Interaction Specificity of Arabidopsis Calcineurin B-Like Calcium Sensors and Their Target Kinases

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Calcium is a critical component in a number of plant signal transduction pathways. A new family of calcium sensors called calcineurin B-like proteins (AtCBLs) have been recently identified from Arabidopsis. These calcium sensors have been shown to interact with a family of protein kinases (CIPKs). Here we report that each individual member of AtCBL family specifically interacts with a subset of CIPKs and present structural basis for the interaction and for the specificity underlying these interactions. Although the C-terminal region of CIPKs is responsible for interaction with AtCBLs, the N-terminal region of CIPKs is also involved in determining the specificity of such interaction. We have also shown that all three EF-hand motifs in AtCBL members are required for the interaction with CIPKs. Several AtCBL members failed to interact with any of the CIPKs presented in this study, suggesting that these AtCBL members either have other CIPKs as targets or they target distinct proteins other than CIPKs. These results may provide structural basis for the functional specificity of CBL family of calcium sensors and their targets.

Ca^{2+} serves as a second messenger in all eukaryotic systems. A typical pathway to transmit the Ca^{2+} signal starts with a protein sensor that binds Ca^{2+} and changes its conformation and function (Chen et al., 1995; Schaad et al., 1996; Ames et al., 1997; Millward et al., 1998; Braunewell and Gundelfinger, 1999). The Ca^{2+} sensor often interacts with other signaling proteins (the "targets") to relay the signal (Vogel, 1994; Crivici and Ikura, 1995; Zielinski, 1998; Iacovelli et al., 1999). Several families of Ca^{2+} sensors have been identified in higher plants. Perhaps the best known is calmodulin (CaM) and CaM-related proteins, which contain four EF-hand domains responsible for Ca^{2+} binding (Zielinski 1998). These Ca^{2+} sensors are small proteins that do not have enzymatic activity by themselves and function by interacting with their target proteins (Zielinski, 1998). The second major class is the CaM-domain protein kinases (CDPK) that contain CaM-like Ca^{2+}-binding domains and a kinase domain in a single protein (Roberts and Harmon, 1992; Harmon et al., 2000). Each individual CDPK protein is expected to detect the changes in the Ca^{2+} parameters and translate these changes into the regulation of a protein kinase activity (Roberts and Harmon, 1992; Harmon et al., 2000).

Two recent studies have identified genes encoding a new family of Ca^{2+} sensors from Arabidopsis (Liu and Zhu, 1998; Kudla et al., 1999). These proteins are similar to both the regulatory B subunit of calcineurin and the neuronal Ca^{2+} sensor (NCS) in animals (Olafsson et al., 1995; Klee et al., 1998). We refer to these unique plant Ca^{2+} sensors as Arabidopsis calcineurin B-like (AtCBL) proteins (Kudla et al., 1999). One member of the *AtCBL* gene family, *AtCBL1*, is highly inducible by stress signals, including drought, cold, and wounding (Kudla et al., 1999). Another member, *AtCBL4* or *Salt Overly Sensitive 3 (SOS3)*, has been shown to play a role in salt resistance of Arabidopsis (Liu and Zhu, 1998). These results strongly suggest that the AtCBL family of Ca^{2+} sensors is involved in plant signal transduction processes in response to stress conditions. Like CaM, calcineurin B, and NCS, AtCBL family members are also small Ca^{2+}-binding proteins that do not have enzymatic activity by themselves and may function by interacting with other signaling proteins.

Using yeast two-hybrid screening, Shi et al. (1999) identified a group of novel protein kinases (CIPKs) as target proteins for AtCBL1. Halfter et al. (2000) similarly found a similar group of protein kinases (SIPs) as targets for AtCBL4 or SOS3. CIPKs/SIPs represent a new subclass of protein kinases that are related to SNF1/AMPK family in the kinase domain but contain a unique regulatory domain in the C-terminal region. The C-terminal regulatory domain is required and sufficient for interacting with AtCBL Ca^{2+} sensors (Shi et al., 1999; Halfter et al., 2000). Halfter et al. (2000) also showed that AtCBL4 interacts with SOS2, a protein kinase that belongs to the CIPK/SIP family. Both SOS3 and SOS2 are involved in salt tolerance of Arabidopsis plants. Therefore, AtCBL/SOS3 interaction with CIPK/SIP may have established a new paradigm for Ca^{2+} signaling in higher plants.

Based on previous studies, both AtCBLs and CIPKs/SIPs clearly represent multi-member protein families, raising an important question on the specificity of interaction and function among members in each protein family. We speculated that the specificity for AtCBL function may stem from several aspects such as expression pattern, subcellular localization,

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interaction affinity with CIPKs, and other targets specific to each isoform (Shi et al., 1999). To assess specificity of AtCBL-CIPK interaction and to identify other isoform-specific interactors, we conducted systematic yeast two-hybrid experiments to screen for interacting proteins with different AtCBL isoforms. Among positive clones identified from all screening experiments, various CIPK members are consistently the most abundant interactors for three AtCBL isoforms (AtCBL1, AtCBL3, and AtCBL4). Another group of three AtCBL members (AtCBL5, AtCBL7, and AtCBL8) interacted with completely different targets other than CIPKs. It is interesting that all the CIPKs identified by yeast two-hybrid screen were present in an N-terminal truncated form. When the full-length proteins of these CIPK members were tested for their interaction with AtCBLs, we observed a strong AtCBL-isoform specificity in the interaction assays. We further addressed the structural basis for these specific interactions.

