trp and His supplemented with 25 mM 3-AT. The array of the yeasts containing the different plasmids is indicated in the scheme on the right. (**C**) *In vitro* interaction of AtCBL2 with a set of representative KinD8 polypeptides. A 5 µg aliquot of recombinant AtCBL2-His_6 per lane (+ AtCBL2) or control samples without recombinant protein (– AtCBL2) were run on two separate SDS–polyacrylamide gels and transferred to PVDF membranes. The first membrane was cut into three pieces (two lanes each) and each piece was incubated for 1 h with the indicated *in vitro* transcribed/translated, ^{35}S-labelled KinD8 polypeptide. After washing, the membranes were analysed for bound kinase polypeptides by autoradiography (left panel). The second membrane was probed with an antiserum produced against AtCBL1 to confirm equal loading in all lanes (right panel). The arrows show the position of AtCBL2-His_6. Molecular weight standards in kilodaltons are indicated on the right.

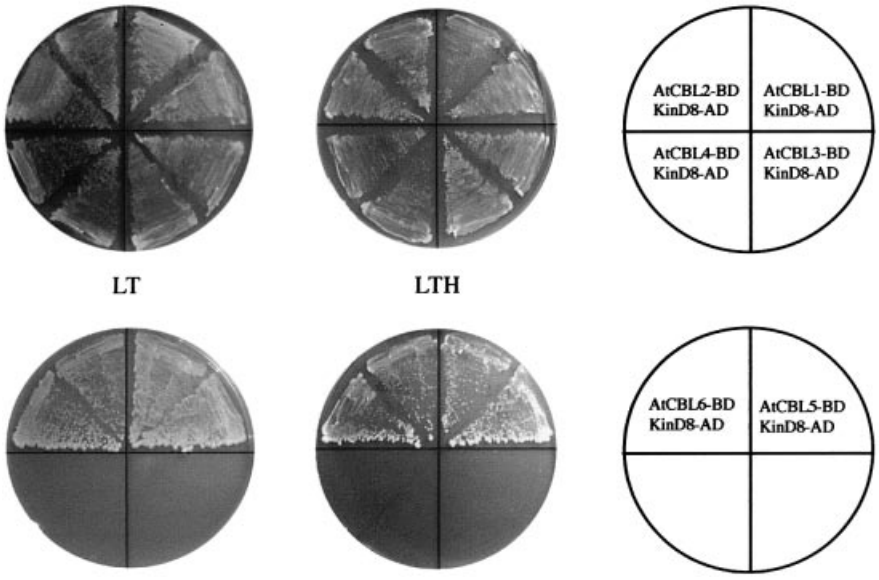


Fig. 3. The NAF domain mediates interaction with all known *Arabidopsis* CBL proteins. The yeast strain SMY3 containing the pGAD.KinD8 construct was transformed with pGBD.BS plasmids expressing the six different CBL proteins. The presence of both plasmids was monitored by growth on synthetic complete media lacking Leu and Trp (LT). Protein interaction was assayed on media lacking Leu, Trp and His, and supplemented with 25 mM 3-AT (LTH). The arrangement of the different yeast strains on the plates is depicted in the schemes on the right.

formation. Exchange of Pro314 by alanine significantly diminished the observed interaction, underscoring the importance of the far N-terminal residues of the interaction domain. In contrast, mutations in the central part of the NAF domain, such as the conversion of Ile322 to aspartic acid or of Ser325 to leucine, interfered only weakly with AtCBL2 interaction or had no effect (Figure 2).

To confirm our two-hybrid results, *in vitro* overlay assays with a representative set of *in vitro* transcribed/translated, ^{35}S-labelled KinD8 polypeptides and recombinant AtCBL2-His$_6$ were performed. While we did not detect binding of KinD86 to AtCBL2-His$_6$, KinD8 and KinD84 specifically interacted with AtCBL2-His$_6$ *in vitro* (Figure 2C), supporting the yeast two-hybrid data.

The NAF domain mediates interaction with all known AtCBL proteins

Comparison of the previously identified CIPKs (AtCIPK1–4; Shi *et al*., 1999) revealed that the NAF domain is highly conserved between these proteins. We therefore investigated the possibility that this domain serves as a general CBL-interacting module and potentially mediates complex formation with different CBL proteins. Since at least AtCBL1–3 show a very high degree of similarity (between 63 and 93% amino acid sequence identity), we attempted to identify additional CBL proteins and include them in this study to broaden the conclusions from these experiments. Database analysis applying the BLASTN and BLASTX algorithms identified two genomic bacterial artificial chromosome (BAC) clones (DDBJ/EMBL/GenBank accession Nos F3D13 and ATFCA6) potentially encoding novel CBL proteins. Both cDNAs were amplified by RT–PCR, cloned and sequenced. AtCBL5 (DDBJ/EMBL/GenBank accession No. AF192885) encodes a predicted polypeptide of 22.2 kDa with 49% amino acid identity and 67% similarity to AtCBL1. The predicted 26 kDa AtCBL6 protein (DDBJ/EMBL/GenBank accession No. AF192884) displays 55% identity and 75% similarity to AtCBL1.

The yeast strain SMY3 containing the pGAD.KinD8 plasmid was transformed with binding domain plasmids encoding AtCBL1–6 and the clones were analysed for *in vivo* interaction in yeast. As depicted in Figure 3, all investigated AtCBLs interacted with KinD8 with comparable intensity, as indicated by growth on selective media and colour lift assays (not shown). In contrast, the controls including calmodulin 4 from *Arabidopsis* induced no reporter gene expression (data not shown). These findings suggest that the NAF domain of AtCIPK1 is necessary and sufficient to mediate interaction with all known plant CBL proteins.

