A Novel Interaction between CCaMK and a Protein Containing the Scythe_N Ubiquitin-Like Domain in Lotus japonicus

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In the Rhizobium-legume symbiosis, calcium/calmodulin-dependent protein kinase (CCaMK) is a key regulator for both rhizobial infection and nodule organogenesis. Deregulation of CCaMK by either a point mutation in the autophosphorylation site or the deletion of the carboxyl-terminal regulatory domain results in spontaneous nodule formation without rhizobia. However, the underlying biochemical mechanisms are poorly understood. Here, using the kinase domain of CCaMK as a bait in yeast two-hybrid screening, we identify a novel protein, CIP73 (for CCaMK-interacting protein of approximately 73 kD), that interacts with CCaMK. CIP73 contains a Scythe_N ubiquitin-like domain and belongs to the large ubiquitin superfamily. Deletion and mutagenesis analysis demonstrate that CIP73 could only interact with CCaMK when the calmodulin-binding domain and three EF-hand motifs are removed from the kinase domain. The amino-terminal 80 amino acid residues (80–160) of CCaMK are required for interacting with CIP73 in yeast cells. On the other hand, protein pull-down assay and bimolecular fluorescence complementation assay in Nicotiana benthamiana show that the full-length CCaMK could interact with CIP73 in vitro and in planta. Importantly, CCaMK phosphorylates the amino terminus of CIP73 in a Ca^{2+}/calmodulin-dependent manner in vitro. CIP73 transcripts are preferentially expressed in roots, and very low expression is detected in leaves, stems, and nodules. The expression in roots is significantly decreased after inoculation of Mesorhizobium loti. RNA interference knockdown of CIP73 expression by hairy root transformation in Lotus japonicus led to decreased nodule formation, suggesting that CIP73 performed an essential role in nodule formation.

In the Rhizobium-legume symbiosis, a new organ, the nodule, is formed to provide a niche for bacterial nitrogen fixation. Initiation and development of nodules relies on a continuous molecular dialogue between the host plant and its rhizobial partner. The legume roots can release metabolites, such as flavonoids or isoflavonoids, which interact with rhizobial nodulation (nod) genes that are required for the synthesis and secretion of bacterial lipochito-oligosaccharide signaling molecules named nod factors (NFs; Long, 1989; Lerouge et al., 1990). The structural features of NFs define the specificity of the interaction between the rhizobia and their plant hosts (Lerouge et al., 1990). Purified NFs, at concentrations as low as 10^{-12} M, are sufficient to trigger many of the early host plant symbiotic responses, including alkalization of the cytosol, depolarization of the plasma membrane, calcium (Ca^{2+}) influx and spiking, and activation of early nodulin genes (Ehrhardt et al., 1996; Felle et al., 1999; Stacey et al., 2006). NFs also induce altered root hair growth and the mitotic activation of inner cortical cells that leads to the formation of nodule primordium (Foucher and Kondorosi, 2000).

In recent years, forward genetics and map-based cloning approaches in the model legumes Lotus japonicus and Medicago truncatula have led to the identification of a set of host genes essential for NF perception and signal transduction (Oldroyd et al., 2005). Receptor-like kinases, such as NFR1 and NFR5 of L. japonicus (Madsen et al., 2003; Radutoiu et al., 2003, 2007) or LYK3 and NFP of M. truncatula (Amor et al., 2003; Arrighi et al., 2006; Smit et al., 2007), with chitin-binding LysM motifs in their extracellular domain, have been postulated as receptors for the chitin-like NFs. Following perception, a series of genes including putative ion channels (DOESN'T MAKE INFECTIONS1 [DMI1])...
A Novel Ubiquitin-Like Protein Interacting with CCaMK