RESULTS

Identification of New Members of AtCBL Family

Physical and functional interactions between AtCBL family Ca^{2+} sensors and their target kinases (CIPKs) have defined a new paradigm for Ca^{2+} signaling in plants (Liu and Zhu, 1998; Kudla et al., 1999; Shi et al., 1999; Halfter et al., 2000). To further understand the functional specificity of various members in the AtCBL family, we identified additional members by searching the Arabidopsis genome database. At least eight different members of AtCBLs were identified. Among them, the cDNA clones for three new members in addition to the previously identified ones (AtCBL1, AtCBL2, AtCBL3, and AtCBL4) were isolated and named AtCBL5, AtCBL7, and AtCBL8, respectively. The deduced amino acid sequences of six members of AtCBL family were compared in Figure 1A. Although all members contain three highly conserved EF-hand motifs, some members have extended N-terminal and C-terminal regions that could be functionally important. For example, three of the six members listed in Figure 1A, AtCBL1, AtCBL4, and AtCBL8, start with a putative myristoylation signature motif [MGXXXSK] (Towler et al., 1988), whereas the other three do not have this sequence motif. It is clear that this difference alone may render distinct subcellular localization to various members.

Interaction Cloning Revealed Specific Interactors for Different AtCBL Members

To understand whether members of AtCBL family perform different or similar function in plants, it is critical to determine whether they interact with same protein targets. As reported earlier (Shi et al., 1999; Halfter et al., 2000), both AtCBL1 and AtCBL4 interact with a number of CIPKs. But it is not known if all AtCBL members interact with all CIPKs, if there is interaction specificity among AtCBLs and CIPKs, or if different AtCBL members actually interact with distinct targets other than CIPKs. To address these possibilities, we performed systematic yeast two-hybrid screens using each of the six AtCBL members as bait. AtCBL1, AtCBL3, and AtCBL4 each interacted with a number of protein kinases that belong to the CIPK family. However, AtCBL5, AtCBL7, and AtCBL8 interacted with proteins other than CIPKs (data not shown). Among the CIPKs isolated, few interacted with all three AtCBL members. These results provided an important clue regarding the specificity of interaction between AtCBLs and CIPKs.

We decided to further address the interaction specificity between CIPKs and AtCBLs. Sequence analysis of these interacting protein kinases indicated that most of them were truncated versions lacking the N-terminal kinase domain, consistent with the fact that the C-terminal region of CIPK1 is responsible for interaction with AtCBLs (Shi et al., 1999). For further analysis, we chose four of the protein kinase clones (CIPK2, 3, 5, and 6) from the yeast two-hybrid screens and isolated the full-length cDNAs. The deduced amino acid sequences of the clones were compared with that of the previously identified CIPK1 gene and shown in the Figure 1B. All of them contain the conserved amino acid residues and the 11 subdomains that are characteristic of the Ser/Thr protein kinase domain (Hanks and Quinn, 1991). Their predicted molecular masses are approximately 50 kD except for CIPK3 (43 kD), which has shorter C-terminal region than other CIPKs. The kinase domains of CIPKs share at least 50% amino acid sequence identity, whereas the C-terminal regions share approximately 30% identity.

Both the N- and C-Terminal Regions of CIPKs Are Involved in Specifying the Interaction with AtCBLs

To analyze the interaction between the full-length protein of CIPKs and AtCBLs, we cloned the full-length CIPKs into the activation domain vector and assayed interaction with various AtCBLs in the DNA-binding domain vector. Several interesting observations are summarized based on the data in Table I. It is clear that each AtCBL member only interacts with a subset of CIPK members. AtCBL1 and AtCBL4 interacted with CIPK1 and CIPK6, respectively. AtCBL3 interacted with four of the five CIPKs tested in this study. As anticipated from the yeast two-hybrid screenings, AtCBL5, 7, and 8 did not interact with any of the five CIPKs. It is more interesting that some CIPK members that interacted with AtCBLs in the N-terminal truncation forms failed to interact with the same AtCBLs when tested in the full-length form. For example, the N-terminal truncated version of CIPK1, 2, 3 were identified by interaction with AtCBL1. However, only CIPK1, but not

Figure 1. Sequence analysis of AtCBLs and CIPKs. A, Amino acid sequence comparison of AtCBL1 (GenBank accession no. AF076251), AtCBL3 (AF076253), AtCBL4 (Y18870), AtCBL5 (AC009519.4), AtCBL7 (AL078465.1), and AtCBL8 (AF069300.1). Solid lines above the sequence indicate the EF-hand motifs. Residues with black background indicate amino acids conserved in at least three genes, and dashes represent gaps to maximize alignment. B, Amino acid sequence comparison of CIPK1 (GenBank accession no. AB022219), CIPK2 (AF286050), CIPK3 (AF286051), CIPK5 (AF285105), and CIPK6 (AF285106). Arrowheads and Roman numerals above the sequences indicate the conserved amino acids and subdomains of Ser/Thr protein kinases, respectively. Solid and dashed underlines indicate regions used to create pGAD.CIPK5C35 and PGAD.CIPK5C20 plasmids, respectively.

other two CIPKs, interacted with AtCBL1 when tested in the full-length form. AtCBL4 protein similarly interacted with the C-terminal domain of both CIPK5 and CIPK6 but interacted only with the full-length protein of CIPK6. These results suggest that the N-terminal region of CIPKs may regulate interaction between AtCBLs and CIPKs.

We then systematically tested the interaction between AtCBLs and the C- and N-terminal region of CIPKs. As shown in Table I, although AtCBL5, 7 and

Table I. *Interaction of CIPKs with AtCBLs in a yeast two-hybrid assay*

The kinase domain (N) or C-terminal region (C) of CIPKs were cloned into the pGAD vectors. Combinations of pGAD.CIPKs and pGBT.CBLs were co-transformed into the Y190 yeast cells. Yeast growth was scored as growth (+) and no growth (−). Numbers in the parentheses indicate units of β-galactosidase activity assayed. Yeast growth and enzyme assay are described in "Materials and Methods."