The NAF domain is a conserved CBL interaction module

Having established that the NAF domain of AtCIPK1 is sufficient to mediate protein interaction with all CBL proteins, it appeared important to investigate whether this domain could be an interaction module characteristic of protein kinases that are targets of this particular class of calcium sensor proteins. As shown in Figure 4A, this motif is conserved in at least all those CIPKs isolated in our initial two-hybrid screen with AtCBL1 as bait. We therefore performed database analysis with the complete AtCIPK1 amino acid sequence and separately with the NAF domain. The N-terminal kinase domain showed a high degree of conservation with SNF-like kinases from a wide range of organisms. In contrast, analysis with the NAF domain revealed homology only to a plant-specific subgroup of these proteins (Figure 4B). These kinases included SNFL2 and 3 from sorghum (Annen and Stockhaus, 1998), PK4 and 7 from rice, and additional kinases from wheat, ice plant and maize (Sano and Youssefian, 1994; Ohba *et al*., 2000). Interestingly, the amino acid residues identified as being important for

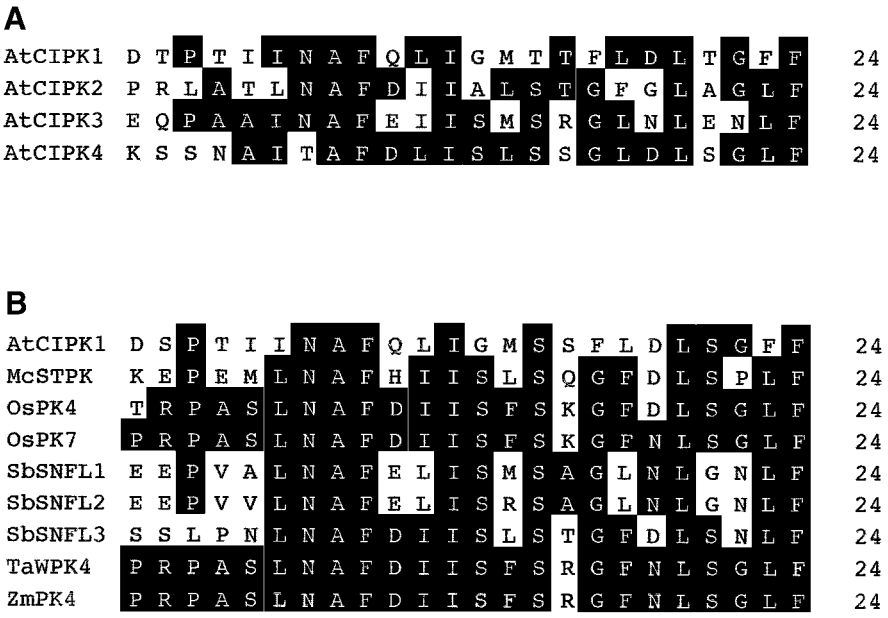


Fig. 4. The NAF domain represents a conserved protein–protein interaction module. (**A**) Alignment of the 24 amino acid polypeptide NAF domain of AtCIPK1, AtCIPK2, AtCIPK3 and AtCIPK4 (DDBJ/EMBL/GenBank accession Nos AF302112, AF286050, AF286051 and AY007221) corresponding to the protein–protein interaction domain identified in the interaction assays. Residues on a black background indicate conserved amino acids. (**B**) Amino acid alignment of the NAF domain of AtCIPK1 with the corresponding domains of WPK4 from wheat (TaWPK4), PK 4 and 7 from rice (OsPK4 and OsPK7), sorghum SNFL1–3 (SbSNFL1–3), PK4 from corn (ZmPK4) and STPK from ice plant (McSTPK). (DDBJ/EMBL/GenBank accession Nos for the complete proteins are: BAA34675.1, BAA83688.1, BAA83689.1, T14735, T14736, T14822, AAF22219.1 and AAD31900.) Amino acid residues conserved in the compared sequences are shown on a black background.

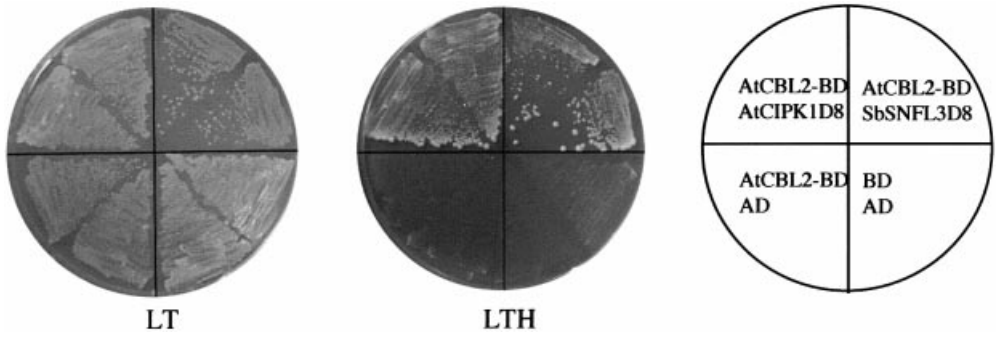


Fig. 5. The NAF domain of heterologous kinases interacts with AtCBL2. The cDNA fragment encoding the NAF domain of sorghum SNFL3 kinase was cloned into the pGAD.GH vector. pGAD.SNFL3D8 and pGBD.AtCBL2 plasmids (and several plasmid combinations used as controls) were introduced into the yeast strain SMY3 and the presence of both plasmids was selected for by growth on media without Leu and Trp (LT). Protein interaction was monitored by growth on media lacking Leu, Trp and His, and supplemented with 25 mM 3-AT (LTH). The plasmid combinations are indicated in the circle on the right.

