

Calcium Sensors and Their Interacting Protein Kinases: Genomics of the Arabidopsis and Rice CBL-CIPK Signaling Networks^{1[w]}

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Calcium signals mediate a multitude of plant responses to external stimuli and regulate a wide range of physiological processes. Calcium-binding proteins, like calcineurin B-like (CBL) proteins, represent important relays in plant calcium signaling. These proteins form a complex network with their target kinases being the CBL-interacting protein kinases (CIPKs). Here, we present a comparative genomics analysis of the full complement of CBLs and CIPKs in Arabidopsis and rice (*Oryza sativa*). We confirm the expression and transcript composition of the 10 CBLs and 25 CIPKs encoded in the Arabidopsis genome. Our identification of 10 CBLs and 30 CIPKs from rice indicates a similar complexity of this signaling network in both species. An analysis of the genomic evolution suggests that the extant number of gene family members largely results from segmental duplications. A phylogenetic comparison of protein sequences and intron positions indicates an early diversification of separate branches within both gene families. These branches may represent proteins with different functions. Protein interaction analyses and expression studies of closely related family members suggest that even recently duplicated representatives may fulfill different functions. This work provides a basis for a defined further functional dissection of this important plant-specific signaling system.

All organisms use a network of signal transduction pathways to cope with their environment, to control their metabolism, and to realize their developmental programs. Calcium has emerged as an ubiquitous second messenger involved in many of these processes (Gilroy and Trewavas, 2001). In plants, intracellular calcium levels are modulated in response to various signals including abiotic stresses, light, pathogens, and hormones (Harper, 2001; Knight and Knight, 2001). In addition, physiological processes like guard cell regulation, root hair elongation, and pollen tube growth are accompanied by distinct spatiotemporal changes in calcium concentration (Evans et al., 2001; Sanders et al., 2002). The specific signatures of these calcium transients can encode information and contribute to the specificity required for efficient stimulus response coupling (McAinsh and Hetherington, 1998; Allen and Schroeder, 2001). Nevertheless, an additional level of regulation in calcium signaling is achieved via the function of calcium-binding proteins (Snedden and Fromm, 1998; Luan et al., 2002; Sanders et al., 2002). These sensor proteins likely recognize specific calcium signatures and relay

these signals into downstream responses such as phosphorylation cascades and regulation of gene expression (Luan et al., 2002).

In the case of plant calcium-dependent protein kinases (CDPKs), which harbor a calcium-binding domain as well as a catalytic Ser/Thr kinase domain, calcium signals can be sensed and transmitted by a single protein (Harmon et al., 2000; Sanders et al., 2002). Functional analyses of different plant CDPKs have provided growing evidence for crucial functions of these proteins in divergent processes like hormone and stress signaling as well as pathogen response (Sheen, 1996; Romeis et al., 2001). Most of the other known calcium sensors, for example, calmodulin (CaM) and CaM-like proteins, do not have an enzymatic activity on their own. Binding of calcium ions to these proteins changes their affinity for and results in a subsequent activation or deactivation of their target proteins (Luan et al., 2002). Recently, the group of plant calcium sensor proteins has been extended by the identification of calcineurin B-like (CBL) proteins from Arabidopsis. These proteins are most similar to both the regulatory B subunit of calcineurin (CNB) and neuronal calcium sensors (NCS) of animals (Kudla et al., 1999). CBL proteins contain EF hand motifs as structural basis for calcium binding and interact specifically with a group of Ser/Thr protein kinases designated as CBL-interacting protein kinases (CIPKs; Shi et al., 1999; Kim et al., 2000). CIPKs most likely represent targets of calcium signals sensed and transduced by CBL proteins. The N-terminal kinase domain of these pro-

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teins is most closely related to sucrose non-fermenting-like and cAMP-dependent kinases from various organisms (Shi et al., 1999). Because of this structural feature, these proteins have also been assigned to the SnRK3 subgroup of plant SNF-like kinases (Hrabak et al., 2003). Subsequent yeast two-hybrid screens and interaction studies uncovered 15 CIPKs from Arabidopsis. Moreover, this analysis identified a conserved 24-amino acid motif within the C-terminal non-kinase region of the CIPKs as sufficient and required to mediate CBL-CIPK interaction (Albrecht et al., 2001). Because of the functionally important and conserved amino acid motif Asn-Ala-Phe, this protein region has been designated as NAF domain (protein families database accession no. PF03822). The high number of identified CBLs and CIPKs suggested a rather complex web of potential interactions. Remarkably, preferential complex formation of individual CBLs with defined subsets of CIPKs has been found to contribute to generating specificity in this signaling network (Kim et al., 2000; Albrecht et al., 2001).

To date, a physiological function has been established only for a few CBL and CIPK proteins. The CBL calcium sensor SOS3 (AtCBL4) and the CIPK-type kinase SOS2 (AtCIPK24) have been identified in genetic screens. They appear to be part of a calcium-regulated signaling pathway that specifically mediates salt stress signaling and adaptation (Liu and Zhu, 1998; Halfter et al., 2000; Liu et al., 2000). Analysis of a *CIPK3* loss-of-function allele established a function of this kinase in regulating abscisic acid (ABA) responses during seed germination and in regulating stress-induced gene expression (Kim et al., 2003). Two independent reverse genetic analyses of *CBL1* function revealed that disruption of the *CBL1* gene renders plants hypersensitive to drought and salt stress and impairs the regulation of early induced transcription factors as well as other stress-responsive genes (Albrecht et al., 2003; Cheong et al., 2003). Moreover, these studies also identified this calcium sensor as a regulator of plant cold stress signaling and adaptation.

The biochemical analyses of CIPKs revealed that both deletion of the CBL-interaction domain and substitution of a critical Thr in the putative activation loop by Asp result in a constitutively hyperactive kinase (Guo et al., 2001; Gong et al., 2002b, 2002c). Phenotypical analyses of plants overexpressing such a constitutively active CIPK (PKS11/CIPK8) suggested a function of the analyzed protein in mediating plant resistance to high concentrations of Glc (Gong et al., 2002a). However, the regulation, function, and interconnections of most of the known CBL and CIPK proteins have not been determined (Luan et al., 2002). Moreover, the identification and analysis of the components of this signaling network has so far been mainly restricted to the species Arabidopsis.

In this study, we present a systematic genome-wide analysis of the Arabidopsis and rice (*Oryza sativa*) CBL and CIPK genes and encoded proteins. Our data provide a framework for future functional dissection of this calcium-regulated plant signal transduction system in both model plants.

RESULTS AND DISCUSSION

The Complement of CBLs and CIPKs Encoded in the Arabidopsis Genome

In our previous studies, we identified and analyzed six CBLs and 15 CIPKs from Arabidopsis (Kudla et al., 1999; Shi et al., 1999; Albrecht et al., 2001). To uncover the full complement of these calcium sensors and their interacting kinases encoded in the Arabidopsis genome, we performed database analyses using different BLAST algorithms (see "Materials and Methods"). Subsequent pair-wise DNA sequence comparisons were applied to identify and exclude double annotations. To distinguish CIPKs from other related SNF-like protein kinases, candidate sequences were additionally analyzed with the "motif" algorithm for the presence of the NAF domain (Albrecht et al., 2001). These analyses led to the identification of 10 loci potentially encoding CBLs and 25 loci that could encode CIPKs (Tables I and II).

We performed a systematic reverse transcriptase (RT)-PCR-based cDNA cloning of all potentially functional CBL and CIPK genes to verify their active expression and to determine the structure of the encoded mRNAs. Our cDNA cloning approach revealed that all genes are transcribed and in several cases led to a correction of the theoretical gene predictions. These data have been incorporated in public databases, and the corresponding nucleotide and protein identification numbers are depicted in Tables I and II.

Our comparison of the genomic loci with their encoded cDNAs revealed that almost all identified Arabidopsis CBL genes harbor six or seven introns in their coding region (Fig. 1A). In the case of *AtCBL10*, an additional intron appears in its N-terminal coding region. This intron lacks any homolog in other *AtCBLs*. Four introns are absolutely conserved in phase and position in all 10 *CBL* genes. Three other introns are structurally conserved, but are absent in either one or two members of the *CBL* gene family, most likely due to intron loss events during evolution. Interestingly, the *AtCBL7* gene, which is located in tandem orientation with *AtCBL3* in the Arabidopsis genome (see below), contains an intron in a position not found in any other *CBL* locus. Remarkably, all *CBL* genes for which the full-length cDNA sequences have been determined (*AtCBL1-4* and *AtCBL9*) harbor introns in their 5'-untranslated region (UTR); one in *AtCBLs1* and 9, and two in *AtCBLs2-4*. Whether this unusual intron composition potentially

Table I. The *Arabidopsis* CBL gene family

Name	Arabidopsis Genome Initiative Identification No. ^a	Nucleotide Identification No. ^b	cDNA ^c	Protein Identification No. ^d	Amino Acid ^e
AtCBL01	At4g17615	AF076251	642	AAC26008	213
AtCBL02	At5g55990	AF076252	681	AAC26009	226
AtCBL03	At4g26570	AF076253	681	AAC26010	226
AtCBL04 ^f	At5g24270	AF192886	669	AAG28402	222
AtCBL05	At4g01420	AF192885	612	AAG28401	203
AtCBL06	At4g16350	AF192884	681	AAG28400	226
AtCBL07	At4g26560	AF290434	645	AAG10059	214
AtCBL08	At1g64480	AF411957	645	AAL10300	214
AtCBL09	At5g47100	AF411958	642	AAL10301	213
AtCBL10	At4g33000	AF490607	741	AAO72364	246

^a Systematic designation given to the genes by the Arabidopsis Genome Initiative (<http://mips.gsf.de/proj/thal/db/index.html>). ^b Nucleotide accession of the experimentally verified cDNA in the National Center for Biotechnology Information GenBank database (<http://www.ncbi.nlm.nih.gov>). ^c Length of the cDNA (coding region) in basepairs. ^d Protein accession in the National Center for Biotechnology Information GenBank database (<http://www.ncbi.nlm.nih.gov>). ^e No. of amino acids of the predicted protein; for alternatively spliced mRNAs, the longest predicted amino acid sequence is given. ^f Alternative name, SOS3 (Liu et al., 1998).

reflects regulatory functions during gene expression remains to be investigated experimentally.