Constructs	pGBT	pGBT-CBL1	pGBT-CBL3	pGBT-CBL4	pGBT-CBL5	pGBT-CBL7	pGBT-CBL8
pGAD-CIPK1	− (<1)	+ (5.1)	+ (28.2)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK1N	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK1C	− (<1)	+ (9.1)	+ (66.5)	+ (2.4)	− (<1)	− (<1)	− (<1)
pGAD-CIPK2	− (<1)	− (<1)	+ (26.7)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK2N	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK2C	− (<1)	+ (5.4)	+ (78.2)	+ (38.2)	− (<1)	− (<1)	− (<1)
pGAD-CIPK3	− (<1)	− (<1)	+ (37.8)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK3N	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK3C	− (<1)	+ (30.0)	+ (149.2)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK5	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK5N	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK5C	− (<1)	+ (81.4)	+ (62.5)	+ (78.5)	− (<1)	− (<1)	− (<1)
pGAD-CIPK6	− (<1)	− (<1)	+ (36.2)	+ (14.1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK6N	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)	− (<1)
pGAD-CIPK6C	− (<1)	+ (32.7)	+ (77.6)	+ (67.6)	− (<1)	− (<1)	− (<1)

8 did not interact with any C-terminal region of CIPK members, the C-terminal region of CIPKs generally interacted with AtCBL1, 3, and 4 at reasonably high yet different strength. One exception to this rule is the C-terminal region of CIPK3, which is much shorter as compared with the C-terminal region of other CIPKs (Fig. 1B). The CIPK3 C-terminal region interacted with AtCBL1 and AtCBL3 but not with AtCBL4. The N-terminal kinase domain of CIPKs did not interact with any AtCBL, consistent with the observation in the previous study (Shi et al., 1999). It is noteworthy that the full-length protein of CIPKs generally interacted with the AtCBL members at lower strength than the C-terminal region alone, suggesting that the N-terminal region of CIPKs hinder the interaction between the C-terminal region and AtCBLs. This inhibitory effect appears to play an important role in specifying the interaction between CIPK and AtCBL members.

Domains in the CIPK N-Terminal Region Determine the Inhibition of AtCBL-CIPK Interaction

Unlike other CIPKs, the full-length protein of CIPK5 did not interact with any of AtCBLs tested in this study, although its C-terminal region strongly interacted with AtCBL1, 3, and 4. These results suggest that the N-terminal region of CIPK5 completely blocked the interaction between its C-terminal region and the AtCBL members. To determine if the inhibitory information resides in the N-terminal region alone, we chose CIPK5 and CIPK6 to conduct domain swapping experiments using the yeast two-hybrid system.

We reasoned that if the N-terminal region alone carries the inhibitory information, the chimeric protein formed by the N-terminal region of CIPK5 and the C-terminal region of CIPK6 (pGAD.CIPK5N-6C) should behave like CIPK5 full-length protein and interact with none of the AtCBLs. The chimera formed by the N-terminal region of CIPK6 and the C-terminal region of CIPK5 (pGAD.CIPK6N-5C), similarly, should interact with AtCBL1, 3, and 4 at reduced level. Results in Figure 2 support our reasoning. The N-terminal region of CIPK5 completely abolished the interaction of CIPK6 C-terminal region with the AtCBLs. The CIPK6 N-terminal region significantly decreased the interaction strength between the CIPK5 C-terminal region and AtCBLs.

To locate the sequences responsible for inhibition of AtCBL interaction, we made a series of deletions in the N-terminal region of CIPK5 as shown in Figure 3. Whereas deletions down to 196 amino acid residue did not change the interaction pattern, removal of additional 29 amino acids began to allow the interaction with AtCBL4 but not with other AtCBLs. These results suggest that the sequence sufficient for inhibiting CIPK5 interaction with AtCBLs is present in a 55-amino acid stretch between 197 and 252. When this region is fused to the C-terminal region of CIPK6, the chimeric protein did not interact with any AtCBLs, further supporting the conclusion. We noted that this region spans subdomain 9, 10, and 11 of the CIPK5 kinase domain and exhibits relatively low sequence conservation among CIPKs compared with other subdomains.

Interaction Domains in CIPKs and AtCBLs

We have shown previously that the C-terminal region of CIPK1 is required and sufficient for interaction with AtCBL1, 3, and 4 (Shi et al., 1999). Sequence alignment of all the identified CIPKs displayed a small conserved region in the AtCBL interacting region as indicated in Figure 1B. This led us to speculate that the small conserved region in the C-terminal region may be directly involved in the physical interaction with AtCBLs. In this study, we tested whether the small conserved motif is in fact sufficient