interaction with CBL proteins appeared to be most conserved in all these different kinases (Figure 4B). We therefore investigated whether or not AtCBL2 would also interact with a NAF domain from a heterologous kinase. The 24 amino acid region corresponding to the NAF domain of sorghum SNFL3 kinase was amplified by PCR, cloned into pGAD.GH vector, sequenced and transformed into yeast containing the pGBD.AtCBL2 plasmid. Growth on selective media (Figure 5) and colour lift assays (not shown) clearly indicated that AtCBL2 interacts with the NAF domain of sorghum SNFL3, although not as strongly as with the corresponding domain of AtCIPK1. This difference may reflect the evolutionary distance between the two species. The fact that both proteins are nevertheless capable of forming a complex suggests that the NAF domain is a common protein interaction module of this class of kinases and therefore defines these proteins as targets of calcium signals transduced by CBL proteins.

Comprehensive two-hybrid screens identify a multitude of CIPKs from Arabidopsis

Recently, we reported the identification of four AtCBL1-interacting kinases (Shi *et al.*, 1999). The existence of at least six different CBL proteins in *Arabidopsis* raised the possibility that even more than four kinases may participate in CBL–CIPK complex formation. We therefore performed comprehensive two-hybrid screens to identify additional target kinases of CBL proteins. The cDNAs coding for AtCBL1, 4 and 6 were cloned into the

pGBD.BS vector and used to screen *Arabidopsis* expression libraries generated from etiolated and light-grown plants. Between 48 and 192 independent colonies from each screen were selected for further characterization. The activation domain plasmids were rescued in *Escherichia coli* and analysed by partial sequence determination of their cDNA inserts. Database analysis indicated that most (up to 80%) of the sequenced clones from each screen encoded potential serine-threonine kinases.

Sequence alignments revealed that the identified 168 cDNA clones define a family of 15 closely related kinases (AtCIPK1–15). All of the analysed clones contained partial cDNAs lacking parts of the N-terminal kinase domain, but encode the C-terminal NAF domain. The distribution of the identified kinases varied considerably between different CBL baits and different cDNA libraries. The largest number of individual kinases (11) was identified with AtCBL1. Some CIPKs (e.g. AtCIPK6 and 9) were identified as potential interactors of all three AtCBLs, while AtCIPK14 was only isolated in a screen with AtCBL6. Complete genomic sequences of these 15 AtCIPKs were obtained by searching the GenBank database. Two additional genomic sequences (DDBJ/EMBL/GenBank accession Nos AC018722 and AC007932) encoding putative AtCIPKs were identified in sequences released by the *Arabidopsis* Genome Initiative. cDNAs representing the complete coding regions of 15 kinases were generated by RT–PCR and were found to encode putative proteins with a molecular weight ranging from 47.8 to 55.0 kDa.

The predicted amino acid sequences of all identified kinases exhibit the typical two-domain structure of this protein family (Figure 6A). Pairwise amino acid alignments revealed a high degree of conservation in the N-terminal kinase domain ranging from 51 to 90% sequence identity and 63–96% similarity. In contrast, the C-terminal non-catalytic region appears to be much less conserved, exhibiting an amino acid identity of 24–58% and a similarity of 36–69%. The only exception is the 24 amino acid NAF domain (58–86% identity, 66–94% similarity), forming an 'island of conservation' in this otherwise variable region. In particular, the amino acid residues identified as critical for AtCIPK1 interaction with CBL proteins appear to be invariant.

Phylogenetic analysis (Figure 6B) revealed that the plant CIPK proteins form a distinct group of kinases clearly separated from other SNF-like proteins. This probably monophyletic subclass of kinases appears to represent a unique family of novel kinases, which specifically evolved in plants. The fact that specific AtCIPKs cluster with kinases from other plant species (e.g. sorghum SNFL2 and 3 or ice plant STPK) may reflect a functional relationship.

Preferential affinity triggers specific CIPK–CBL complex formation

The substantial number of identified CBL and CIPK proteins suggests a complex scenario of many possible combinations of different CBL calcium sensor molecules with different CIPK proteins. In addition, the expression pattern of previously described CBL genes and of genes coding for NAF domain-containing kinases may indicate a function for these proteins in distinct cellular processes. However, it is likely that at least some of the CBL–CIPK complexes will be assembled in temporal and local competition. We therefore investigated whether or not preferential interaction of specific CBL proteins with defined kinases could contribute to the required specificity in decoding and transducing the various calcium signals.

Activation domain plasmids expressing the complete coding regions of the kinases were introduced into yeast strains containing pGBD.AtCBL1–6 plasmids. As depicted in Figure 7A, most of the investigated potential complex-forming combinations showed a differential reporter gene activation, indicating preferential complex formation. Of the kinases investigated, AtCIPK1 interacted exclusively with AtCBL1, 2 and 3. Complex formation with AtCBL2 and AtCBL3 was only observed for AtCIPK4, 7, 12 and 13. AtCIPK6 interacted preferentially with AtCBL2 and to some extent also with AtCBL1 and 3. AtCBL5 showed protein interaction only with AtCIPK2 and AtCIPK11. AtCIPK9 appeared to form a complex exclusively with AtCBL2, but not with any other calcium sensor protein investigated. Although a partial cDNA clone encoding AtCIPK14 was isolated in a two-hybrid screening with AtCBL6, the corresponding full-length protein did not interact preferentially with this protein. Instead, weak reporter gene activation was observed for all six AtCBL–AtCIPK14 combinations. Moreover, AtCBL6 did not appear to interact strongly with any of the analysed kinases. These differences between the full-length proteins and the truncated variants may highlight the importance of the tertiary structure of the complete kinase proteins for the interaction with defined CBLs. None of the analysed kinases showed interaction with AtCBL4/SOS3, which is known to interact with the SOS2 kinase (Halfter *et al*., 2000). To corroborate the yeast results, we investigated the interaction of a set of representative AtCBLs (AtCBL2 and 6) and AtCIPKs (AtCIPK1, 13 and 14) *in vitro* as described above (see Figure 1C). Whereas AtCBL2-His_6 associated with all three selected kinases, we could not detect any interaction with AtCBL6-His_6 (Figure 7B). Interestingly, the differential affinity of AtCBL2-His_6 for the tested kinases already observed in the yeast two-hybrid assay was also found *in vitro* (Figure 7B). Taken together, our data indicate that different full-length CIPK proteins exhibit a preferential interaction affinity for defined CBL proteins.