In contrast to the CBL genes, only eight of 25 *AtCIPK* genes harbor multiple intron sequences (Fig. 1B). Again, phase and position of these introns, if present, appear to be rather conserved. The position of seven introns is absolutely invariant. The other six

introns are only absent in one to three intron-containing members of the CIPK gene family. One exception is represented by an additional intron in the *AtCIPK23* gene, which is not shared by any other CIPK gene. Moreover, the *AtCIPK16* gene harbors only a single intron not found in other CIPK genes. This might indicate a recent intron acquisition in this

Table II. The *Arabidopsis* CIPK gene family

Name	Arabidopsis Genome Initiative Identification No. ^a	Nucleotide Identification No. ^b	cDNA ^c	Protein Identification No. ^d	Amino Acid ^e
AtCIPK01	At3g17510	AF302112	1,335	AAG28776	444
AtCIPK02	At5g07070	AF286050	1,371	AAF86506	456
AtCIPK03	At2g26980	AF286051	1,326	AAF86507	441
AtCIPK04	At4g14580	AY007221	1,281	AAG01367	426
AtCIPK05	At5g10930	AF285105	1,296	AAF86504	431
AtCIPK06	At4g30960	AF285106	1,326	AAF86505	441
AtCIPK07 ^f	At3g23000	AF290192	1,290	AAK16682	429
AtCIPK08	At4g24400	AF290193	1,338	AAK16683	445
AtCIPK09	At1g01140	AF295664	1,350	AAK16684	449
AtCIPK10	At5g58380	AF295665	1,440	AAK16685	479
AtCIPK11	At2g30360	AF295666	1,308	AAK16686	435
AtCIPK12	At4g18700	AF295667	1,470	AAK16687	489
AtCIPK13	At2g34180	AF295668	1,509	AAK16688	502
AtCIPK14 ^g	At5g01820	AF295669	1,329	AAK16689	442
AtCIPK15 ^h	At5g01810	AF302111	1,266	AAK16692	421
AtCIPK16	At2g25090	AY030304	1,410	AAF19215	469
AtCIPK17	At1g48260	AY036958	1,299	AAK64513	432
AtCIPK18	At1g29230	AY034099	1,563	AAK59695	520
AtCIPK19	At5g45810	AY030303	1,452	AAK50347	483
AtCIPK20	At5g45820	AY035225	1,320	AAK61493	439
AtCIPK21	At5g57630	AY034100	1,251	AAK59696	416
AtCIPK22	At2g38490	AF450478	1,338	AAL47845	445
AtCIPK23	At1g30270	AY035226	1,449	AAK61494	482
AtCIPK24 ⁱ	At5g35410	AF395081	1,341	AAK72257	446
AtCIPK25	At5g25110	AF448226	1,467	AAL41008	488

^a Systematic designation given to the genes by the Arabidopsis Genome Initiative (<http://mips.gsf.de/proj/thal/db/index.html>). ^b Nucleotide accession of the experimentally verified cDNA in the National Center for Biotechnology Information GenBank database (<http://www.ncbi.nlm.nih.gov>). ^c Length of the cDNA (coding region) in basepairs. ^d Protein accession in the National Center for Biotechnology Information GenBank database (<http://www.ncbi.nlm.nih.gov>). ^e No. of amino acids of the predicted protein; for alternatively spliced mRNAs, the longest predicted amino acid sequence is given. ^f Alternative name, AtSR2 (Chikano et al., 2001). ^g Alternative name, AtSR1 (Chikano et al., 2001). ^h Alternative name, ATPK10 (Mizoguchi et al., 1994). ⁱ Alternative name, SOS2 (Halfter et al., 2000).

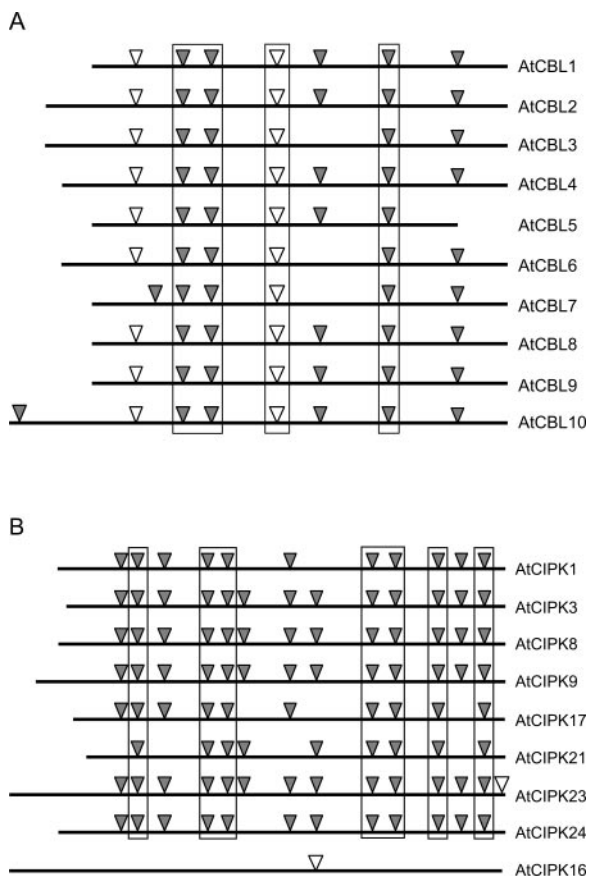


Figure 1. Intron-exon structure of the Arabidopsis *CBL* (A) and *CIPK* (B) genes. Introns located within the coding region of the genes are schematically depicted as triangles. Gray triangles refer to introns located between two codons (phase 0), whereas white triangles indicate introns positioned between the first and second nucleotide of a codon (phase 1). Introns, which are conserved within one gene family, are framed.

gene. In addition, we identified a single intron in the 5'-UTR of *AtCIPK15*, which otherwise does not harbor intron sequences in its coding region. Taken together, these findings suggest the preservation of a relatively constant exon-intron composition of the *CBL* and *CIPK* genes during the evolution of the Arabidopsis genome.

In addition, we assayed the Arabidopsis *CBLs* and *CIPKs* for the occurrence of alternative mRNA splicing. To this end, the cDNA sequences obtained during this study were compared with all available full-length cDNA and expressed sequence tag (EST) sequence data. These investigations identified alternatively spliced mRNAs for *AtCBL2*, *AtCBL4*, and *AtCBL10* as well as for *AtCIPK3* and *AtCIPK9*. All alternatively spliced cDNA sequences have been deposited in the respective GenBank entries. The nucleotide identification numbers of the analyzed ESTs and cDNA sequences are listed in Supplemental Table SI. The differential splicing events of the *AtCBL10* and *AtCIPK3* transcripts are exemplarily depicted in Figure 2. In the case of *AtCBL10*, the pro-

cessing of an alternative exon leads to two potential protein isoforms with different N-terminal sequences (*AtCBL10-1*, MEQVSSRSSSLT...; *AtCBL10-2*, MTTGRPNILALKISTRSSSLT...). The differential splicing of *CBL2* and *CBL4* transcripts affects the composition of their mature 5'-UTR. Interestingly, one spliced intron in the 5'-UTR of *AtCBL4/SOS3* encompasses 3.7 kb of genomic sequence, a fact that needs to be considered when attempting to study expression of this gene by the analyses of promoter-reporter gene constructs in transgenic plants. For *AtCIPK3*, we detected three alternatively spliced forms, which differ in the C-terminal part of the protein. An alignment of these three protein sequences is depicted in Supplemental Fig. S1. These isoforms arose by differential splicing of intron 11 within the otherwise completely processed full-length mRNAs. In isoform *AtCIPK3-2*, the recognition of an alternative splice site leads to an RNA lacking parts of exon 12 and the complete exons 13 and 14. Therefore, the potentially specified *AtCIPK3-2* protein would suffer a truncation of 69 amino acids. In the *AtCIPK3-3* isoform, retaining of intron 11 results in an in-frame stop codon within this intron sequence. We identified three differentially spliced transcript forms of *AtCIPK9*, which are generated by alternative splicing of introns 7 and 13. Compared with *AtCIPK9-1*, the resulting variants would encode proteins either lacking two amino acids (*AtCIPK9-2*) or harboring four additional amino acids (*AtCIPK9-3*). Only a relative low number of full-length ESTs or cDNAs have been available for our analyses of potential alternative splicing events. This limits the statistical reliability of any prediction about the ratios of certain transcripts in planta. Nevertheless, the only four cDNAs identified for *AtCBL10* represent two alternatively spliced forms (*AtCBL10-1*, two cDNAs; *AtCBL10-2*, two cDNAs), and the six identified *AtCIPK3* cDNAs represent three isoforms (*AtCIPK3-1*, three cDNAs; *AtCIPK3-2*, one cDNA; *AtCIPK3-3*, two cDNAs). The rather even distribution of differentially spliced mRNAs argues against a for-

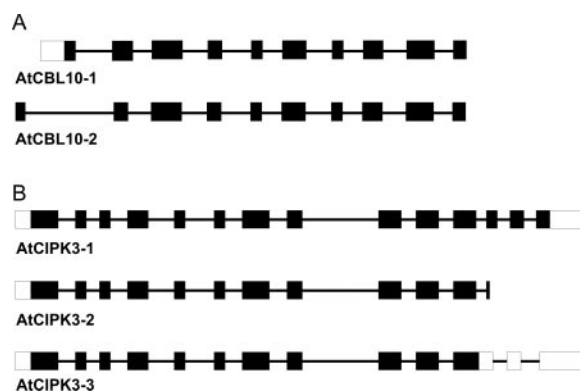


Figure 2. Schematic depiction of alternatively spliced *AtCBL10* (A) and *AtCIPK3* (B) transcripts. The white-boxed regions refer to untranslated mRNA sequences, whereas black boxed domains indicate translated exon sequences. Introns are presented as black lines.

tuitous detection of “mis-spliced” RNAs and makes it more likely that these alternatively spliced mRNAs are functional. Currently, it remains to be established how widespread the occurrence of alternative splicing is within the CBL and CIPK families and what the functional significance of the resulting protein isoforms could be. However, our identification of multiple differentially spliced isoforms is likely to add an additional level of complexity to the regulation of this signaling system.