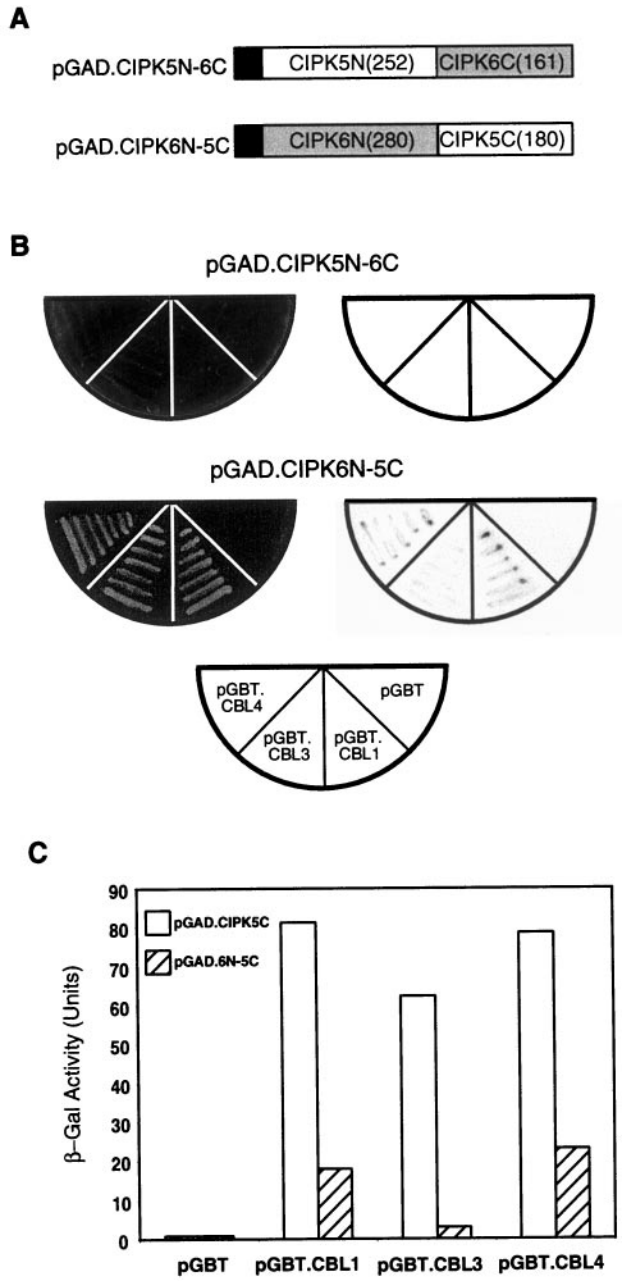

Figure 2. The kinase domain of CIPK5 interferes with the interaction between AtCBLs and the C-terminal region of CIPKs. A, Schematic diagram of the chimeric constructs. The pGAD.CIPK5N-6C and pGAD.CIPK6N-5C plasmids were created as described in "Materials and Methods." White and light gray boxes represent CIPK5 and CIPK6, respectively. B, Yeast two-hybrid assays. The chimeric plasmids were transformed into the Y190 yeast cells, which contain each of the pGAD plasmids indicated in the half circle at the bottom. The half circles at left indicate growth of the yeast cells on SC-His-Leu-Trp medium. The half circles at right show the filter-lift assay. C, Measurement of β-galactosidase activity. Three individual transformants were used to measure the β-galactosidase activity as described in "Materials and Methods." Each value represents the average.

for interaction with AtCBLs using the yeast two-hybrid system. For this purpose, we constructed two plasmids pGAD.CIPK5C35 and pGAD.CIPK5C20, each of which contains a small fragment from the conserved C-terminal region of CIPK5 (35 and 20 amino acids, respectively) in the activation domain vector. Then the two plasmids were tested for interaction with AtCBL1, 3, and 4. The CIPK5 protein was selected because its C-terminal region interacts strongly with all the three AtCBLs. As shown in Figure 4A, both 35 and 20 amino acid fragments interacted with AtCBL4 but not with AtCBL1 and AtCBL3. Compared with the interaction strength between AtCBL4 and the entire C-terminal region of CIPK5 (CIPK5C), the two fragments interacted with AtCBL4 at significantly reduced levels (Fig. 4B). These results suggest that the conserved domain in the C-terminal region of CIPKs may be sufficient for interacting with certain AtCBL member(s) but may need surrounding sequences to have an optimum interaction.

We also dissected the structural requirement in the AtCBLs for interaction with CIPKs. The AtCBL3 protein was taken as an example because it interacted with multiple CIPKs and had an extended N-terminal sequence. It is interesting that almost any deletion in the AtCBL3 sequence abolished interaction with CIPKs as shown in Figure 5A. One exception was the deletion of first 30 amino acids from the N terminus of AtCBL3. When co-transformed with pGAD.CIPKs, this N-terminal deleted form supported yeast growth on the selection medium as the full-length AtCBL3 (Fig. 5B). However, interaction strength measured by the filter-lift assay and β-galactosidase solution assay was dramatically decreased except for interaction with CIPK6 (Fig. 5C). These results suggest that the deleted region of the protein is required for interaction with CIPKs. These regions include the EF-hand domains and the short extensions at both C and N termini. This rigorous sequence requirement for AtCBL3 probably explains why AtCBL7 did not interact with any of the AtCBL3-interacting kinases, even though it shares al-

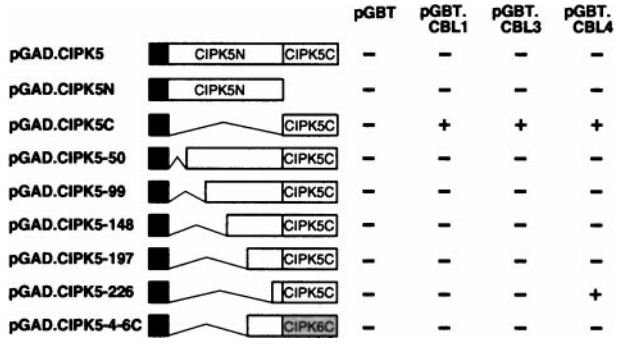

Figure 3. Identification of inhibitory domain in the N-terminal region of CIPK5. A series of N-terminal deletion mutants of CIPK5 were cloned into the pGAD vector and transformed into Y190 yeast cells containing pGBT, pGBT.CBL1, pGBT.CBL3, or pGBT.CBL4. Co-transformed yeasts were assayed for bait-prey interactions by determining their growth and measuring β-galactosidase activity. The plus signs indicate both yeast growth and color development in the filter-lift assay. The minus signs represent no growth. Other symbols are the same as in Figure 2.