Discussion

Recent studies identified a new group of calcium sensor proteins (AtCBLs) from *Arabidopsis* most closely related to calcineurin B and NCS from animals (Liu and Zhu, 1998; Kudla *et al*., 1999). Despite their similarity to animal calcineurin B, subsequent analyses revealed that the AtCBL proteins interact with a novel group of serine-threonine protein kinases (AtCIPKs) (Shi *et al*., 1999; Halfter *et al*., 2000).

The aims of this study were to examine the molecular basis of CBL–CIPK interaction and, in particular, to elucidate whether specific complex formation of these calcium sensor proteins with their target kinases could provide a mechanism to decipher and transmit cellular calcium signals precisely. We identified a 24 amino acid domain of AtCIPK1 as being required and sufficient to

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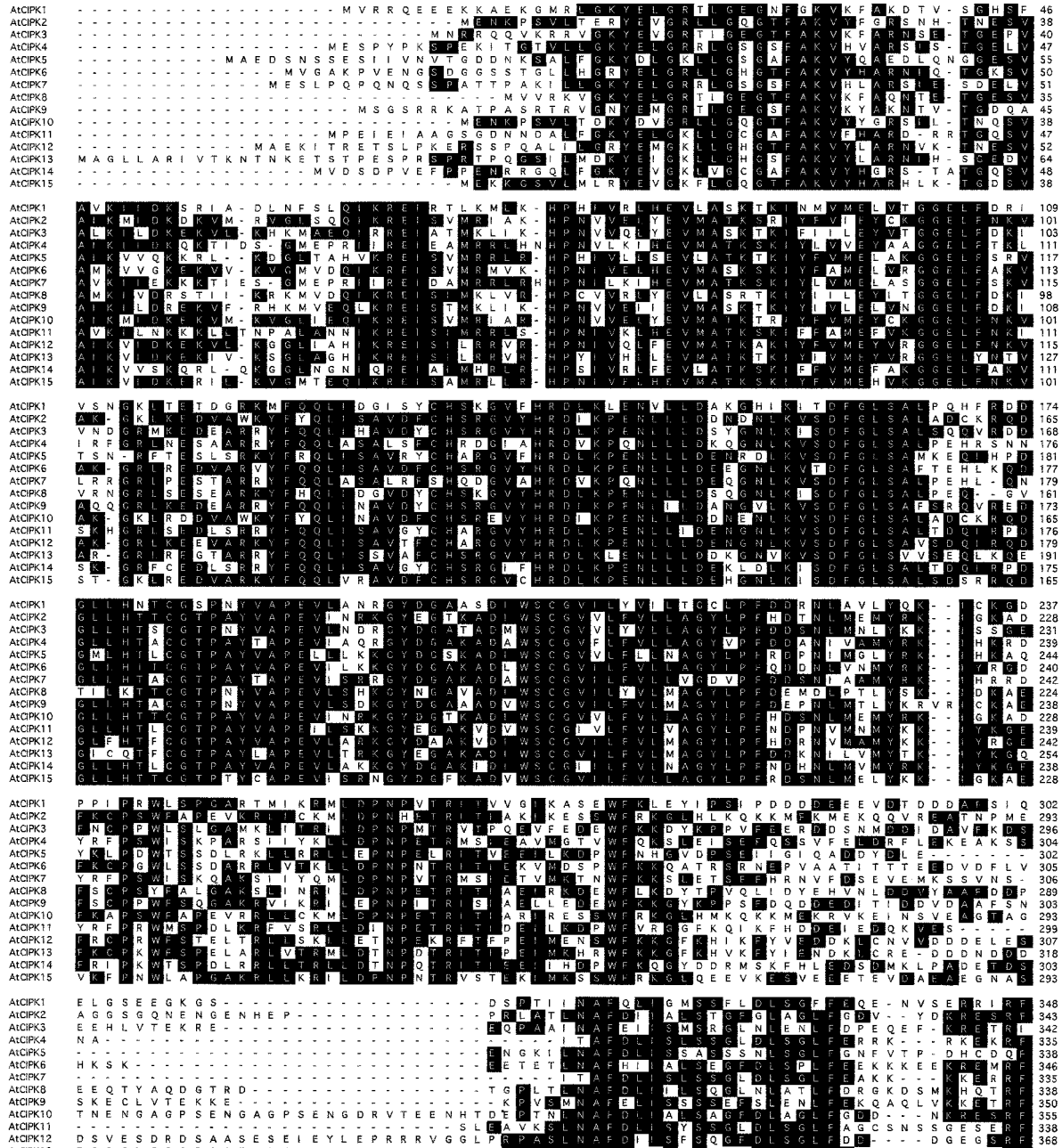
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AtCIPK15