Chromosomal Distribution and Evolution of CBLs and CIPKs

Analyses of the almost completely sequenced Arabidopsis genome have revealed that segmental duplications of chromosomal regions during evolution have significantly shaped the current structure of this genome (Grant et al., 2000; Bowers et al., 2003). These processes have been discussed as important mechanisms that significantly contributed to the evolution of Arabidopsis gene families (Blanc et al., 2000). We therefore analyzed the genomic distribution and evolutionary history of the CBL and CIPK genes. Most of the CBL genes are concentrated on chromosomes IV and V (Fig. 3A). While chromosomes II and III do not harbor CBL loci, *AtCBL8* represents the only CBL gene located on chromosome I. In contrast, the 25 CIPK genes are distributed among all five Arabidopsis chromosomes (Fig. 3B).

To uncover locus duplication events, we performed whole-genome sequence comparisons of all CBLs and CIPKs covering 100 kb of additional sequence surrounding each gene (see “Materials and Methods”). For the CBLs, these analyses identified three interchromosomal translocations resulting in two duplicated CBL genes (Fig. 3A; Supplemental Fig. S2). Two independent duplications affecting different parts of chromosomes IV and V led to the formation of the *AtCBL1/AtCBL9* and *AtCBL2/AtCBL3* gene pairs, respectively (Supplemental Fig. S2). In contrast, a duplication affecting regions of chromosomes IV and I was followed by loss of one CBL gene copy (Supplemental Fig. S2). Therefore, the *AtCBL8* gene, whose ancestor has been involved in this rearrangement, at present represents a singular CBL lacking a closely related counterpart. We also identified a single local tandem duplication leading to the formation of the *AtCBL3/AtCBL7* gene pair. Interestingly, the two gene pairs *AtCBL9* and *AtCBL3* that arose by segmental duplications represent the most closely related CBL pairs. They exhibit a significantly higher degree of conservation than the *AtCBL7* pair encoded by tandemly oriented genes. While both the *AtCBL1/AtCBL9* and the *AtCBL2/AtCBL3* protein pairs share 89% and 92% identical amino acid residues, *AtCBL3* and *AtCBL7* exhibit only 60% sequence identity (Supplemental Table SII). This might point to a relatively ancient origin of this tandemly

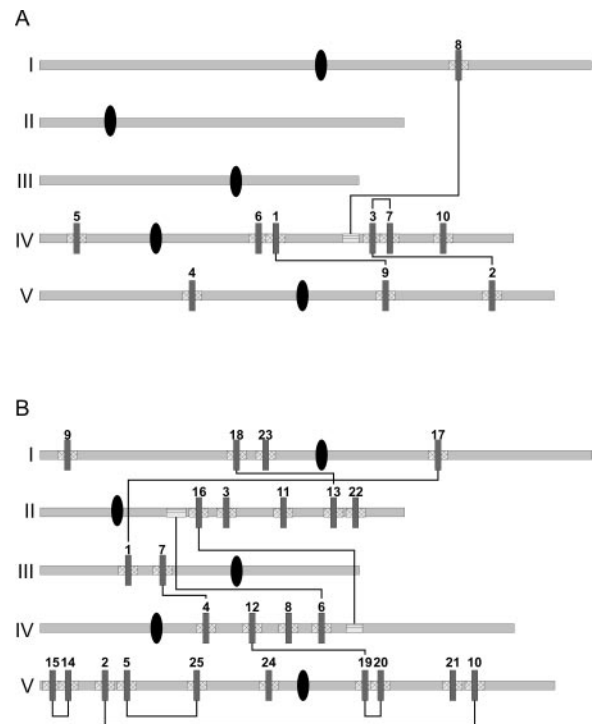


Figure 3. Chromosomal distribution and schematic representation of segmental chromosomal duplications affecting CBL (A) and CIPK (B) loci. Roman numerals indicate the chromosome numbers, whereas vertical bars labeled with Arabic numerals represent the respective Arabidopsis CBL or CIPK genes. Checkered boxes refer to the chromosomal regions analyzed in detail to identify duplication events. Duplications are indicated by lines connecting the affected loci. Horizontally striped boxes label duplication events, which were followed by subsequent CBL or CIPK gene losses. Centromeric regions are indicated by ellipses.

oriented gene pair as compared with the identified segmental duplications.

Our whole-genome sequence comparison for the 25 CIPK loci uncovered altogether eight segmental duplication events (Fig. 3B; Supplemental Fig. S3). Six of these chromosomal rearrangements led to the presence of pairs of closely related CIPKs in the genome, whereas two loci duplications were followed by subsequent deletions of the affected kinases during the evolution of the Arabidopsis genome. In addition, we identified two CIPK gene pairs (*AtCIPK14/AtCIPK15* and *AtCIPK19/AtCIPK20*), which arose by tandem duplications. As observed for the CBLs, all segmentally duplicated CIPK gene pairs (*AtCIPK1/AtCIPK17*, *AtCIPK2/AtCIPK10*, *AtCIPK4/AtCIPK7*, *AtCIPK5/AtCIPK25*, *AtCIPK12/AtCIPK19*, and *AtCIPK13/AtCIPK18*) encode the most closely conserved protein pairs, whereas proteins encoded by tandemly oriented genes (*AtCIPK14/AtCIPK15* and *AtCIPK19/AtCIPK20*) exhibit a lower degree of conservation (Supplemental Table SIV). Taken together, our analysis of the chromosomal evolution of the CBL and CIPK gene families indicates that segmental duplications have predominantly contributed to the current

complexity of both Arabidopsis gene families. Only two of the 10 Arabidopsis *CBL* genes and four of the 25 *AtCIPKs* are encoded in tandem orientation. In contrast, four *AtCBLs* and 12 *AtCIPKs* were identified as segmentally duplicated loci, indicating the importance of this evolutionary mechanism for the growth and diversification of these gene families. A recent genome-wide analysis of *NBS-leucin rich repeat (LRR)* genes in Arabidopsis (Meyers et al., 2003) reported frequent microscale rearrangements and extensive duplications as driving forces for the evolution of this large gene family. Moreover, these amplification processes led to the clustering of *NBS-LRR* sequences in specific chromosomal regions. Our analysis indicates that the evolution of the *CBL* and *CIPK* genes in Arabidopsis did not follow such a pattern. One potential explanation for this difference could be a strong selection pressure for gene dosage effects of *CBLs* that would select against extensive duplications of functional identical genes. Moreover, each newly formed *CIPK* would have to co-evolve with the corresponding *CBL(s)* regulating this kinase. These functional constraints might have influenced the evolutionary processes forming the extant *CBL-CIPK* signaling network.

To extend our studies on *CBLs* and *CIPKs* beyond Arabidopsis, we searched the publicly available EST (and genomic) databases for homologous sequences in other organisms. For *CBLs*, the applied criteria included a significantly higher similarity with the Arabidopsis *CBLs* than with *CaM* as well as the *CBL*-specific spacing of the EF hand motifs. Homologous *CIPK*-type kinases from other species were defined by both the similarity with the *AtCIPKs* and the presence of the NAF domain. Although these analyses systematically excluded partial ESTs covering only N-terminal parts of the encoded proteins, we nevertheless identified ESTs encoding *CBLs* and *CIPKs* in plant species like *Medicago truncatula* (nine different *CBLs* and 11 distinct *CIPKs*), wheat (*Triticum aestivum*; 11 *CBLs* and 29 *CIPKs*), barley (*Hor-*

deum vulgare; nine *CBLs* and 14 *CIPKs*), soybean (*Glycine max*; seven *CBLs* and 13 *CIPKs*), the gymnosperm *Pinus* sp. (two *CBLs* and seven *CIPKs*), and the moss *Physcomitrella patens* (four *CBLs* and three *CIPKs*). The accession numbers of the identified ESTs are displayed in the Supplemental Table S1b. The occurrence of multiple isoforms of *CBLs* and *CIPKs* in all analyzed plant species indicates that these proteins generally form a complex signaling network. We did not identify EST or genomic sequences encoding *CBLs* or *CIPKs* outside the plant kingdom, suggesting that the function of these signaling components is confined to plants but arose early in plant evolution.

We next investigated the occurrence of *CBLs* and *CIPKs* in the extensively sequenced genome of the monocotyledonous plant rice. To this end, we separately analyzed the available sequence data for the two rice subspecies *O. sativa* subsp. *japonica* and *O. sativa* subsp. *indica* (for details see "Materials and Methods"). In both genomes, we identified the same set of 10 calcium sensor proteins and 30 interacting kinases. The general features of the rice *CBLs* and *CIPKs* are summarized in Tables III and IV. The available sequences of both rice species do not cover their complete genomes and are only partially assembled. Nevertheless, the consistency of identified *CBL* and *CIPK* genes suggests that we uncovered the full complement of both gene families in rice. The rice genome is not only significantly larger than the Arabidopsis genome but also predicted to encode a greater number of genes (32,000–55,000 as compared with 27,300 in Arabidopsis; Goff et al., 2002; Yu et al., 2002; Wortman et al., 2003). As reported for several other gene families, for example, P-type ATPases and ABC transporters (Baxter et al., 2003; Jasinski et al., 2003), this general increase in the number of genes in rice obviously did not significantly affect the complexity of the *CBL* and *CIPK* gene families.

Comparison of the exon-intron composition of Arabidopsis and rice genes revealed a rather high degree

Table III. The rice *CBL* gene family

Name	Clone Identification No. ^a	Clone Identification No. ^b	Position ^c	Introns ^d	cDNA ^e	Amino Acid ^f
OsCBL1	AC084763	(AAAA01004016)	94244-103391	7	639	213
OsCBL2	AL713904	(AAAA01001368)	5789-9843	7	678	226
OsCBL3	AC092390	(AAAA01005520)	28690-34606	7	675	225
OsCBL4	AC097111	(AAAA01001069)	26564-28044	7	633	210
OsCBL5	AP003504	(AAAA01005392)	59100-61578	7	642	214
OsCBL6	AL713906	(AAAA01000906)	69628-73277	7	678	226
OsCBL7	AP004212	(AAAA01011541)	16316-18296	7	642	214
OsCBL8	CLC10421	(AAAA01023033)	30197-32277	7	642	214
OsCBL9	AP002908	(AAAA01061971)	121268-123750	8	870	290
OsCBL10	AP002901	(AAAA01012074)	128202-130821	8	747	249

^a Nucleotide accession of a bacterial artificial chromosome clone coding for a rice *CBL* (<http://www.ncbi.nlm.nih.gov>). ^b Alternative rice bacterial artificial chromosome clone from the second subspecies (accessions beginning with AAAA.. refer to *O. sativa* subsp. *indica*, all other accessions indicate *O. sativa* subsp. *japonica*). ^c Gene position on the corresponding rice bacterial artificial chromosome clone listed in the "Clone Identification No." column. ^d No. of introns within the coding region. ^e Length of the cDNA (coding region) in basepairs. ^f No. of amino acids of the predicted protein.