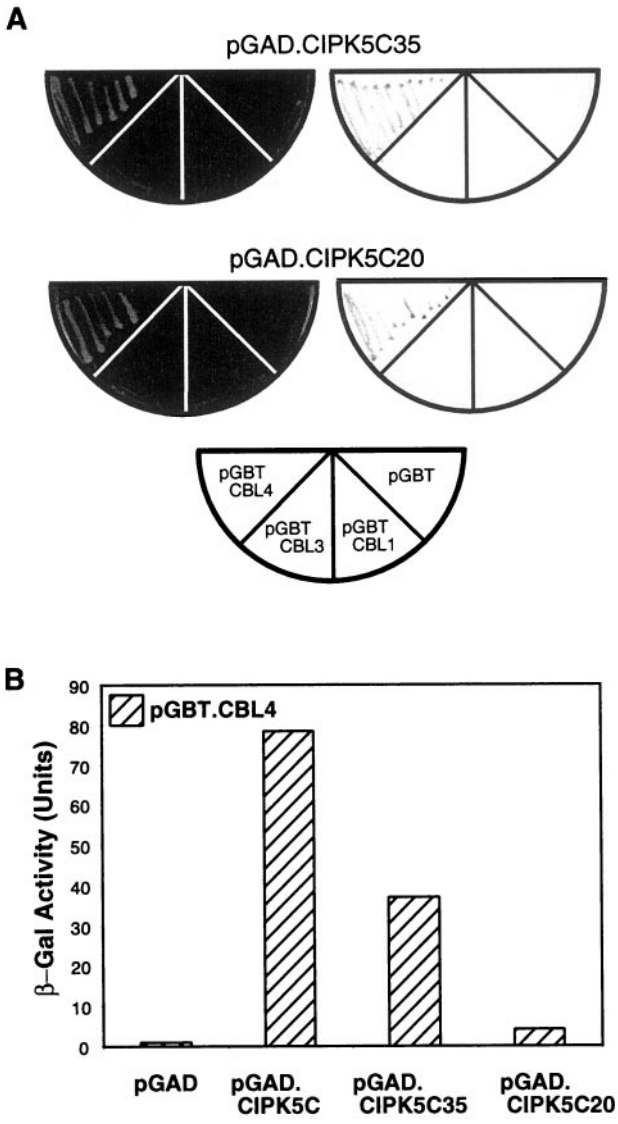

Figure 4. The small conserved domain in the C-terminal region of CIPK5 interacts with AtCBL4 at reduced strength. A, Yeast growth and filter-lift assays. The pGAD.CIPK5C35 and pGAD.CIPK5C20 plasmids were created as described in "Materials and Methods." The plasmids were then co-transformed into the Y190 yeast strains with pGBT, pGBT.CBL1, pGBT.CBL3, and pGBT.CBL4. The half circles at left indicate yeast growth on the selection medium. The half circles at right show the filter-lift assay results. The half circle at bottom represents the arrangement of the yeast strains containing the indicated plasmids. B, Measurement of β-galactosidase activity from the yeast strains containing pGBT.CBL4 and the indicated pGAD plasmids. Three individual transformants were used to measure the β-galactosidase activity as described in "Materials and Methods." Each value represents the average.

most 80% overall sequence identity with AtCBL3. The sequence in the N-terminal extension of AtCBL7 apparently is different from that of AtCBL3. This finding may also provide an explanation for the fact that AtCBL5, 7, and 8 failed to produce any CIPK-like clones from the yeast two-hybrid screenings.

DISCUSSION

Ca^{2+} serves as a ubiquitous second messenger in plant signal transduction (Bush, 1995; Ehrhardt et al., 1996; Franklin-Tong et al., 1996; Knight et al., 1996, 1997; Felle and Hepler, 1997; Holdaway-Clarke et al., 1997). Change in Ca^{2+} concentration is often detected by Ca^{2+}-binding proteins (as Ca sensors) that include CaM, CDPK, and CBL. In response to a change in

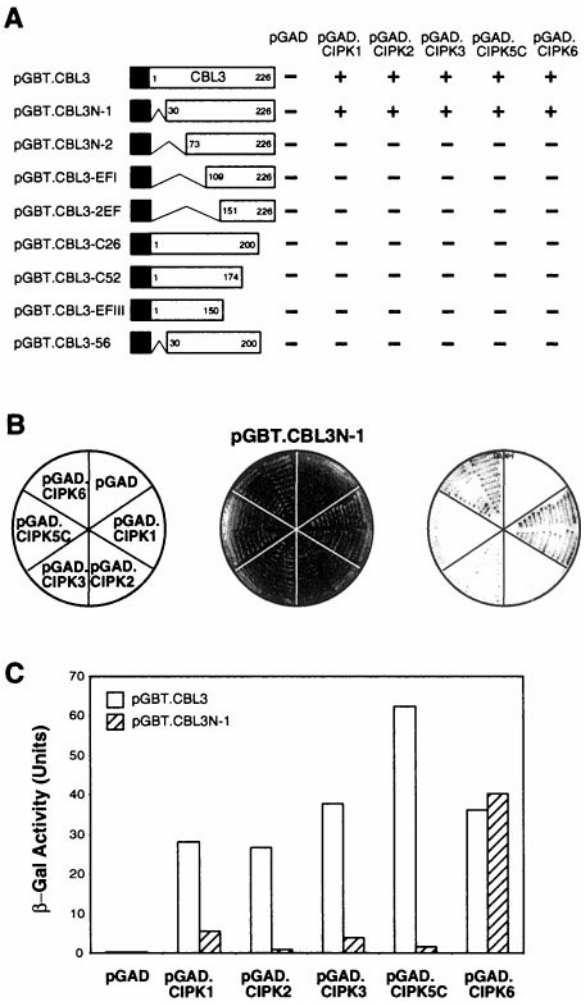

Figure 5. Small deletions in either N- or C-terminal ends of AtCBL3 dramatically changes its ability to interact with CIPKs. A, Yeast growth on the selection medium. Solid black boxes indicate the DNA-binding domain in the pGBT vector. Numbers in the open boxes represent the beginning and the ending positions of AtCBL3 protein fragments. Each deletion construct was transformed into the Y190 yeast strains containing pGAD, pGAD.CIPK1, pGAD.CIPK2, pGAD.CIPK3, pGAD.CIPK5C, and pGAD.CIPK6, respectively. Yeast growth was monitored on the selection medium and indicated as growth (+) and no growth (−). B, Filter-lift assay of pGBT.CBL3N-1. The pGBT.CBL3N-1 plasmid was transformed into the yeast cells, which contain each of the indicated pGAD plasmids (left circle). The circles in the middle and at right show the results of yeast growth and filter-lift assay, respectively. C, Measurement of β-galactosidase activity. Three individual transformants were used to measure the β-galactosidase activity as described in "Materials and Methods." Each value represents the average.

Ca^{2+} concentration, all of these Ca^{2+} sensors may be activated and begin to function in the same or distinct pathways. In addition, each of these three types of Ca^{2+} sensors forms a large family with many similar but distinct members. How do all these individual Ca^{2+} sensors function in a plant cell? For CaM and CBL, they function by interacting with their target proteins. Based on the current research, the members in the CaM family often interact with the same or similar protein targets (Zielinski, 1998). Previous studies on AtCBL family also suggest that AtCBLs interact with a family of kinases called CIPKs or SIPs (Shi et al., 1999; Halfter et al., 2000). In the present study, we sought to determine the interaction specificity among AtCBL and CIPK family members to provide molecular basis for the functional diversity of AtCBL/CIPK pairs.