RESULTS

Characterization of a CCaMK-Associated Protein from *L. japonicus*

CCaMK is characterized by an N-terminal Ser-Thr kinase domain, an autoinhibitory domain, a CaM-binding domain, and a C-terminal neural visinin-like domain with three EF hands. Using the full-length CCaMK as a bait, the Y2H system identified a homologous protein, CIP73, as a CCaMK interacting protein. CIP73 is a protein of approximately 73 kDa, containing a Scythe_N ubiquitin-like domain and belonging to the large ubiquitin superfamily. It interacts with CCaMK through its Scythe_N domain, resulting in the formation of spontaneous nodules in *M. truncatula*. However, unlike the mutation in the autophosphorylation site, the deletion mutants are sufficient to allow bacterial entry. Both types of mutations can induce nodules when inoculated with rhizobia (*Gleason et al., 2006*). These results indicate that both the CaM-binding and EF-hand domains are essential for rhizobial infection but are independent for nodule organogenesis. Recently, the epistatic relationships between symbiotic genes show that a gain-of-function CCaMK/CASTOR mutation suppresses loss-of-function common symbiotic genes required for the generation of Ca\(^{2+}\) spiking not only for nodule organogenesis but also for successful infection of rhizobia and arbuscular mycorrhiza (AM) fungi. These findings demonstrate a key role of CCaMK in the coordination of nodule organogenesis and infection (*Hayashi et al., 2010*; *Madsen et al., 2010*). However, one question still remains: how does CCaMK differentially activate organogenesis and the infection-related pathway? Further genetic studies and yeast two-hybrid (Y2H) interaction screening have identified a protein, CYCLOPS/IPD3, in both *L. japonicus* and *M. truncatula*. CYCLOPS is a phosphorylation target of CCaMK. The CaM-binding and EF-hand domains are required for the CCaMK-CYCLOPS interaction in yeast. The cyclops mutants block symbiotic infection but are dispensable for nodule organogenesis. It has been proposed that CYCLOPS forms an ancient, preassembled signal transduction complex with CCaMK that is specifically required for infection, whereas organogenesis likely requires additional yet-to-be identified CCaMK-interacting partners or substrates (*Yano et al., 2008*).

In this work, using the sole kinase domain of CCaMK as bait by the Y2H interaction screening approach, we identified a novel protein named CIP73 (for CCaMK-interacting protein of approximately 73 kDa) that contains a Scythe_N ubiquitin-like domain and belongs to the large ubiquitin superfamily. Contrary to CCaMK-CYCLOPS interaction, CIP73 can only interact with the kinase domain of CCaMK. The CaM-binding and EF-hand domains inhibit the CCaMK-CIP73 interaction, whereas organogenesis likely requires additional yet-to-be identified CCaMK-interacting partners or substrates (*Yano et al., 2008*). Considering the results presented in the literature, the authors propose that CIP73 may be a new regulator of nodule organogenesis.
IPD3 in *Medicago* (Messinesse et al. 2007). To identify new interacting partners for CCaMK in this study, the kinase domain of CCaMK was used as a bait to screen a root cDNA Y2H library of *L. japonicus* constructed in the prey vector pGADT7-Rec (Zhu et al., 2008). Interactions were tested in repeated experiments on stringent selective medium (synthetic dextrose [SD]-Trp-Leu-His-Ade). Two independent positive yeast colonies were revealed, and the two cDNAs showed identical nucleotide sequence coding the C-terminal region (413–691 amino acids) of a gene.

Using 5' RACE, a full-length cDNA (GenBank accession no. GU980966) was identified. It contained an open reading frame of 2,076 nucleotides encoding a protein of 691 amino acids with a predicted molecular mass of approximately 73 kD (Supplemental Fig. S1). This protein was designated as CIP73 (Fig. 1A). The protein sequence analysis revealed that CIP73 contained a C-terminal ubiquitin homology region that is similar to the N terminus of Scythe in *Xenopus* and Bat3 (HLA-B-associated transcript 3) in human (Banerji et al., 1990; Thress et al., 1998). This domain was named the Scythe_N domain (Fig. 1A). The proteins containing the Scythe_N domain are widely present in animals and regulate apoptosis in a variety of settings (Thress et al., 1998; Desmots et al., 2005). Besides the Scythe_N ubiquitin-like domain, CIP73 bears only limited resemblance to identified proteins with well-known functions. PSORT (Horton et al., 2007) analysis revealed a potential nuclear localization sequence (NLS; 686–689, KRQK) located in the C terminus.