Table IV. The rice CIPK gene family

Name	Clone Identification No. ^a	Clone Identification No. ^b	Position ^c	Introns ^d	cDNA ^e	Amino Acid ^f
OsCIPK01	AP002482	(AAAA01013723)	115507-120039	12	1,383	461
OsCIPK02	AP003757	(AAAA01003105)	48660-49991	–	1,329	443
OsCIPK03	AP003818	(AAAA01000189)	85611-88557	12	1,335	445
OsCIPK04	AL837528	(AAAA01012226)	145269-146633	–	1,362	454
OsCIPK05	AP003052	(AAAA01008215)	13923-12538	–	1,383	461
OsCIPK06	AAAA01000701	(CLB3888)	17837-19192	–	1,353	451
OsCIPK07	AAAA01012457	(CL017116)	3882-4219	–	1,335	445
OsCIPK08	AP003449	(AAAA01001679)	74597-82361	13	1,326	441
OsCIPK09	AC104427	(AAAA01000865)	32041-35803	13	1,368	456
OsCIPK10	AC131175	(AAAA01023197)	68596-69961	–	1,353	451
OsCIPK11	AP003230	(AAAA01008532)	102353-103861	–	1,506	502
OsCIPK12 ^g	AP003256	(AAAA01013578)	38475-36913	–	1,560	520
OsCIPK13	AP003052	(AAAA01023672)	2348-3883	–	1,533	511
OsCIPK14	BX000511	(AAAA01002327)	111991-113310	–	1,317	439
OsCIPK15	BX000512	(AAAA01013485)	111783-112587	–	1,302	434
OsCIPK16	AAAA01003250	(CL01893)	7861-9231	–	1,368	456
OsCIPK17	AAAA01004506	(CL003998)	10945-14154	11	1,362	454
OsCIPK18	AAAA01001065	(CL24701)	22322-23695	–	1,371	457
OsCIPK19 ^h	AB011967	(AAAA01011190)	3641-5267	–	1,527	509
OsCIPK20	AAAA01036134/ AAAA01006662	(HTC152200-B01)	1-1361/1360-1401	–	1,398	466
OsCIPK21	AP003749	(AAAA01019996)	64996-69312	12	1,491	497
OsCIPK22	AAAA01005133	(CL008902)	3820-5175	–	1,353	451
OsCIPK23	AP003703	(AAAA01001330)	16522-20217	13	1,350	450
OsCIPK24	AAAA01002034	(CL024530)	1909-7271	13	1,359	453
OsCIPK25	AAAA01000413	(CL018106)	16907-18457	–	1,542	514
OsCIPK26	AAAA01001395	(HTC020888-A01)	25212-26693	–	1,479	493
OsCIPK27	CL000365	(AAAA01015351)	11848-13185	–	1,335	445
OsCIPK28	AAAA01001836	(CLB9204)	11694-13001	–	1,305	435
OsCIPK29	AP003757	(AAAA01003105)	32507-33838	–	1,329	443
OsCIPK30	AP003256	(AAAA01019275)	30049-28619	–	1,428	476

^a Nucleotide accession of a bacterial artificial chromosome clone coding for a rice CIPK (<http://www.ncbi.nlm.nih.gov>). ^b Alternative rice bacterial artificial chromosome clone from the second subspecies (accessions beginning with AAAA.. refer to *O. sativa* subsp. *indica*, all other accessions indicate *O. sativa* subsp. *japonica*). ^c Gene position on the corresponding rice bacterial artificial chromosome clone listed in column "Clone Identification No." ^d No. of introns within the coding region. ^e Length of the cDNA (coding region) in basepairs. ^f No. of amino acids of the predicted protein. ^g Alternative name, OsPK07 (Ohba et al., 2000). ^h Alternative name, OsPK04 (Ohba et al., 2000).

of structural conservation (data not shown). The rice genes *OsCBL1* to 7 all harbor seven introns, which are absolutely invariant in phase and position when compared with the respective conserved seven introns present in six of the 10 *AtCBLs*. This situation suggests that the single intron losses observed in *AtCBL3*, 5, 6, and 7 occurred after the divergence of both evolutionary lineages. These seven introns are conserved in *OsCBL9* and *OsCBL10*. Interestingly, these two genes share the additional first intron within their N-terminal extension with their closely related counterpart *AtCBL10*.

As observed for the Arabidopsis CIPKs, the members of the rice CIPK gene family can also be divided into intron-less (eight *OsCIPKs*) and intron-harboring (22 *OsCIPKs*) representatives (Table IV). The latter exhibit a high degree of intron conservation with their relative Arabidopsis gene family members and harbor 11 to 13 introns, which are all conserved in phase and position. Gene structures with 11 or 12 introns remaining can clearly be attributed to single or double intron loss events. For example, *OsCIPK1*

and *OsCIPK21* have lost just one of these conserved introns (homologous to intron 8 in *AtCIPK8*).

In our analysis, we identified the two genes *OsCIPK14* and *OsCIPK15* as located on different chromosomes in the both rice subspecies. Both predicted proteins are almost identical and differ only by a single amino acid substitution and in their C-terminal nine amino acids. Therefore, this CIPK pair might represent an example for a very recent duplication event in this species. Our genomic analyses of rice CBL and CIPK genes did not detect any tandemly duplicated *OsCBL* genes. Of the 30 *OsCIPKs*, three gene pairs appeared to be organized in tandem orientation (*OsCIPK5/13*, *OsCIPK12/30*, and *OsCIPK2/29*). Moreover, these gene pairs are only relatively distantly related, suggesting a rather ancient origin of the duplications (see below). Therefore, despite the larger genome size of rice as compared with Arabidopsis, microscale recombination events do not seem to have significantly affected the evolution of both rice gene families.

To further elucidate the relationships of CBLs and CIPKs, we performed phylogenetic analyses to infer clustering patterns reflecting sequence similarity and evolutionary ancestry. Plant CaM as well as animal CNB and NCS were included as outgroups in the analysis of the calcium sensor proteins. The resulting unrooted phylogenetic tree is depicted in Figure 4. (To maintain clarity, this tree does not present bootstrap values. A cladogram displaying all bootstrap values is presented in Supplemental Fig. S4.) Plant CBLs clearly form a monophyletic group separated from CaMs and calcium sensor proteins from animals. Moreover, Arabidopsis CBL pairs (*AtCBL1/AtCBL9* and *AtCBL2/AtCBL3*) resulting from segmental duplications of chromosomal regions appear to be most closely related, again pointing to a relatively recent formation of these gene pairs. In contrast, the CBL pair *AtCBL3/AtCBL7*, which evolved by tandem duplication, is far less related.

The evolutionary comparison of rice and Arabidopsis CBL genes clearly points to the occurrence of multiple species-specific duplication events. Of course, these duplications could also be diagnostic for larger evolutionarily related groups, like monocotyledonous and dicotyledonous species. However, the lack of additional plant genome sequences currently prevents a further investigation of this issue. This situation will most likely impede a precise prediction of common CBL gene functions based solely on sequence information from rice and Arabidopsis. For example, the two closely related calcium sensor proteins pairs *AtCBL2/3* and *OsCBL2/3* result from duplication events that occurred independently in both species analyzed. *OsCBL9* and *10* are clearly

related to *AtCBL10* by both sequence similarity and intron composition. However, it is currently impossible to address whether the rice-specific duplication leading to *OsCBL9* and *10* could result in functional overlaps or, alternatively, reflects a functional specialization. Likewise, the single *OsCBL1* gene is most closely related to the duplicated *AtCBL1* and *AtCBL9* genes, which probably fulfill different functions in Arabidopsis (see below). Nevertheless, in combination with additional information, such as CBL-CIPK interaction specificity and gene expression data, knowledge of these evolutionary relations will help to design reverse genetic strategies to unravel CBL gene functions.

Interestingly, the distribution of myristoylated Arabidopsis and rice CBL proteins coincides with two neighboring branches in the dendrogram. The only predicted non-myristoylatable calcium sensor in these branches (*AtCBL8*) still harbors a recognizable degenerated myristoylation consensus sequence. This might suggest a single early event during the evolution of the CBL genes leading to the loss of this lipid modification in the remaining CBL lineages. Such a diversification would have enabled the separate evolution of membrane-associated and membrane-independent calcium-signaling pathways.

Plant SnRK1 and SnRK2 kinases were included as outgroups in the phylogenetic analysis of the Arabidopsis and rice CIPKs. The resulting unrooted phylogenetic tree is depicted in Figure 5. (To maintain clarity this tree does not present bootstrap values. A cladogram displaying all bootstrap values is presented in Supplemental Fig. S5.) This dendrogram clearly indicates a monophyletic origin of the com-

Figure 4. Phylogenetic relationships of Arabidopsis and rice CBLs with related calcium-binding proteins. Alignment of full-length protein sequences and phylogenetic analyses were performed as described in "Materials and Methods." The accession numbers of the Arabidopsis and rice CBLs are presented in Tables I and III. Arabidopsis CaM 1 (*AtCaM1*, accession no. NP_198594), human CaM 1 (*HsCaM1*, accession no. AAA51918), yeast CNB (*ScCNB*, accession no. P25296), human NCS 1 (*HsNCS1*, accession no. NP_NP055101), and human recoverin (*HsRCV1*, accession no. BAA19460) were included in the analyses as the most closely related non-CBL-type calcium-binding proteins. Stars indicate myristoylated *AtCBLs* and potentially myristoylated *OsCBLs*.