As a step toward this goal, we have screened a yeast two-hybrid library using several AtCBL members as baits to identify interacting clones. Each AtCBL member appeared to interact with a number of protein targets. Three of the AtCBLs (AtCBL1, 3, and 4) predominately target a group of protein kinases called CIPKs, whereas other three members (AtCBL5, 7, and 8) did not interact with any CIPKs or CIPK-like molecules. Moreover, the three CIPK-interacting AtCBLs showed significantly different interaction strength toward individual CIPKs. These results have clearly demonstrated isoform specificity that governs the interaction between AtCBLs and CIPKs. Of course, the expression pattern and the subcellular localization of AtCBLs and CIPKs will also contribute to their final pairing in vivo. Distinct AtCBL-interaction profile of each CIPK will probably specify the regulation and therefore the function of CIPK members. The strong interaction between AtCBL4/SOS3 and SOS2 kinase presents an example how interaction affinity could determine the functional specificity (Halfter et al., 2000). AtCBL1-CIPK1 interaction in vivo strongly suggests that AtCBL1-CIPK1 function together in cellular processes including stress responses (Shi et al., 1999; G. Pandey, S. Luan, unpublished data).

We also investigated the structural basis for interaction specificity between CIPKs and AtCBLs. Our studies demonstrated that both the N-terminal kinase domain and the C-terminal regulatory domain in CIPKs play a role in specifying interactions with AtCBLs. The C-terminal region contains the structural domains responsible for actual interaction and confers only partial specificity toward different AtCBL members. This partial specificity is further enhanced by the N-terminal region that inhibits interaction between CIPK C-terminal regions and AtCBLs. For example, the C-terminal region of CIPK 2 interacted with AtCBL1, 3, and 4 at different strength. The interaction strength with AtCBL3 is much higher than with AtCBL1 and AtCBL4. When tested in full-length protein, CIPK2 interacted with only AtCBL3, indicating that weaker interactions between its C-terminal region and the other two AtCBLs were eliminated by N-terminal inhibition.

The degree of inhibition conferred by the N-terminal region differs among the CIPK members. The strongest inhibition was observed with the N-terminal region of CIPK5 that blocked the strong interactions between its C-terminal region and all three AtCBLs. Deletion analysis of the CIPK5 N-terminal region further identified a small region (55 amino acids in length), which is sufficient to inhibit the interaction with AtCBLs. This domain is located between subdomain 9 and 11 of the CIPK5 kinase domain. When this small region is fused to the CIPK6 C-terminal region, it prevented the chimeric protein from interacting with AtCBLs. Since the sequences in these subdomains are less conserved among the CIPK members, it is possible that the inhibition diversity in the N-terminal regions comes from sequence variation in these subdomains.

Regarding the structural requirement for AtCBLs to interact with CIPKs, we found that almost all sequence information in AtCBL3 is necessary. Besides the three EF-hand motifs in the center of the AtCBL sequences, each AtCBL member has a variable N- and C-terminal extension. We noted that the N-terminal extension in AtCBL3 is conserved, to some degree, in AtCBL1 and AtCBL4, but not in AtCBL5, AtCBL7, and AtCBL8 (Fig. 1A). This sequence diversity could explain why AtCBL5, AtCBL7, and AtCBL8 did not interact with any of the CIPKs tested in this study. Although we cannot rule out the possibility that a group of unidentified CIPKs serve as targets for these AtCBL members, it is more likely that AtCBL5, AtCBL7, and AtCBL8 may have protein targets other than CIPKs. The results from yeast two-hybrid screenings using these AtCBLs as baits further supported this possibility. So far, none of the interacting clones identified by AtCBL5, 7, or 8 represents any CIPK-like proteins (data not shown). Further analyses of these non-CIPK interactors will provide important information toward understanding the functional diversity of AtCBL family.

Besides interaction specificity among AtCBL and CIPK family members, several lines of evidence support that individual AtCBL and CIPK members may have distinct functions in plants. Genetic analysis indicates that AtCBL4/SOS3 is involved in ionic stress resistance in Arabidopsis. This function is clearly specific to AtCBL4/SOS3 and is not complemented by other family members (Liu and Zhu, 1998). The fact that AtCBL1 gene expression is highly induced by cold, wounding, and drought implicates AtCBL1 in osmotic and mechanical stress responses (Kudla et al., 1999). Expression of several other AtCBL members also show distinct pattern suggesting that they are expressed and function differently in plant development and stress responses (Kudla et al., 1999; Y.H. Cheong, S. Luan, unpublished data).

As we discussed earlier (Shi et al., 1999), the structural features among the AtCBL family members also provide important basis for functional diversity. Perhaps the most significant structural feature is the myristoylation motif in AtCBL1, AtCBL4, and AtCBL8. With myristoylation, these members may relocate to the cell membrane and therefore change their function (Calvert et al., 1995). The other members that do not have a myristoylation motif appear to lack a mechanism to become membrane associated. This and other structural differences will certainly make them functionally differ from each other.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening and Assays

The Arabidopsis λ-ACT cDNA expression library (CD4-22) constructed by Kim et al. (1997) was obtained from Arabidopsis Biological Resource Center (Columbus, OH). The two-hybrid library screening was carried out essentially according to Durfee et al. (1993). Briefly, the plasmid library was obtained from the phage library by in vivo excision and used to transform Y190 strain that expresses a bait protein. Transformants were plated onto the synthetic medium (SC) that lacks Leu, Trp, and His (SC-Leu-Trp-His). To inhibit background growth of yeast cells, 25 mM 3-amino-1,2,4-aminotriazole was also supplemented to the medium. Colonies that appeared within 5-d incubation were selected for further analyses. For yeast two-hybrid interaction assays, genes of interest were first cloned into either the activation domain (pGAD.GH) or the DNA-binding domain (pGBT9.BS) vectors. Then, the two plasmids were introduced into yeast strain Y190 carrying two reporter genes by the lithium acetate method (Ito et al., 1983; Schiestl and Gietz, 1989). Yeast cells carrying both plasmids were selected on the synthetic medium lacking Leu and Trp (SC-Leu-Trp). The yeast cells were streaked on the SC-Leu-Trp-His plate to determine the expression of *HIS3* nutritional reporter. The β-galactosidase expression of the His^+ colonies was analyzed by filter-lift assays as described below.