A search for CIP73 sequences in plant databases identified CIP73-like proteins from *M. truncatula*, Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), *Ricinus communis*, and *Populus trichocarpa* (Supplemental Fig. S2). Proteins containing the Scythe_N-like domain are also widely present in plant genomes, but their functions remain unknown. Homology analysis showed that the N-terminal ubiquitin-like domain was more closely related to the Scythe_N domain in animals than to LjUbiquitin (Fig. 1C).

**CIP73 Interacts with CCAK in the Y2H System**

To confirm the interaction between CIP73 and CCaMK, a series of CCaMK deletions and point mutants were fused to the GAL4 DNA-binding domain (BD). A full-length CIP73, CIP73-N (residues 1–413), and CIP73-C (residues 414–691) were fused to the GAL4 activation domain (AD; Fig. 2). Using the Y2H approach, we detected that CIP73 only interacted with the N terminus of CCaMK (residues 1–300) but not with the full-length CCaMK or the fragment containing the CaM-binding domain (residues 1–340). Moreover, CIP73 did not interact with several CCaMK mutants: a point mutation in the autophosphorylation site (T265I) that causes nodule development in the absence of rhizobia (Tirichine et al., 2006), a kinase-defective mutant (G30E), and a CaM-binding deleted mutant (Fig. 2A). The further analysis of deletion constructs revealed that the N-terminal 80 amino acid residues (80–160) of CCaMK was sufficient to...
interact with CIP73 in the Y2H system (Fig. 2B). However, CCaMK<sup>1–300</sup> interacted with CIP73-C and did not interact with CIP73-N (Fig. 2B). The full-length CIP73 and the N terminus of CIP73 fused to the GAL4 BD showed autoactivation in yeast (Supplemental Fig. S3A). The results of the Y2H analysis revealed that CIP73 could form a homodimer and that CIP73-C (414–691) is necessary for self-interaction (Supplemental Fig. S3B).

**CIP73 Interacts with CCaMK in Vitro and in Planta**

We have demonstrated that the full-length CCaMK could not interact with CIP73 in yeast cells (Fig. 2A). Next, we tested whether the full-length CCaMK can interact with CIP73 by in vitro protein pull-down assay and bimolecular fluorescence complementation (BiFC) assay. In most cases, the interaction between a protein kinase and its substrate is transient and difficult to detect in the Y2H system unless a mutated protein kinase and its substrate is transient and difficult to detect in the Y2H system. However, protein pull-down assay or immuno-precipitation could detect such interactions (Patharkar and Cushman, 2000; Lee et al., 2003).

The full-length CCaMK and a series of CCaMK deletion mutants were expressed as glutathione S-transferase (GST) fusion proteins. CIP73-C (414–691) was expressed with 6× His tag in *Escherichia coli* for the interaction assays in vitro. The His-tagged CIP73-C protein was incubated with different bead-bound GST-CCaMK truncated proteins or GST alone. After washing with buffer, proteins retained to the beads were separated by SDS-PAGE. Immunoblot analysis showed that His-CIP73-C associated with the beads, demonstrating that CIP73-C was able to interact with all deletion mutants and the full-length CCaMK (Fig. 3A).

It has been shown that CCaMK interacted with CYCLOPS in the nucleus of *Nicotiana benthamiana* using the BiFC assay (Yano et al., 2008). Taking advantage of a multicolor BiFC system (Waadt et al., 2008), we can simultaneously visualize the multiple protein interactions in the same cell. The constructs SCFP<sub>N</sub>−CCaMK and CIP73::SCFP<sub>N</sub> were cotransformed and transiently expressed in *N. benthamiana* epidermis cells to confirm CIP73 protein interactions in planta. Strong cyan fluorescence was observed in the nucleus when CIP73 and CCaMK fused to the C-terminal and N-terminal halves of cyan fluorescent protein (CFP), respectively (Fig. 3B, a–c). The SCFP<sub>C</sub>−CCaMK and CYCLOPS::Venus<sub>N</sub> constructs were coexpressed in *N. benthamiana* epidermis cells, and strong green fluorescence was observed in the nucleus (Fig. 3B, d–f). However, only weak marginal background fluorescence was detected when all three constructs were infiltrated at the same time (data not shown). These results indicated that CCaMK could interact with either CIP73 or CYCLOPS in living plant cells. However, whether they could form a ternary complex remained to be tested.