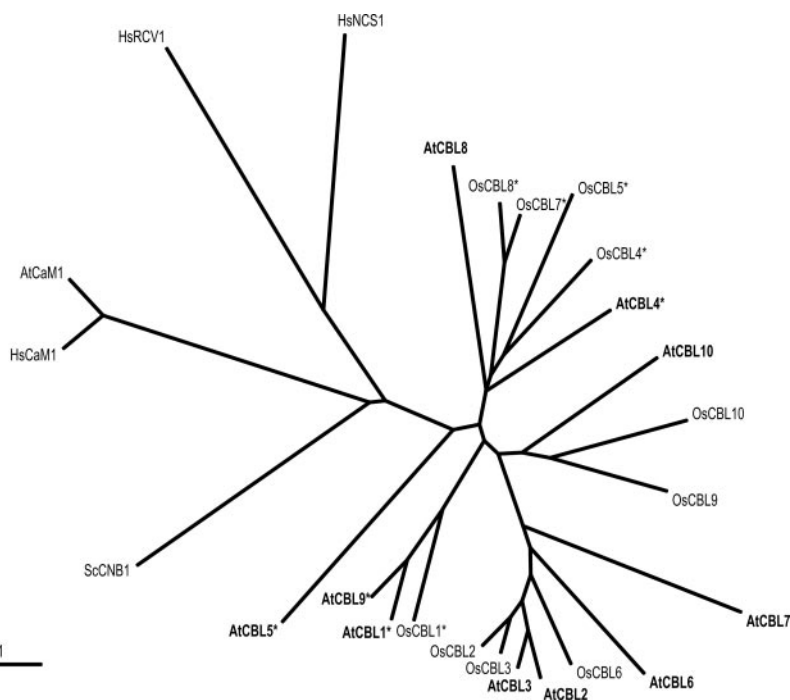
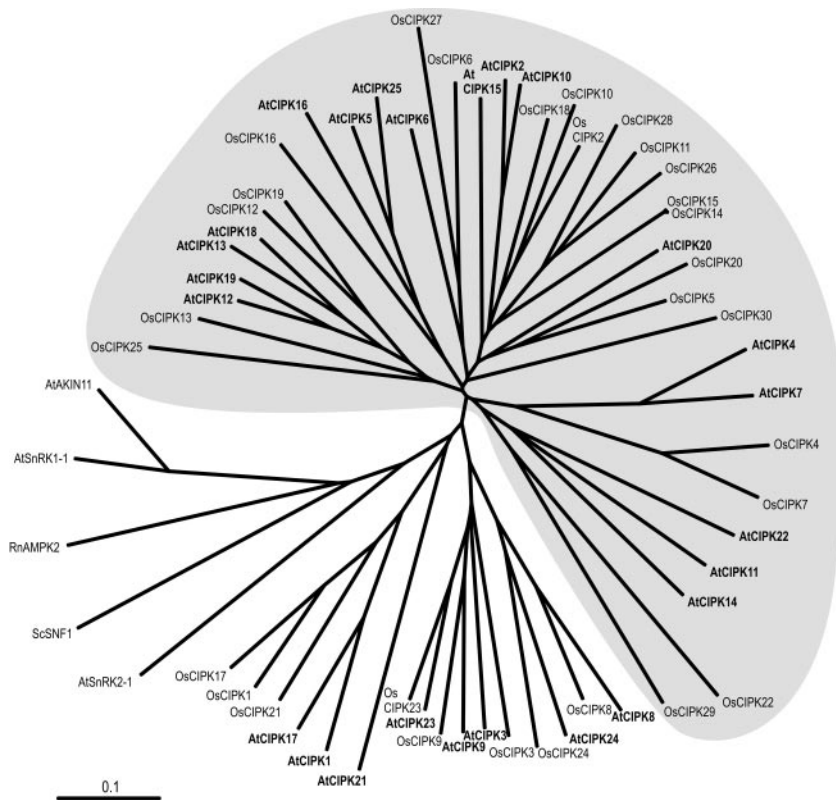


Figure 5. Phylogenetic relationships of Arabidopsis and rice CIPKs with related SNF-like kinases. Alignment of full-length protein sequences and phylogenetic analyses were performed as described in "Materials and Methods." The sub-group of CIPK not harboring intron sequences is depicted on gray background. The accession numbers of the Arabidopsis and rice CIPKs are presented in Tables II and IV. Related SNF-like or cAMP-dependent protein kinases from Arabidopsis (*AtSnRK2-1*, accession no. NP_196476; *AtSnRK1-1*, accession no. NP_566130; *AtKIN11*, accession no. T52633), yeast (*ScSNF1*, accession no. A26030), and rat (*RnAMPK2*, accession no. Q09137) were included in the analyses as the most closely related non-CIPK-type protein kinases.



plex family of plant CIPKs. Interestingly, members of both the rice and the Arabidopsis CIPKs form two distinct subgroups based on their sequence similarity. This clustering of CIPKs strictly coincides with the presence (or absence) of introns in the genes of these subgroups, indicating that the ancestors of both groups have been present in the genome before the evolutionary separation of the mono- and dicotyledonous lineages. Moreover, several groups of closely related Arabidopsis and rice CIPKs form well-supported separate branches. This might suggest a relatively early diversification of the *CBL* and *CIPK* gene families during plant evolution.

The resolution of evolutionary relationships between individual CIPK proteins within these branches varies significantly. For example, the rice and Arabidopsis representatives of *CIPK8*, *9*, *23*, and *24* are clearly recognizable as distinct orthologous gene pairs. This might allow for prediction of similar functions of their gene products in both species. In contrast, in both the *CIPK4/7* and the *CIPK1/17/21* branches, our analysis indicates parallel species-specific duplication events. This prevents an interspecific gene-to-gene assignment of distinct CIPKs. However, it appears noteworthy that in rice and Arabidopsis, the evolution of these branches followed the same pattern resulting in the closely related gene groups *CIPK4/7* and *CIPK1/17/21* in both species. The slightly increased number of CIPKs in rice compared with Arabidopsis can be largely attributed to rice-specific duplications in the evolutionary

branch encompassing *AtCIPK2*, *6*, *15*, and *20* and the respective rice homologs. Although this branch just represents four CIPKs from Arabidopsis, it encompasses 12 members of the rice kinase family. It needs to be analyzed if the amplification of this rice CIPK subgroup just results in functional overlaps or instead points to an evolutionary specialization. Finally, our data indicate that the complexity of this signaling network is not restricted to Arabidopsis and therefore is likely to reflect functional diversification.

Comparative Analyses of the Proteins

The CBL protein family appears to be rather conserved in size and structure. Most of the genes encode predicted polypeptides ranging from 23.5 to 26 kD in Arabidopsis and 23.9 to 25.9 kD in rice (Tables I and III). The only noted exceptions are represented by *AtCBL10* and the recently duplicated *OsCBL9* and *OsCBL10*, which, due to an N-terminal extension, have predicted molecular masses of 31.7, 32.9 and 29.9 kD, respectively. Although the N termini of *AtCBL10* and *OsCBL9/10* do not harbor any discernible sequence or signal motif, it appears conceivable that this domain is of functional relevance. The amino acid sequence identity of different CBLs ranges from 29% to 92% (33%–93% similarity) in Arabidopsis and from 40% to 92% (44%–94% similarity) in rice (Supplemental Tables SII and SIII). In Arabidopsis, the most closely related proteins *AtCBL1* and *AtCBL9* as well as *AtCBL2* and *AtCBL3*

share 89% and 92% identical (91% and 93% similar) amino acid residues, respectively. Even the most divergent protein pair AtCBL5 and AtCBL10 exhibits 29% sequence identity (33% similarity). The rather conserved structure of these calcium sensor proteins might point to a very similar mode of action and likely reflects their conserved interaction with the CIPKs as their common target proteins.

Almost all known animal and yeast calcium sensor proteins of the NCS and calcineurin type are modified by the 14-carbon saturated fatty acid myristate. Covalent linkage of myristate via an amide bond to the Gly in the N-terminal sequence MGXXXS/T is catalyzed by *N*-myristoyl transferase and occurs cotranslationally after removal of the initiator Met by *N*-aminopeptidase. In many systems, *N*-myristoylation promotes protein-protein or protein-membrane interactions (Resh, 1999). Myristoylation of the mammalian calcium sensor recoverin is a prerequisite for the occurrence of the calcium-myristoyl switch leading to a calcium-dependent membrane association of recoverin (Ames et al., 1997). Our analysis revealed that four of the 10 Arabidopsis CBL proteins harbor a conserved myristoylation motif (Fig. 6A). In rice, five CBL proteins (OsCBL1, 4, 5, 7, and 8) represent potential substrates for this lipid modification. Myristoylation has been shown to be required for the function of AtCBL4/SOS3, although the exact role of myristoylation of this protein remained a mystery (Ishitani et al., 2000). Moreover, AtCBL1, 5, and 9 appear to become myristoylated when translated *in vitro* in a rabbit reticulocyte lysate (O. Batistic and J. Kudla, unpublished data). On the other hand, interaction of AtCBL1 with its target kinases occurs independently of myristoylation (Albrecht et al., 2001). Therefore, a possible function of CBL myristoylation could be the membrane targeting of CBL-CIPK complexes. The hydrophobicity of a myristoylated peptide alone is usually not sufficient to anchor the protein to the plasma membrane. Stable membrane association of myristoylated proteins is often accompanied by palmitoylation of an adjacent Cys residue (Resh, 1999). This palmitoylatable Cys residue is present in all four myristoylated CBLs from Arabidopsis as well as in all potentially myristoylated OsCBLs, suggesting that these proteins could be membrane targeted by virtue of dual lipid modification (Fig. 6A).

Calcium-binding proteins contain EF hand motifs as structural basis for calcium binding. Each EF hand consists of a loop of 12 amino acids flanked by two α -helices. A single Ca^{2+} ion is bound to each EF hand via the loop domain. In CaM, which harbors four EF motifs, these sites act pair wise resulting in a cooperativity of calcium binding (Haeseleer et al., 2002). Calcium sensors of the NCS/recoverin/guanylate cyclase activating protein-type exhibit a different mode of calcium binding, although they harbor four EF hands like CaM (Burgoyne and Weiss, 2001). In

recoverin, attachment of calcium is commonly initiated only on EF hands 2 and 3 and leads to significant conformational changes of the affected protein. Because of a Cys-Pro substitution in the 12 amino acid loop within the first EF hand, this motif is nonfunctional in terms of calcium binding in all NCSs. Instead, this EF hand can mediate the interaction with their target proteins, like for example guanylate cyclase in the case of GCAP-2 (Ermilov et al., 2001). Several members of the NCS/recoverin/GCAP calcium sensor group exhibit variations from the canonical EF hand sequence in single EF hands (Haeseleer et al., 2002). These mutations modulate but do not abolish the calcium-binding affinity of each specific calcium sensor, thereby providing a mechanism to differentially decipher various calcium signals.