mediate interaction with AtCBL2 *in vitro* and *in vivo*. Because of its prominent conserved amino acids Asn-Ala-Phe, we refer to this domain as the NAF domain. Mutational analysis of single residues within this peptide revealed that substitution of certain conserved amino acids either completely abolished or significantly diminished interaction with AtCBL2. Since two of these effective substitutions (Pro314 to alanine and Phe337 to proline) are located very close to the borders of the investigated peptide, it appears likely that the identified NAF domain represents a minimum protein interaction module. Secondary structure computation for the C-terminal region of AtCIPK1 revealed a long helical structure, which potentially could provide the structural basis for the observed interaction. A reliable three-dimensional structural prediction for the identified 24 amino acid interaction module is currently not feasible since there are no known related structures available in the databases. Therefore, resolution of the detailed structure of this novel protein–protein interaction motif awaits further investigation. Animal calcineurin B, which is closely related to AtCBL2, interacts with a long α-helical region of calcineurin A (Griffith *et al*., 1995). The small size of the NAF domain makes a similar mode of interaction between AtCBL2 and AtCIPK1 relatively unlikely. Recoverin, another animal calcium sensor protein with significant similarity to AtCBL1, interacts in a calcium-dependent manner with rhodopsin kinase (Chen *et al*., 1995). However, our comparison of rhodopsin kinase with AtCIPK1 (data not shown) revealed no sequence similarities within the regions mediating protein–protein interaction, indicating that the observed AtCBL2–AtCIPK1 complex formation relies on a novel mode of interaction. Neuronal calcium sensor NCS-1, another member of the recoverin-type calcium-binding protein family, can substitute or potentiate calmodulin but not calcineurin B effects *in vivo* and *in vitro* (Schaad *et al*., 1996). Therefore, NCS-1 function and most probably its binding preferences appear to be more closely related to those of calmodulin. This is a feature not shared by CBL proteins. The obvious sequence conservation of the identified NAF domain of AtCIPKs, which binds AtCBL-type calcium sensor proteins, suggests a rather specific mode of protein–protein interaction as the molecular basis for the observed complex formation.

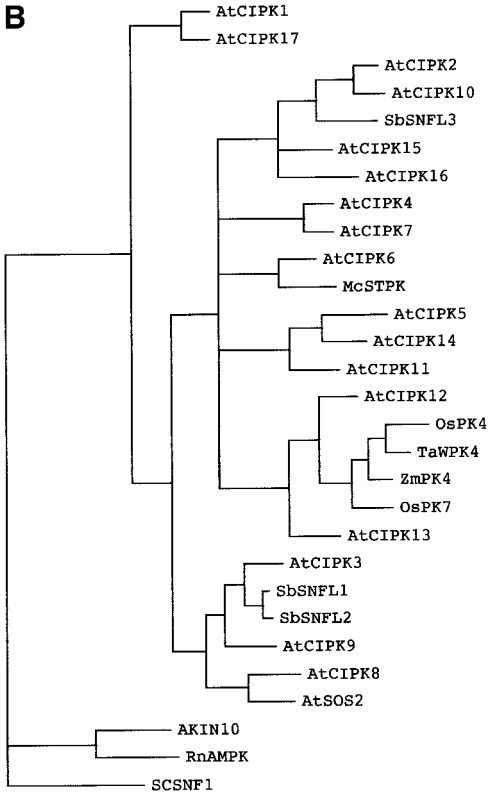

Fig. 6. Sequence comparison and phylogenetic analysis of CIPK proteins. (**A**) Amino acid alignment of CIPK proteins from *Arabidopsis*. The depicted proteins correspond to the following DDBJ/EMBL/GenBank accession Nos: AtCIPK1 (AF302112), AtCIPK2 (AF286050), AtCIPK3 (AF286051), AtCIPK4 (AY007221), AtCIPK5 (AF285105), AtCIPK6 (AF285106), AtCIPK7 (AF290192), AtCIPK8 (AF290193), AtCIPK9 (AF295664), AtCIPK10 (AF295665), AtCIPK11 (AF295666), AtCIPK12 (AF295667), AtCIPK13 (AF295668), AtCIPK14 (AF295669) and AtCIPK15 (AF302111). The alignment was generated using the CLUSTAL method with DNASTAR software. Amino acids conserved in the 15 compared sequences are shown on a black background. The numbers on the right indicate the amino acid position. Dashes mark gaps introduced to improve the alignment. (**B**) Phylogenetic analysis of plant kinases containing the NAF domain. The alignment is based on the 444 amino acid complete coding region of AtCIPK1. The phylogenetic tree shown is a bootstrap parsimony 50% consensus tree. Phylogenetic analysis was performed with the PAUP 4.0 program package (http:/www.Ims.si.edu/PAUP/). For DDBJ/EMBL/GenBank accession numbers of AtCIPKs, see above. The other plant protein kinases containing a NAF domain are described in the legend to Figure 4B. Yeast SNF kinase (SCSNF1, accession No. NP010765), rat AMPK-activated protein kinase (RnAMPK, Q09137) and *Arabidopsis* SNF1-related protein kinase 10 (AKIN10, Q38997) were included in the analysis as representative related kinases.

The identified NAF domain of AtCIPK1 is sufficient to mediate complex formation with all known CBL proteins from *Arabidopsis*. Moreover, the NAF domain from heterologous protein kinases, such as SNFL3 from sorghum, also interacts with AtCBL2 from *Arabidopsis*. This suggests that the NAF module represents a general and conserved CBL interaction domain of protein kinases involved in calcium signalling. Any kinase possessing a NAF domain therefore can be expected to be a potential target of CBL proteins. Consequently, this would indicate that the specific cellular processes mediated by this kinase, as well as the substrates of this kinase, are subject to regulation by calcium signalling.