**CIP73 Expression and Subcellular Localization**

Using semiquantitative reverse transcription (RT)-PCR, we examined the CIP73 and CCaMK mRNA levels in different organs of *L. japonicus*. Both CIP73 and CCaMK mRNA were preferentially expressed in roots as compared with leaves and stems. But unlike CCaMK, which had a high mRNA level in nodules, the CIP73 mRNA level was very low in nodules (Fig. 4A, left). After inoculation with *Mesorhizobium loti*, CIP73 exhibited an expression pattern very similar to that of CCaMK, with a transient decrease in expression after inoculation (Fig. 4A, right). This postinoculation decrease was also observed in IPD<sub>3</sub> (Messinese et al., 2007). These results indicated that CIP73 mRNA was constitutively expressed in uninoculated roots and down-regulated by inoculation of *M. loti*.

Sequence analysis showed that there was a putative NLS segment located in the C terminus of CIP73 (Fig. 1A). This suggested that CIP73 might be located in the nucleus of living cells. To determine the subcellular localization of CIP73, a construct containing CIP73

![Figure 2](image-url)
fused in-frame with GFP (GFP::CIP73) was made under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This construct was transiently expressed in onion (Allium cepa) epidermal cells via particle bombardment. Confocal laser-scanning microscopic examination revealed that cells expressing the control 35S::GFP exhibited strong fluorescence both in the cytoplasmic and nuclear compartments (Fig. 4B, a–c). In contrast, when fused with the full-length CIP73 protein, the GFP signal was mainly concentrated in the nuclear compartment (Fig. 4B, d–f). These data indicated that CIP73 localized to the nucleus of onion epidermal cells.

To examine the intracellular distribution of CIP73 in L. japonicus roots, we transformed the 35S::GFP and CIP73 (486–691)::GFP constructs into L. japonicus by means of Agrobacterium rhizogenes. GFP localization was scored on 3-week-old transformed hairy roots. Similar to the results obtained with onion epidermal cells, 35S::GFP hairy roots exhibited GFP fluorescence in both the cytoplasmic and nuclear compartments (Fig. 4C, a–c), while GFP signal of the CIP73 (486–691)::GFP hairy roots was mainly concentrated in nucleus in L. japonicus (Fig. 4C, d–f).

CIP73 Is a New Phosphorylation Substrate of CCaMK in Vitro

It has been demonstrated that CCaMK was able to phosphorylate CYCLOPS in vitro (Yano et al., 2008). This indicated that CIP73 might be another potential phosphorylation substrate for CCaMK. To further investigate the biochemical functions of the interaction between CCaMK and CIP73, we performed the phosphorylation and autophosphorylation assay between CCaMK and CIP73 in vitro. The nonspecific substrate casein was used as a control. Our result showed that the N terminus of CIP73, which did not interact with CCaMK in the Y2H assay, was phosphorylated by CCaMK. On the other hand, the C terminus of CIP73, which showed interaction with CCaMK in the Y2H assay, was not phosphorylated by CCaMK (Fig. 5A). These results were quite different from what was observed with CYCLOPS. The N terminus of CYCLOPS, which interacted with CCaMK in the Y2H assay, was phosphorylated by CCaMK. The C terminus of CYCLOPS, which did not interact with CCaMK in the Y2H assay, was not phosphorylated by CCaMK (Yano et al., 2008). Thus, our data suggested that the biological function of the interaction between CCaMK and CIP73 was different from that of CCaMK and CYCLOPS both in vitro and probably also in planta, not only in yeast cells.

The autophosphorylation activity of CCaMK was increased in the presence of Ca^{2+} alone, whereas substrate (CIP73) phosphorylation was accelerated by the addition of CaM (Fig. 5B). These phosphorylation effects were very similar to CYCLOPS and myelin basic protein, which were phosphorylated by CCaMK. Thus, these data suggested that CIP73 is a new phosphorylation substrate of CCaMK in vitro.