Our comparative analyses of the EF hand composition in the Arabidopsis CBL protein family revealed extensive similarities to the NCS-type calcium sensors. All 10 AtCBL proteins harbor four EF hand motifs (Fig. 6A). Interestingly, the size of the linker regions between the EF hand loops is absolutely conserved in all proteins and appears to be unique to this family of calcium sensor proteins. EF1 and EF2 are 22 amino acids apart, whereas 25 amino acids separate EF2 from EF3, and 32 amino acids are inserted between EF3 and EF4. The number of EF hands as well as their spacing is also absolutely conserved in all predicted OsCBL proteins (data not shown). Size variation of CBL proteins is therefore exclusively caused by extension or reduction of the N- and C-terminal regions (Fig. 6A). This might suggest a conserved three-dimensional structure of all plant CBLs. Also, like in NCS proteins, the first EF hand, although conserved in all CBLs, appears to be unable to bind Ca^{2+} ions. This is caused by either single-amino acid substitutions at critical calcium-binding positions (either positions X, Y, and Z or $-Y$, $-X$, and $-Z$; for the Arabidopsis representatives, see Fig. 6B) or larger structural changes (Fig. 6B). In most cases, the oxygen-containing calcium-binding residues at the Y and/or X positions in this EF loop are altered to Ala, Val, Ile, or Cys (as observed in EF1 of GCAP proteins). In addition, in AtCBL5, the essential amino acid at the $-Z$ position is replaced by the positively charged residue Lys. Both AtCBL6 and AtCBL7 display structural changes in the first EF hand. In EF1, four amino acids of the canonical loop in AtCBL6 appear to be deleted. In AtCBL7, EF1 harbors five additional amino acids between the fourth and fifth amino acids (Z position) of the loop, thereby dividing the EF hand motif. Furthermore, several amino acids have been deleted from the entering helix, which could lead to a protein with a different structural conformation in the region of EF1.

AtCBL6, AtCBL7, and AtCBL8 also contain additional mutations in other EF hands (Fig. 6B). In the EF3 loop of AtCBL8, the normally conserved Y residue is changed to the non-oxygen-containing amino

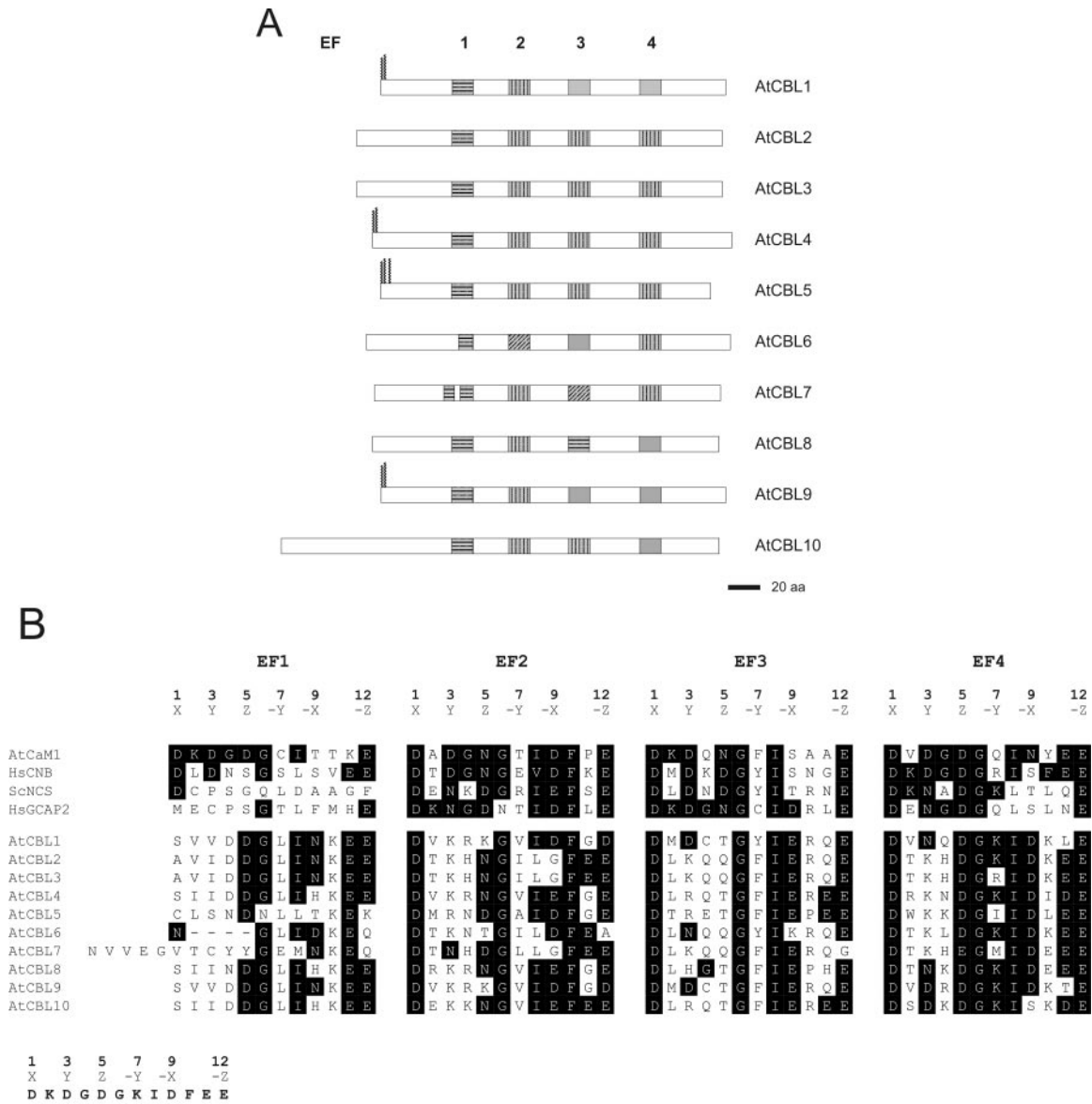


Figure 6. Analyses of EF hand motif distribution and composition. A, Schematics of the domain and motif organization of AtCBLs. The numbering of the EF hands is depicted on the top of the figure. Short jagged lines indicate experimentally verified myristoylation sites and longer jagged lines point to potential palmitoylation motifs. Gray boxes represent EF hands with a conserved canonical amino acid composition, whereas horizontally striped boxes indicate EF hands with single or double non-oxygen-containing amino acid substitutions. Vertically striped boxes depict EF hands with single or double basic amino acid substitutions and diagonally striped boxes stand for EF hands with basic amino acid and non-oxygen-containing amino acid substitutions. B, Detailed comparisons of EF hand motif sequences of the AtCBLs with EF hands from other calcium-binding proteins. The canonical EF hand consensus sequence and coordinates are depicted in the left bottom corner of the figure. Amino acid residues, which match this consensus sequence within one distance unit, are black shaded. The EF hand consensus sequence was calculated based on the calcium-binding EF hands of human CNB (HsCNB, accession no. AAB08721), GCAP-2 (HsGCAP2, accession no. Q9UMX6), yeast NCS-1 (HsNCS, accession no. NP_055101), and Arabidopsis CaM 1 (AtCaM1, accession no NP_198594) and all canonical calcium-binding EF hands of the AtCBLs.

acid His. In AtCBL6, the conserved Glu at the -Z position of EF2 is altered to Ala, and at the same position in the EF3 loop of AtCBL7, the calcium-binding residue is changed to Gly. Both mutations could lead to a change in the loop conformation and therefore to a markedly reduced affinity for calcium (Lewit-Bentley and Réty, 2000). In addition, we de-

tected a remarkable variation of the EF hand sequences in all AtCBL proteins (Fig. 6B). Most of the observed deviations from the canonical EF hand sequence are predicted to result in a reduced affinity toward calcium ions. For example, in the remaining EF hands of AtCBL2, 3, 4, and 5, the oxygen-containing amino acid at the Y position is replaced by

the positively charged amino acid Lys or Arg. The same mutation is also observed in EF2 and EF4 of AtCBL6, EF3 and EF4 of AtCBL7, EF2 of AtCBL8, and EF2 and EF3 of AtCBL10. Moreover, in the binding loop of EF2 of AtCBL1 and AtCBL9, the oxygen amino acid at the Z position is changed to Lys (Fig. 6B). Taken together, this analysis revealed that in AtCBL1 and AtCBL9, EF binding loops 3 and 4 represent a normal canonical sequence. The canonical EF hand sequence can also be identified in the EF-binding loop 3 of AtCBL6, EF2 of AtCBL7, and EF4 of AtCBL8 and AtCBL10. Therefore, the CBL protein family can be divided into proteins with one canonical EF hand (AtCBL6, 7, 8, and 10), with two canonical EF hands (AtCBL1 and 9) and without any canonical EF hand (AtCBL2, 3, 4, and 5; Fig. 6A). A similar variation of EF hand sequences appears to occur in the predicted rice CBL proteins (data not shown). These differences in the EF hand composition in individual CBLs could lead to different affinities toward calcium ions. Whether such differences in calcium-binding affinity contribute to deciphering the different calcium signals in response to various environmental stimuli awaits further experimental analyses. So far, no quantitative Ca^{2+} -affinity studies have been reported for any CBL protein, and side-by-side comparisons of Ca^{2+} binding have not been performed. AtCBL1 has been shown to bind calcium in vitro in gel-overlay assays and displays a shift during electrophoresis after calcium binding (Kudla et al., 1999). Moreover, in vitro studies suggest that interaction of AtCBL1 with AtCIPK1 requires micromolar levels of Ca^{2+} (Shi et al., 1999). Up to now, the function of CBL1-CIPK1 interaction in regulating CIPK1 kinase activity has not been investigated, because the identity of CIPK1 substrates has remained elusive. In contrast, in vitro studies of SOS3/AtCBL4 revealed that this protein binds calcium in overlay assays, but that this binding is too weak to induce a mobility shift during electrophoretic separation (Ishitani et al., 2000). Moreover, at least in vitro, interaction of SOS3/AtCBL4 with SOS2/AtCIPK24 appears to occur calcium independently (Halfter et al., 2000). Nevertheless, in vitro, the autophosphorylation activity of SOS2/AtCIPK24 and its kinase activity toward synthetic substrates are further stimulated by interaction with both SOS3/AtCBL4 and calcium (Guo et al., 2001, Gong et al., 2002c). Therefore, it appears conceivable that significant variations in Ca^{2+} affinity of individual CBLs and variation in Ca^{2+} dependence of CBL-CIPK interaction and activation might exist. This potential aspect of Ca^{2+} regulation in this signaling system clearly deserves more attention.