Which cellular processes could represent the 'areas of operation' of CBL-interacting protein kinases? The AtCBL4/SOS3-interacting kinase SOS2 has been identified as a component of salt stress-induced signal transduction in *Arabidopsis* (Halfter *et al*., 2000; Liu *et al*., 2000). Other kinases containing a NAF domain were isolated as SNF-like kinases from a number of species including sorghum, wheat, corn and rice (Sano and Youssefian, 1994; Annen and Stockhaus, 1998; Ikeda *et al*., 1999; Ohba *et al*., 2000). The expression patterns of these kinases in response to different environmental stimuli appeared to be surprisingly diverse considering the high degree of conservation among the analysed proteins. For example, expression of WPK4 and OsPK4 was up-regulated in response to cytokinins, light and nutrient deprivation, whereas ZmPK4 mRNA levels specifically increased after cold

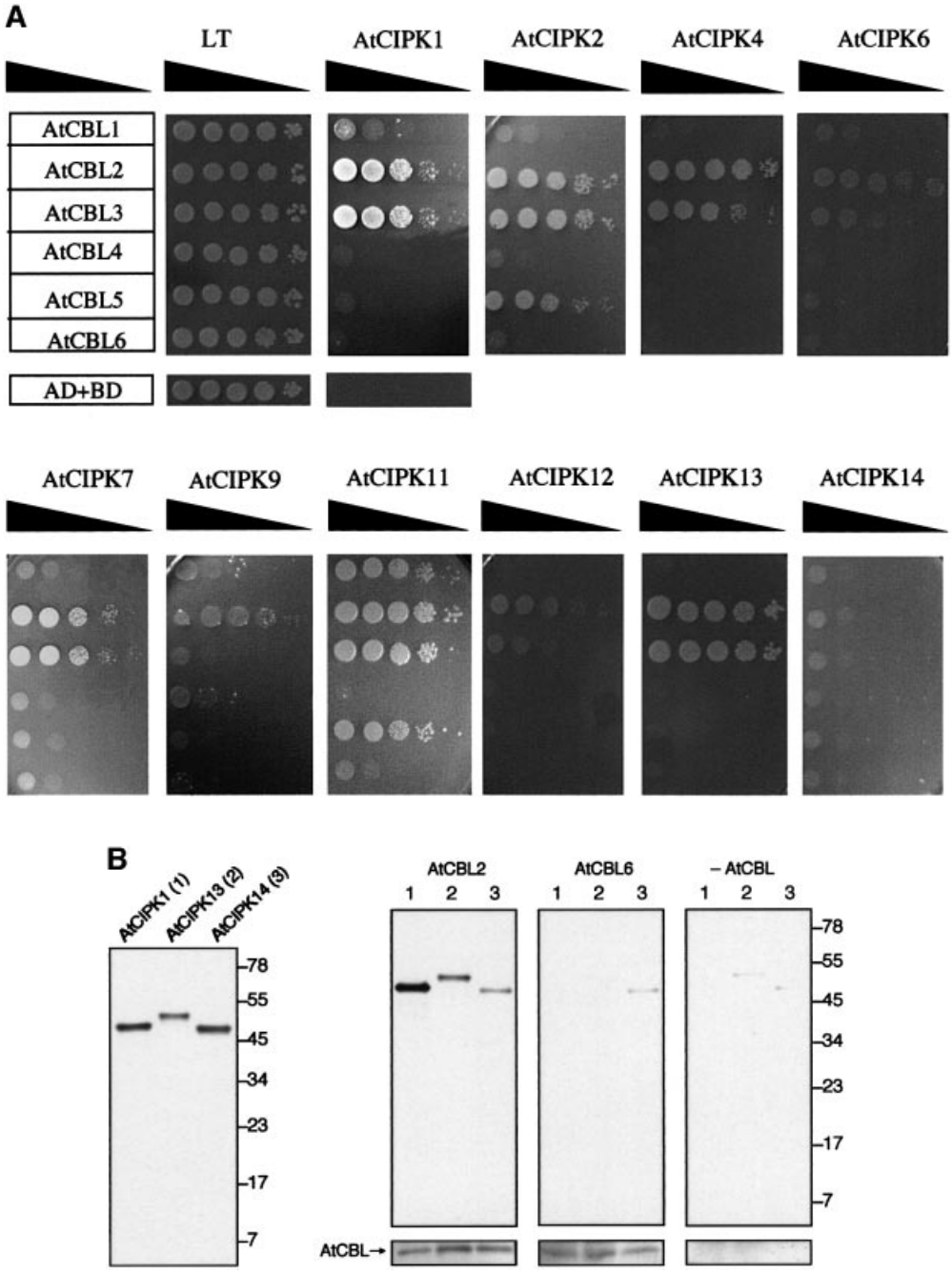

Fig. 7. Specific formation of CBL–CIPK complexes in yeast two-hybrid assays and *in vitro*. (**A**) Yeast SMY3 strains containing the indicated plasmid combinations were grown in SC medium without Leu and Trp to an OD_{600} of 2.0, and 10 µl aliquots of different dilutions (1, 10^{-1}–10^{-4}) were spotted onto selective and non-selective plates (non-selective medium, SC –Leu, –Trp; selective medium, SC –His, –Leu, –Trp, supplemented with 25 mM 3-AT). The combination of plasmids is indicated on the left (pGBD.AtCBL1–6) and at the top (different pGAD.AtCIPKs). Decreasing cell densities in the dilution series are illustrated by narrowing triangles. LT depicts a representative dilution series on non-selective plates. All experiments were carried out at least in duplicate. (**B**) *In vitro* interaction of AtCBL2 and AtCBL6 with AtCIPK1 (lanes 1), AtCIPK13 (lanes 2) and AtCIPK14 (lanes 3). Left panel: autoradiograph of *in vitro* transcribed/translated AtCIPKs. The indicated kinases were produced as described in Figure 1C. A 2.0 µl aliquot of each of the translation reactions was separated by SDS–PAGE followed by autoradiography. Right panel: the indicated ^{35}S-labelled AtCIPKs and recombinant His_6-tagged AtCBL2 or AtCBL6 were co-incubated for 2 h on ice. As a control, the assay was also performed without added CBL protein (– AtCBL). Co-purification of CBL–CIPK complexes and autoradiography of the samples were carried out as described in Figure 1C. Affinity-purified AtCBL proteins were detected by Ni-NTA conjugate recognizing the His_6 tag. Molecular weight standards in kilodaltons are indicated on the right.

stress. This divergence in expression patterns of CIPKs is paralleled by differential expression of the CBL genes. For example, AtCBL1 is the only known CBL gene from *Arabidopsis* whose expression responds strongly to cold, drought and wounding. Taken together, these data suggest that different CBL and CIPK proteins function in specific signalling and/or adaptation processes in plants.

In this study, we have isolated 15 CBL-interacting kinases as potential CBL targets using AtCBL1, 4 and 6 as baits. All *Arabidopsis* CIPKs share a typical two-domain structure comprising a C-terminal catalytic domain with high similarity to yeast SNF and mammalian AMP-dependent kinases, and a less conserved unique C-terminal region responsible for interaction with CBL proteins. This C-terminal domain does not exhibit significant similarity to other SNF or SNF-like kinases. This fact, taken together with the observed interaction with CBL calcium sensor proteins, makes it highly unlikely that the cellular function of CIPKs is related to that of SNF/SNF-like kinases. Rather, it appears reasonable to assume that CIPKs represent a novel class of serine-threonine kinases transmitting cellular calcium-mediated responses comparable with the distinct family of plant calcium-dependent protein kinases (CDPKs). This family of kinases is defined by a C-terminal calmodulin-like calcium-binding regulatory domain (Harmon *et al*., 2000). The plethora of CDPK isoforms identified in plants has led to the assumption that most of the calcium-regulated protein kinase activities in plants are mediated by CDPKs (Sanders *et al*., 1999). Considering the large number of distinct CIPKs described here, this view may need to be revised. Our data suggest that CIPKs, like CDPKs, contribute substantially to the cellular 'tools' deciphering various calcium signals in plant cells.