CIP73 RNA Interference Roots Are Impaired in Nodulation

In order to explore the possible function of CIP73 during the nodulation process, RNA interference (RNAi)
experiments were carried out. We transformed the L. japonicus hairy roots with two CIP73-specific RNAi constructs under the control of the CaMV 35S promoter by A. rhizogenes LBA1334, as described in “Materials and Methods.” The transformed hairy roots were selected by GUS staining, and nontransformed roots were excised from the seedling. The identified plants were then transferred to pots filled with vermiculite and sand (1:1) and grown in a growth chamber. After 5 d, the transgenic hairy roots were inoculated with M. loti MAFF303099 and grown for 4 weeks for observation of growth and nodulation phenotypes (Fig. 6, A–C). Expression of CIP73 in transgenic hairy roots was examined by real-time RT-PCR. The level of CIP73 transcripts in the transgenic roots was 20% to 50% of that of the control roots (Fig. 6E). The average number of nodules formed on control hairy roots was 6.7, while that on the two RNAi-transformed plants RNAi-1 and RNAi-2 was 1.7 and 2.5, respectively. Statistical analysis showed that the nodule number with the two RNAi constructs was significantly different from the controls (P < 0.03, n = 17 and P < 0.01, n = 19, respectively; Fig. 6D). Also, we observed that approximately 30% of the CIP73 RNAi roots did not develop any nodule, whereas 100% of the control roots contained nodules.

As root growth defects may influence nodule number, we examined the growth phenotype of CIP73 RNAi roots that had not been inoculated with M. loti under the same conditions as the nodulation assay. As shown in Supplemental Figure S5, the growth of CIP73 RNAi roots was not significantly different with the vector control. We also examined the growth of the CIP73 RNAi and control roots on half-strength Murashige and Skoog (MS) and Broughton and Dilworth agar plates, and no obvious difference was observed (data not shown).

Rhizobial and Mycorrhizal Infection Phenotypes of CIP73 RNAi Roots

CCaMK is suggested to be involved not only in the rhizobial infection through infection threads (ITs) but...
also in nodule organogenesis. CYCLOPS is also known to be required for the initiation of ITs but not nodule organogenesis (Yano et al., 2008). To determine how the decreased nodule formation was mediated by CIP73 RNAi, we individually analyzed the rhizobial infection process by using lacZ-labeled M. loti to visualize IT formation at 7 d post inoculation. In CIP73 RNAi roots, ITs could initiate from curled root hair tips (Fig. 7B), grew through well-elongated root hair to root epidermis (Fig. 7C), and further penetrated into the root cortex (Fig. 7D). Also, some ITs could reach into the nodule primordia tissues and rhizobia could release from ITs into the nodule cells. However, the number of ITs that reached the cortex and nodule primordia was significantly reduced compared with the control roots (Fig. 7E). These data indicated that the impaired nodulation in CIP73 RNAi roots is probably because of cortical events associated with nodule organogenesis but not rhizobial infection.

As both CCaMK and CYCLOPS also control mycorrhization, we next determined the mycorrhization phenotype of CIP73 RNAi roots. In the CIP73 RNAi-1 and RNAi-2 roots, 2 weeks after inoculation with Glomus intraradices, the AM fungi penetrated into the outer cell layers, colonized the root cortex, and formed arbuscules and vesicles, and the hyphal, arbuscular, and vesicular colonizations did not differ from the vector control (Supplemental Fig. S6). These results indicated that the impaired nodulation in CIP73 RNAi roots is probably because of cortical events associated with nodule organogenesis but not rhizobial infection.

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**DISCUSSION**

In the *Rhizobium*-legume symbiosis, CCaMK is a central regulator of nodule organogenesis and rhizobia infection (Gleason et al., 2006; Tirichine et al., 2006). CYCLOPS, an interacting partner with CCaMK, is a phosphorylation substrate of CCaMK (Yano et al., 2008). In this report, we identified a novel CCaMK-interacting protein, CIP73, in *L. japonicus*. CIP73 contained a Scythe_N ubiquitin-like domain. Similar to CYCLOPS, CIP73 was also a phosphorylation target of CCaMK in vitro (Fig. 5A). CaM could enhance the level of substrate phosphorylation in the presence of Ca\(^{2+}\), and substrate (CIP73) phosphorylation was accelerated by the addition of Ca\(^{2+}\)/CaM. [See online article for color version of this figure.]
different from the interaction between CCaMK and CYCLOPS in yeast cells. The site between the CaM-binding domain and the second EF hand was required for the CCaMK-CYCLOPS interaction in yeast. In contrast, CIP73 could interact with CCaMK only when both the CaM-binding domain and three EF-hand motifs were removed from the kinase domain. This was equivalent to a constitutively active kinase resulting in spontaneous nodule development in the absence of rhizobia (Gleason et al., 2006). CCaMK was required for both nodule organogenesis and rhizobial infection. It has been shown that the CaM-binding and EF-hand domains are essential for rhizobial infection but not for nodule organogenesis. CYCLOPS was also identified to be specifically required for rhizobial infection but not for nodule organogenesis (Yano et al., 2008). These biologic functions of CCaMK and CYCLOPS might be partially explained by their interaction identified in yeast cells. To this extent, CIP73 could only interact with the kinase domain of CCaMK, indicating that CIP73 is possibly required for nodule organogenesis.