Interestingly, CBL proteins resulting from gene duplications (AtCBL1 and AtCBL9) harbor an absolutely identical amino acid composition in the EF hand loops 1 and 2. Because EF hands in animal NCSes and CNBs form functional pairs, it appears possible that the N-terminal parts of AtCBL1 and 9

fulfill identical functions (e.g. protein-protein interaction via EF1). Contrarily, the two EF hands at the C-terminal ends of these proteins have diverged during evolution, resulting in EF motifs with potentially distinct calcium-binding affinities. Therefore, the differences in the last two EF hand loops could lead to a different affinity toward calcium ions and consequently could convey different functions of the highly related calcium sensors AtCBL1 and AtCBL9.

Like the CBLs, the 25 AtCIPK and the predicted 30 OsCIPK proteins display a high degree of conservation. In Arabidopsis, the proteins exhibit an overall amino acid sequence identity ranging from 33% to 78%. The sizes of the predicted proteins vary from 46 to 59 kD (Table II; Supplemental Table SIV). Pair-wise amino acid alignments of the highly conserved N-terminal kinase domain revealed 51% to 90% sequence identity (63%–96% similarity). In contrast, the C-terminal non-catalytic region appears to be much less conserved, exhibiting an amino acid identity of 24% to 58% (36%–69% similarity). The only exception is represented by the 24-amino acid NAF domain (58%–86% identity; 66%–94% similarity). A similar degree of conservation in size and structure is also typical for the rice CIPKs (Table IV; Supplemental Table). All CIPK proteins harbor the 11 conserved subdomains typical for Ser/Thr kinases. Biochemical analyses of AtCIPKs expressed in bacteria revealed a rather low enzymatic activity of the recombinant protein (Shi et al., 1999; Guo et al., 2001). For several AtCIPKs, it has been established experimentally that a substitution of a conserved Thr (within the activation loop) to Asp results in a hyperactive enzyme (Gong et al., 2002b, 2000c). Thus phosphorylation-dependent activation of CIPKs thus might provide a mechanism to convey the cellular cross-talk with other signaling systems like CDPKs and mitogen-activated protein kinases.

To investigate the potential localization and modification of the CIPKs, we performed bioinformatics analyses using the PSORT and the MOTIF algorithms. None of the CIPKs appears to harbor any conserved lipid modification (e.g. myristoylation, palmitoylation, and farnesylation) motif or displays any detectable unambiguous cellular sorting sequence. Therefore, the determination of the sub-cellular targeting of the CIPKs will require experimental confirmation for each of these kinases. Nevertheless, a combination of CBL localization data together with information about the specificity of CBL-CIPK complex formation should allow experimentally testable predictions about the sub-cellular targeting of specific CIPKs.

Sequence Similarity of CBLs and CIPKs Does Not Necessarily Result in Functional Redundancy

Our previous yeast two hybrid analyses on a subset of Arabidopsis CBLs and CIPKs have established that specific CBL-CIPK complex formation contributes to

generating specificity in this signaling network and that this differential calcium sensor/kinase affinity is also reflected in *in vitro* experiments with purified proteins (Albrecht et al., 2001). To exemplarily unravel the interaction preferences of two closely related CBL proteins with the complete set of Arabidopsis CIPKs, we studied AtCBL1 and AtCBL9 interactions with AtCIPK1 to AtCIPK25 in yeast two-hybrid interaction analyses. To this end, full-length cDNAs of AtCBL1, AtCBL9, and all AtCIPKs were cloned in the respective DNA-binding domain and GAL4 activation domain plasmids. After transformation into the yeast strain SMY3, interaction was monitored by growth on interaction-selecting media lacking His. As shown in Figure 7, in these analyses, AtCBL1 exhibited a significant interaction only with a subset of six CIPKs (AtCIPK1, 7, 8, 17, 18, and 24).

For four additional CIPKs, we observed a weak tendency of complex formation, whereas 12 CIPKs did not show any affinity toward AtCBL1. Our data did not reproduce the AtCBL1-AtCIPK15 interaction reported by Guo et al. (2002; in this study designated as ScaBP5 and PKS3). This difference could, for example, result from the different vector systems and procedures used in both studies.

Interestingly, the observed preferential complex formation does not appear to coincide with the phylogenetic relationships of the AtCIPKs, because, for example, members of the evolutionary separated branches of intron-harboring (AtCIPK1, AtCIPK8, AtCIPK17, and AtCIPK24) as well as intron-free CIPKs (AtCIPK7 and AtCIPK18) interact with AtCBL1. In addition, of the closely related duplicated CIPK pairs, only AtCIPK1/AtCIPK17 exhibited a

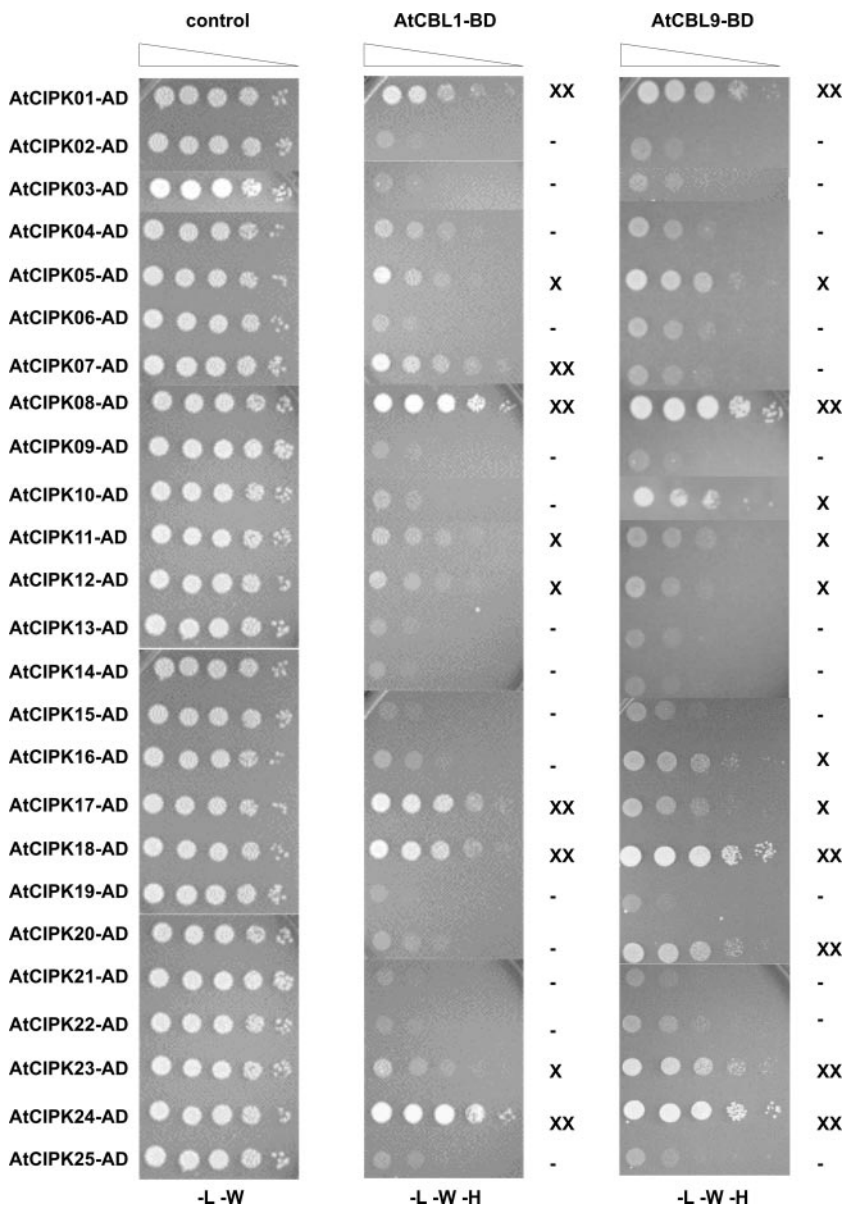


Figure 7. Comparative yeast two-hybrid interaction analysis of AtCBL1 and AtCBL9 with all Arabidopsis CIPKs. The yeast strain SMY3 containing the indicated plasmid combinations were grown on SC medium $-L$ and $-W$ to an A_{600} of 2. Ten microliters of 10-fold dilution series was spotted onto selective ($-H$, $-L$, and $-W$) and nonselective ($-L$ and $-W$) media. Decreasing cell densities in the dilution series are illustrated by narrowing triangles. LW depicts a representative dilution series on nonselective plates. The observed interaction intensities are illustrated at the right. XX indicates strong interaction (growth detectable to at least 10^{-4} dilution). X indicates weak interaction (growth detectable at 10^{-3} dilution; -, no interaction).

similar affinity toward AtCBL1, whereas, for example, the pairs AtCIPK4/AtCIPK7 and AtCIPK13/AtCIPK18 displayed a rather different interaction profile. These data indicate, that sequence similarity and evolutionary history are not sufficient to predict CBL-CIPK interactions. Therefore, the exact structural features determining the specificity of complex formation remain to be uncovered. In addition, these findings might suggest that even a high conservation of certain CBL or CIPK family members does not necessarily indicate functional redundancy.

In our analyses, AtCBL9 displayed strong interaction with six different CIPKs and some degree of interaction with additional six CIPKs (Fig. 7). Of the strongly interacting kinases, only a subset of four CIPKs (AtCIPK1, 8, 18, and 24) exhibited a similar affinity toward both CBL proteins. In contrast, AtCIPK7 and AtCIPK17 preferentially interact with AtCBL1, whereas AtCIPK21 and AtCIPK23 appear to interact more efficiently with AtCBL9.