Assays of β-Galactosidase

Transformants were streaked onto the selective medium (SC-Leu-Trp) and grown for 1 d at 30°C. Filter-lift assays for blue color development were performed overnight at 30°C as described by Breeden and Nasmyth (1985). For quantitative assays, transformants were grown at 30°C to mid-log phase (optical density $[OD]_{600}$ = 0.5–1.0) in the SC-Leu-Trp liquid medium. Activity of β-galactosidase was measured at OD_{574} using chlorophenol red-β-D-galactopyranoside as the substrate according to Durfee et al. (1993) and expressed in units. The equation below was used to calculate β-galactosidase activity: units of β-galactosidase activity = $1{,}000 \times OD_{574}/(V \times T \times OD_{600})$, in which V is volume of culture in milliliters, T is reaction time in minutes, and OD_{600} is yeast cell density.

Cloning of the Full-Length CIPK cDNAs

To isolate the full-length cDNAs encoding CIPK2, 3, 5, and 6, the ^{32}P-labeled probes were prepared from AtCBL-interacting CIPK clones, which encode only the C-terminal regions of the four CIPKs. The λ-PRL Arabidopsis cDNA library (CD4-7) obtained from Arabidopsis Biological Resource Center was screened essentially as described in Kim et al. (1998). Approximately 2×10^5 plaque forming units were transferred onto nylon membranes (Hybond N^+, Amersham, UK) and hybridized with the probes. Hybridization was performed at 65°C for 20 h in 0.5 M Na_2HPO_4 (pH 7.2) and 7% (w/v) SDS with gentle agitation. Following the hybridization, filters were washed twice in solution I containing 5% SDS (w/v), 1 mM EDTA, and 0.04 M Na_2HPO_4 (pH 7.2) and once in solution II containing 1% SDS (w/v), 1 mM EDTA, and 0.04 M Na_2HPO (pH 7.2) for 15 min at 65°C. Positive plaques were selected and purified through three rounds of screening. Plasmids containing the inserts were in vivo excised from the positive plaques using a *Escherichia coli* strain DH10B(ZIP) according to the instruction manual from GIBCO BRL (Grand Island, NY). DNA sequences were determined with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and were analyzed with software from DNASTAR (Madison, WI).

Construction of Plasmids

The pGBT.CBL1 plasmid was constructed as described previously (Kudla et al., 1999). To generate pGBT.CBL3, the coding region of the AtCBL3 cDNA was PCR-amplified with a pair of primers CBL3-2 and CBL3-3. The PCR product was digested with *Eco*RI/*Sal*I and ligated into pGBT9.BS. To make plasmids pGBT.CBL3N-1, pGBT.CBL3N-2, pGBT.CBL3-EFI, and pGBT.CBL3-2EF, PCR fragments were produced from AtCBL3 cDNA with four forward primers (CBL3-22, CBL3-21, CBL3-15, and CBL3-16, respectively) and a reverse primer (CBL3-2). Following *Eco*RI/*Sal*I digestion, they were cloned into pGBT9.BS. To create plasmids pGBT.CBL3-C26, pGBT.CBL3-C52, and pGBT.CBL3-EFIII, we carried out PCRs on AtCBL3 cDNA using a forward primer CBL3-3 and three reverse primers (CBL3-20, CBL3-18, and CBL3-14, respectively) and cloned the fragments into pGBT9.BS. Primer CBL3-22 and CBL3-20 were used to make pGBT.CBL3-56.

To construct pGBT.CBL4, PCR was performed on the full-length AtCBL4 cDNA using primers P47 and pGEX3′. The PCR fragment was digested with *Bcl*I/*Sal*I and cloned into pGBT9.BS. We identified additional AtCBL members (AtCBL5, 7, and 8) by searching the Arabidopsis genome database using AtCBL1, 3, and 4 as query sequences. The cDNAs containing each of the coding regions for AtCBL5, 7, and 8 were PCR-amplified with three pairs of primers (CBL5F/5R, CBL7F/7R, and CBL8F/8R, respectively) using 2 μL of phage stock of the Arabidopsis cDNA library (Arabidopsis Biological Resource Center, Columbus, OH) as template. Each of the resulting PCR products was cloned as an *Eco*RI/*Sal*I fragment into pGBT9.BS and named pGBT.CBL5, pGBT.CBL7, and pGBT.CBL8, respectively.

Plasmids pGAD.CIPK1, pGAD.CIPK1N, and pGAD.CIPK1C were constructed and reported previously (Shi et al., 1999). To create plasmids pGAD.CIPK2, pGAD.CIPK3, pGAD.CIPK5, and pGAD.CIPK6, we performed PCRs on the full-length CIPK cDNAs using four individual pairs of primers (CK2-3/5, CK3-3/4, CK5-1/2, and CK6-1/2, respectively). Each of the PCR fragments was digested with *Bam*HI/*Sal*I and cloned into pGAD.GH. To generate the C-terminal deletion mutant constructs pGAD.CIPK2N, pGAD.CIPK3N, pGAD. CIPK5N, and pGAD.CIPK6N, the following pairs of primers CK2-3/6, CK3-3/7, CK5-1/4, and CK6-1/4 were used. Each PCR fragment was cloned in the *Bam*HI/*Sal*I site of the pGAD.GH plasmid. To make plasmids pGAD.CIPK2C, pGAD.CIPK3C, pGAD.CIPK5C, and pGAD.CIPK6C, PCRs were performed using four individual pairs of primers, CK2-7/5, CK3-6/4, CK5-11/2, and CK6-9/2, respectively. The PCR products were cloned into pGAD.GH digested with *Bam*HI and *Sal*I.