The number of distinct CIPKs reported here raises the question of how specificity in forwarding different calcium signals to their subsequent response elements is achieved. As known for other protein families, this is likely to be realized by expression of some of these genes in specific tissues, under certain physiological conditions or at distinct developmental stages. A potential regulatory mechanism, i.e. calcium-dependent reversible membrane association of CIPKs by means of myristylation, has already been suggested for AtCBL1 and 4 (Shi *et al*., 1999; Halfter *et al*., 2000). We propose that specific formation of defined CBL–CIPK complexes contributes to generating specificity in decoding various calcium signals occurring in parallel within the plant cell. The immense number of possible CBL–CIPK combinations *in planta* will make it difficult to explore functional interactions of specific CBL proteins with defined target kinases. Specific channelling of calcium signals in plant cells appears to be especially important since, for example, the calcium transients induced by salt stress and cold are similar (Knight *et al*., 1996, 1997) but the final induction of downstream responses needs to be stimulus specific. The interaction specificity of the calcium sensor AtCBL4/SOS3 with the CIPK SOS2 (Halfter *et al*., 2000) could channel such a 'salt-specific' pathway. The separation of drought- and cold-induced signalling from this 'salt-specific' pathway could be achieved via the preferential interaction of AtCBL1 (which is induced by drought and cold but not salt stress) with AtCIPK1. Interestingly, we also observed an interaction of AtCBL1 with AtCIPK11. It is tempting to speculate that both kinases represent targets of AtCBL1 and that the substrate specificity of these kinases provides an additional 'molecular switch' to specifically relay the signal to downstream targets specific for drought or cold responses. At least some of the CIPKs, such as AtCIPK1 and SOS2, appear to exhibit a high substrate specificity, since for example they did not phosphorylate common kinase substrates *in vitro* (Shi *et al*., 1999; Halfter *et al*., 2000). A high substrate specificity of individual CIPKs could also explain the fact that, to date, more CIPKs than CBLs have been identified. Several CIPKs could receive the calcium signal from one and the same CBL protein, but subsequently forward this input precisely to their highly specific target substrates.

Although the interactions observed in two-hybrid assays require further confirmation *in planta*, it appears likely that the observed preferential complex formation of AtCBLs with their target kinases reflects a mechanism contributing to the specificity of calcium signals, which might occur spatially and temporally in parallel in plant cells. In addition, differential cofactor dependence of the CIPKs may contribute as a potential mechanism to establish specificity. AtCIPK1 exhibits a rather unusual Mn^{2+} dependence for kinase activity *in vitro* (Shi *et al*., 1999). In contrast, the SOS2 kinase required only Mg^{2+} as a cofactor in *in vitro* activity assays (Liu *et al*., 2000). The influence of the calcium concentration as a potential regulator of CBL–CIPK interaction is currently only understood fragmentarily, but again diversity between different CBL–CIPK complexes is emerging. While AtCBL1–AtCIPK1 complex formation is strictly calcium dependent *in vitro*, interaction of AtCBL4/SOS3 with the SOS2 kinase does not require calcium under *in vitro* conditions (Shi *et al*., 1999; Halfter *et al*., 2000).