Although the interaction observed in yeast does not necessarily mean that these proteins also interact in planta, these data nevertheless indicate that even if several kinases are expressed simultaneously in a single cell, differential interaction affinity can facilitate an efficient signal channeling. Moreover, despite the high similarity of AtCBL1 and AtCBL9, our data suggest that both calcium sensor proteins could target specific kinases with different efficiency. Kinases that are expressed in the same cells and interact equally strongly with both CBL proteins may potentially represent signaling nodes for different stimuli received by the two CBL proteins.

To further address this aspect, we comparatively analyzed the expression patterns of the closely related *AtCBL1* and *AtCBL9* genes in response to different environmental cues. Two-week-old seedlings were challenged with cold, drought, or salt stress, and RNA was extracted from samples harvested at the indicated time points (Fig. 8). Semiquantitative RT-PCR analyses were performed with gene-specific primers to determine the expression levels of *AtCBL1*, *AtCBL9*, and, as a constitutively expressed

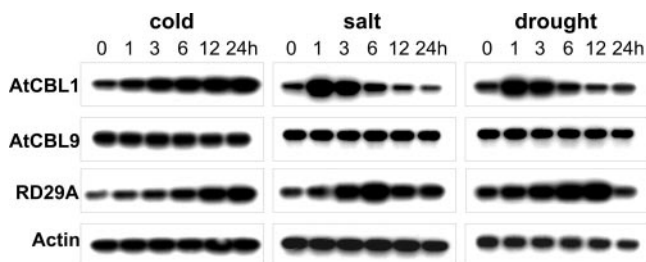


Figure 8. Comparative expression analysis of *AtCBL1* and *AtCBL9* in response to abiotic stresses. Semiquantitative RT-PCRs were performed with gene-specific primers using cDNAs synthesized from RNA samples isolated from tissues harvested at the indicated time points of cold, salt, or drought treatments. For experimental details, see “Materials and Methods.”

control, *actin2*. The expression of the stress-induced *RD29A* gene was analyzed as a control for the effectiveness of the performed treatments. These analyses revealed that whereas *AtCBL1* gene expression was strongly induced under these conditions, *AtCBL9* expression did not respond to these stimuli, providing additional evidence for different functions of the two proteins. This assumption is further corroborated by the recent finding that loss of *AtCBL1* gene function impairs the ability of the affected mutant plants to respond to drought, cold, and salt stress (Albrecht et al., 2003; Cheong et al., 2003). These findings suggest that CBL1 represents an upstream regulator of stress gene expression and serves as a rate-limiting factor in multiple abiotic stress response pathways. Interestingly, despite the observed effects of both *cb11* knock-out mutants on salt, drought, and cold stress responses, these mutant were indistinguishable from wild-type plants with regard to their responsiveness to the phytohormone ABA. This observation points to a function of CBL1 upstream or independent of any modulation of stress signaling by ABA. In contrast, in another investigation of CBL1 function (there designated as SCaBP5) the authors postulated a function of this calcium sensor protein as a global regulator of ABA responses (Guo et al., 2002). These contradictory findings might be explained by the RNA interference-based experimental approach pursued in the study by Guo et al. (in contrast to the T-DNA induced loss-of-function allele studies in the two other reports; discussed in detail by Albrecht et al. [2003]). Accordingly, our preliminary characterization of a T-DNA-induced *cb19* knock-out mutant revealed a specific impairment of ABA responses in these plants (C. D’Angelo and J. Kudla, unpublished data).

Taken together, our data suggest that functional redundancy might only occur to a limited extent within the CBL-CIPK signaling network. Therefore, its components should be amenable to functional analyses by reverse genetic approaches using T-DNA or transposon-induced knock-out lines. Our genomic and bioinformatics analyses of CBL and CIPK genes and proteins presented in this work can provide an important foundation for the further functional dissection of these important plant-specific signaling pathways.

MATERIALS AND METHODS

Database Searches and Computational Analyses of CBL and CIPK Sequences and Genomic Loci

The complement CBL and CIPK sequences encoded in the Arabidopsis genome were identified by sequence comparisons of previously identified AtCBLs and AtCIPKs with entries in the GenBank database (<http://www.ncbi.nlm.nih.gov>) and the Arabidopsis MIPS database (<http://mips.gsf.de/proj/thal/db/index.html>) using BLASTP and TBLASTN and the “motif” algorithms (Altschul et al., 1990, 1997; Falquet et al., 2002). CBL and CIPK encoding sequences from rice (*Oryza sativa*) were identified using the same algorithms to search the rice genome sequences deposited in three different databases: (a) the nonredundant rice sequence subset of the Gen-

Bank database, containing the results of the International Rice Genome Sequencing Project (<http://www.ncbi.nlm.nih.gov/blast/>), (b) the draft rice genome sequence of the Torrey Mesa Research Institute (<http://portal.tmri.org/rice/RicePublicAccess.html>; Goff et al., 2002), and (c) the draft rice genome sequence of the Chinese Academy of Sciences (<http://www.ncbi.nlm.nih.gov/blast/Genome/PlantBlast.shtml>; Yu et al., 2002). The first two sequence sets were derived from *O. sativa* subsp. *japonica* and the third from *O. sativa* subsp. *indica*. If possible, predictions of *OsCBL* and *OsCIPK* coding sequences were verified with available EST and full-length cDNA sequences. For verification and identification of special features in the UTRs or of splicing variants of the analyzed genes, BLASTN searches for corresponding cDNA full-length and EST sequences were performed in the GenBank database.

Deduced protein sequences were aligned with ClustalX (Thompson et al., 1997). Members of the CBL gene family were identified based on sequence conservation compared with other calcium-binding proteins as well as strictly conserved distances between the four EF hand loops. In addition to sequence conservation, the presence of the NAF domain (Albrecht et al., 2001) was the decisive criterion to include a kinase sequence into the CIPK gene family. Resulting multiple alignments were processed with BIOEDIT to determine the homology scores. For construction of phylogenetic trees, the CBL or CIPK amino acid sequences were aligned together with chosen representatives from related gene families. Tree reconstruction using the neighbor-joining method and confirmation of tree topology by bootstrap analysis (1,000 replicates) were performed with ClustalX (default settings). Analysis of chromosomal segment duplications affecting CBL or CIPK loci was performed as described (Kolukisaoglu et al., 2002).

cDNA Cloning, Sequence Analyses, Plasmid Constructions, and Yeast Two-Hybrid Assays

To identify conserved protein modification motifs and targeting sequences, the CBL and CIPK proteins were analyzed with the PROSITE (Falquet et al., 2002) and PSORT (<http://psort.nibb.ac.jp/>) programs, respectively. Potential N-terminal palmitoylation sites were identified by comparison of the N-terminal sequences with the N-terminal sequences of the known myristoylated and palmitoylated proteins Ara6 (Ueda et al., 2001; accession no. NP_567008) and lck (Paige et al., 1993; Yurchak and Sefton, 1995; accession no. I48845).

The identification and cloning of *AtCBL1* to 6 and *AtCIPK1* to 15 has been described previously (Albrecht et al., 2001). A complete list of primers used for RT-PCR, cDNA cloning, and expression studies during the course of this work is provided in Supplemental Table SVI. RNA isolation, RT-PCR, plasmid constructions for *AtCBL7* to 10 and *AtCIPK18* to 24, and yeast two-hybrid studies were performed as previously described (Albrecht et al., 2001). All yeast two-hybrid studies were performed in duplicate.

Structural and Motif Analysis of Proteins

The position of the EF hands within the CBL proteins was determined by comparing the complete sequence of all AtCBL proteins with the EF consensus sequence, available at PROSITE (PDOC00018). Comparisons of AtCBL EF hands 1, 2, 3, and 4 were performed based on the calculated consensus sequence. These consensus sequence was calculated with the ClustalW algorithm (Thompson et al., 1994) based on an alignment of all EF hands from HsCNB, AtCaM, and the three calcium-binding EF hands from HsNCS-1, HsGCAP-2, and the canonical EF hands identified in the different AtCBLs.

Stress Treatments and Quantitative RT-PCR Analyses

In all stress experiments described, tissue samples of Arabidopsis cv Wassilewskija were taken after 0, 1, 3, 6, 12, and 24 h, immediately frozen in liquid nitrogen, and stored at -80°C until use. Cold treatments were performed with 14-d-old soil-grown seedlings, which were transferred from 21°C to 2°C and kept under these conditions for 24 h in the dark. For drought assays, plants were grown hydroponically in Magenta boxes for 14 d on one-half-strength Murashige and Skoog medium with 0.5% (w/v) Suc. Magenta boxes were transferred into a flow chamber, and plants were exposed to a stream of dry air in a flow bench for 20 min (resulting in approximately 10% fresh weight loss) and then returned into Magenta

boxes. Afterward the Magenta boxes were closed and samples were taken at the described time points. For salt stress response analyses, the hydroponic cultivation medium was supplemented with 100 mM NaCl, and samples were taken at the respective time points.

RNA for quantitative RT-PCR analyses was isolated from 150-mg tissue samples using TriFast solution (Peqlab, Erlangen, Germany). cDNA syntheses were performed using 3 μg of random hexamer primer and 200 units of Moloney murine leukemia virus RT (Promega, Madison, WI) for 1 h at 42°C . Two hundred nanograms of cDNA was used as template in all PCR reactions with gene-specific primers. In preliminary experiments, we first determined for each gene to be analyzed the cycle number that was within the linear range of PCR product amplification. Subsequently, PCR amplifications (1 min, 94°C ; 1 min 30 s, 58°C ; 1 min, 72°C ; and volume, 50 μL) were performed for 23 cycles. The six transcripts (corresponding to each time point) were assayed simultaneously in separate tubes using identical cDNA aliquots and the respective gene-specific primers. Aliquots (10 μL) of each amplification reaction were separated by agarose gel electrophoresis and transferred onto nylon membranes. All RT-PCR reactions were performed in triplicate. Hybridizations were carried out with an equimolar mixture of DNA fragments specific for the *AtCBL1*, *AtCBL9*, *Atactin2*, and *RD29A* genes and radioactively labeled with a Ready Prime kit (Amersham Biosciences, Uppsala). For signal detection and quantification, membranes were either exposed to x-ray films or evaluated by phosphorimaging.

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