The C-terminal region of CIPK6 was amplified using primers CK6-5 and CK6-6. The resulting PCR fragment was digested with *Sal*I/*Apa*I and inserted in frame at the end of the CIPK5 N-terminal region in pGAD.CIPK5N to create pGAD.CIPK5N-6C. A pair of primers CK5-9/10 were used to amplify the CIPK5 C-terminal region. The PCR fragment was ligated into the *Sal*I/*Apa*I site of pGAD.CIPK6N to produce pGAD.CIPK6N-5C. The 5′-deletion mutants of pGAD.CIPK5 were created by PCRs using a series of forward primers with a reverse primer CK5-2. The forward primers CK5-5, 6, 7, 8, and 12 were used to generate pGAD, CIPK5-50, pGAD,CIPK5-99, pGAD,CIPK5-148, pGAD, CIPK5-197, and pGAD,CIPK5-226, respectively. To produce pGAD.CIPK5-197-6C, we carried out a PCR on pGAD.CIPK5N-6C using primers CK5-8 and CK6-6. The PCR fragment was digested with *Bam*HI/*Apa*I and cloned into pGAD.GH. To make plasmids pGAD.CIPK5C35 and CIPK5C20, PCR fragments were produced from the CIPK5 cDNA with a forward primer (CK5-19) and two reverse primers (CK5-20 and CK5-21, respectively). Following *Bam*HI/*Sal*I digestion, they were cloned into pGAD.GH. All the PCRs were carried out using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) to enhance fidelity. All the constructs above were verified by DNA sequencing.

Oligonucleotide Primers

Primers used in this study are listed below, with restriction enzyme sites underlined: CBL3-2, 5′-TTGGTCGACTCAGGTATCTTCCACCTGCG-3′; CBL3-3, 5′-TATGAATTCCATGTCGCAGTGCATAGACG-3′; CBL3-14, 5′-TTAGTCGACAGCCACCACCATTTGCTTCA-3′; CBL3-15, 5′-TTTGAATTCGCGTGCTCTCTCTGTCTTTC-3′; CBL3-16, 5′-AATGAATTCTACTCTTGCTGAGTCCGGCA-3′; CBL3-18, 5′-ATTGTCGACTTCCTCAAATGTCTTGTCGA-3′; CBL3-20, 5′-AATGTCGACTAGAAGGGAAGGATGCCTGA-3′; CBL3-21, 5′-AACGAATTCTCAGTTAGCCTTGTTCAAGAC-3′; CBL3-22, 5′-ATAGAATTCCCTTGGTGACCCTGAACTTC-3′; P47, 5′-GGTGTGATCAAATGGGCTGCTCTG-3′; PGEX3′, 5′-CCGGGAGCTGCATGTGTCAGAGG-3′; CBL5F, 5′-AAGGAATTCAATGTTGGCAATTCG-3′; CBL5R, 5′-GATGTCGACTAGTCTTCAACTTCA-3′; CBL7F, 5′-ACGGAATTCCATGGATTCAACAAG-3′; CBL7R, 5′-ATTGTCGACCTCAGGTATCTTCCAC-3′; CBL8F, 5′-TAGAATTCAATGGGATGTGTTTGC-3′; CBL8R, 5′-CAGTCGACTTACTTCAAGAAAGGG-3′; CK2-3, 5′-TATGGATCCGATGGAGAACAAACCAAGTG-3′; CK2-5, 5′-CTAGTCGACCTATGATGGTTCTTGCTCTC-3′; CK2-6, 5′-TTAGTCGACCTGACTTGTTGTTTCTCCAT-3′; CK2-7, 5′-AATGGATCCGATGGAGAAACAACAAGTCA-3′; CK3-3, 5′-AGTGGATCCAATGAATCGGAGACAGCAAG -3′; CK3-4, 5′-AATGTCGACTCATACATCACTTTGCTGTT-3′; CK3-6, 5′-AAAGGATCCCGAGGAGAGGGATGATTCAA-3′; CK3-7, 5′-TTGGTCGACTTGAATCATCCCTCTCCTCG-3′; CK5-1, 5′-GAGGGATCCGATGGGAAGGTTATTAGGCA-3′; CK5-2, 5′-AATGTCGACTCAACAATCCTCGGAAGAAG-3′; CK5-4, 5′-ATAGTCGACATCCGTCGATCTGGATCTAC-3′; CK5-5, 5′-ATTGGATCCTTCAATCATGAAGCTCGTTC-3′; CK5-6, 5′-ATTGGATCCTGCTCGTAGATATTTCCAGC-3′; CK5-7, 5′-TATGGATCCGGAACAGATTCTTCAAGACG-3′; CK5-8, 5′-ATAGGATCCATGTTTGCCGTTTCAAGATG-3′; CK5-9, 5′-TATGTCGACTCTCGATTCCGGCGATTATG-3′; CK5-10, 5′-AATGGGCCCTCAACAATCCTCGGAAGAAG-3′; CK5-11, 5′-ATAGGATCCGCTCCGGAAAAACTTCACTC-3′; CK5-12, 5′-TATGGATCCGGAAGCGAGGAGGTTGATTT-3′; CK5-19, 5′-AATGGATCCCAACGCTTTCGAATTCATAT-3′; CK5-20, 5′-AATGTCGACGCCGACGACCGTGACGTAAA-3′; CK5-21, 5′-ATTGTCGACCTCGAACAAGCTCGATAAGT-3′; CK6-1, 5′-AATGGATCCCATGGTCGGAGCAAAACCGG-3′; CK6-2, 5′-AATGTCGACTCAAGCAGGTGTAGAGGTCC-3′;CK6-4, 5′-TATGTCGACTCTCTTCCTTCGACTTGTGC-3′; CK6-5, 5′-AATGTCGACGGAAGACGTTAAACGCGTTTC-3′; CK6-6, 5′-AATGGGCCCTCAAGCAGGTGTAGAGGTCC-3′; CK6-9, 5′-ATAGGATCCACAAGCAACGAGATCAAGAAA-3′.

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