The identification of a novel family of protein kinases as targets of CBL-type calcium sensor proteins adds a new level of complexity to the elaborate cellular network deciphering calcium signals. The recent finding that, despite the existence of multiple isoforms of AtCBLs and AtCIPKs, mutations in single genes reveal analysable phenotypes (Liu and Zhu, 1998; Liu *et al*., 2000; V.Albrecht and J.Kudla, unpublished) will facilitate the exploration of these sophisticated signalling mechanisms by means of reverse genetic approaches in the near future.

Materials and methods

General methods

Molecular biology techniques were performed using standard protocols (Sambrook *et al*., 1989). Total RNA was isolated with Trifast solution (Peqlab) and poly(A) RNA was purified by applying 1 mg of total RNA to oligo(dT) columns (Gibco-BRL). For cDNA synthesis, 3 μg of poly(A) RNA were primed with 3 μg of random hexamer primer and reverse transcription was performed using the Omniskript RT Kit (Qiagen). A list of primers used in the course of this work can be obtained upon request.

Cloning, sequence analysis and plasmid constructions

Genomic sequences encoding AtCBL5 and AtCBL6 were identified by searching the GenBank database with the AtCBL1 cDNA sequence applying the BLASTN and BLASTX algorithms, respectively. Genomic sequences for various AtCIPKs isolated in the two-hybrid screens were also obtained from this database. PCR was performed with Pwo-Polymerase (Peqlab) and primers designed to amplify the complete coding regions and to introduce appropriate restriction sites for cloning into the target plasmids. The amplification products were cloned into the desired plasmids and sequenced with the ALF system (Amersham Pharmacia). All the constructs were verified by sequencing of both DNA strands and subsequent database comparison using the BLAST program. For two-hybrid screens and assays, the vectors pGBT9.BS and pGAD.GH were used (Elledge *et al*., 1991). For additional two-hybrid screens, the pBI880 vector was used to generate bait plasmids (Wang *et al*., 1998). All constructs for protein expression were cloned into pET24b+ (Novagen) for expression of CIPK proteins and peptides, or pQE31 (Qiagen) for CBL proteins.

Yeast two-hybrid screens and interaction assays

The yeast strains Y190, SMY3 (Cardenas *et al*., 1994) and PJ69-4A (James *et al*., 1996) were used for two-hybrid screens and quantitative interaction assays, respectively. The GAL4 activation domain-tagged pACT (Kim *et al*., 1997) and pBI771 (Wang *et al*., 1997) cDNA expression libraries were amplified and used for two-hybrid screens. Yeast transformation and two-hybrid screens were performed as described by Kim *et al*. (1997). Between 48 and 192 independent plasmids per screen were rescued in *E.coli* and analysed by partial sequence determination.

For additional quantitative interaction assays, plasmid combinations were transformed into yeast and quantitative *o*-nitrophenyl-β-D-galactopyranoside assays were performed as described (Kudla *et al*., 1999). Each quantitative assay was reproduced in at least three independent experiments.

Expression and purification of recombinant AtCBL proteins

The coding regions of AtCBL2 and AtCBL6 cDNAs were cloned into pET24b(+) (Novagen). Overexpression and purification of His_6-tagged proteins was performed on Ni-NTA–Sepharose under denaturing conditions as described by the manufacturer (Qiagen). His_6-tagged CBL proteins were renatured by overnight dialysis at 4°C against 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1 mM dithiothreitol (DTT). Protein assays for determination of CBL protein concentrations were carried out as described (Harter *et al*., 1993).

Co-affinity purification and overlay assays

cDNA fragments of AtCIPK1, AtCIPK13 and AtCIPK14 were amplified by PCR and subcloned into pET24b(+), to generate constructs expressing AtCIPKs without a His_6 tag. With 1 µg of DNA of each construct, *in vitro* transcription–translation reactions were performed in the presence of 20 µCi of L-[^{35}S]methionine (Amersham Pharmacia) using the TNT T7 Quick Coupled Transcription/Translation System (Promega).

For co-affinity purification assays, 2 µg of His_6-tagged CBL proteins were mixed with 15 µl of *in vitro* translatate and 20 µl of Ni-NTA beads in 100 µl of binding buffer (50 mM Tris–HCl pH 6.7, 100 mM NaCl, 0.05% Tween-20, 0.2 mM $CaCl_2$, 10 mM imidazole). After incubation for 2 h at 4°C, the beads were washed four times with 0.5 ml of binding buffer. Co-purified proteins were eluted with 50 µl of elution buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 50 mM EDTA). Two 20 µl samples were resolved on two different SDS–polyacrylamide gels (Harter *et al*., 1993). One gel was autoradiographed with Kodak BioMax MR film. The proteins of the second gel were transferred onto PVDF membranes (Millipore). Western blot analysis for the detection of His_6-tagged AtCBL proteins was carried out as described previously (Harter *et al*., 1993) using an AtCBL1 antiserum produced in mice or Ni-NTA conjugate (Qiagen).

For overlay assays, 5 µg of AtCBL2 were separated by SDS–PAGE and transferred onto PVDF membranes (Harter *et al*., 1993). The membranes were blocked with TBST (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 3% bovine serum albumin. The membranes were incubated for 2 h with 25 µl of *in vitro* translatate in a total volume of 2.5 ml of binding buffer without imidazole. Finally, the membranes were washed three times with 5 ml of binding buffer without imidazole, air-dried and autoradiographed with Kodak BioMax MR film.

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