Although the full-length CCaMK did not interact with CIP73 in yeast cells, their interaction was detected using in vitro protein pull-down assay and BiFC assay in N. benthamiana (Fig. 3). Both CIP73 and CYCLOPS could interact with CCaMK within the nucleus of heterozygous N. benthamiana cells. However, whether both the CCaMK-CYCLOPS and CCaMK-CIP73 interactions could occur simultaneously in a single cell remained to be tested. To address this question, the multicolor BiFC approach was used (Waadt et al., 2008). Coinfiltration of A. tumefaciens strains carrying SCFP<sub>C</sub>::CCaMK and CIP73::SCFP<sub>N</sub> constructs into N. benthamiana leaves resulted in the formation of cyan BiFC complexes in the nucleus, whereas coexpression of SCFP<sub>C</sub>::CCaMK and CYCLOPS::Venus<sub>N</sub> resulted in the formation of green BiFC complexes that were also localized in the nucleus. However, when all three constructs were infiltrated at the same time, only weak marginal background fluorescence was detected (data not shown). It is possible that CIP73 and CYCLOPS were competing for CCaMK. This results in a reduced fluorescence emission that could not be detected by the system. Alternatively, the undetectable signal might be due to the low transformation efficiency for this triple infiltration.

In M. truncatula, CCaMK (DMI3) with both C-terminal and N-terminal GFP fusions, driven by the 35S promoter, show strong nuclear localization in epidermal root cells (Kaló et al., 2005), and a similar localization was observed in root hair cells when the N-terminal GFP fusion was driven by the DMI3 promoter (Smit et al., 2005). CIP73 has a putative NLS segment located in the C terminus (Fig. 1A), suggesting that CIP73 might also be located in the nucleus. We first attempted to detect CIP73 with C-terminal GFP fusion under the control of the 35S promoter in L. japonicus hairy roots and onion epidermal cells. However, no fluorescence was detected in these cells. In contrast to the full-length CIP73, the C terminus (486–691) of CIP73 with C-terminal GFP fusion showed strong fluorescence in nuclei both in L. japonicus hairy roots (Fig. 4C, d–f) and onion epidermal cells (Supplemental Fig. S4, D–F). Murakami et al. (2006) also showed strong nuclear localization of CIP73 in L. japonicus epidermal cells and root hair cells. In conclusion, the interaction between CCaMK and CIP73 in yeast cells is conserved in higher plants. However, the C terminus of CIP73 is required for nuclear localization in higher plants, whereas the C terminus of CIP73 is not required for nuclear localization in yeast cells. This suggests that the interaction between CCaMK and CIP73 might be modulated by the presence of the NLS segment in the C terminus of CIP73.
reported that no fluorescence could be detected in *L. japonicus* hairy roots for NSP2 with a C-terminal GFP fusion. On the other hand, CIP73 with an N-terminal GFP fusion in pMON30060 vector showed strong nuclear localization in onion epidermal cells (Fig. 4B, d–f). At this stage, we cannot explain this strange phenomenon. Continued work to test CIP73 with C- and N-terminal GFP fusions in *L. japonicus* root or other organs by regenerating plants via somatic embryogenesis is likely to solve the problem.

mRNA examination in different organs showed that the expression of CIP73 was organ regulated. The mRNA level was much higher in uninoculated roots as compared with leaves and stems. This suggested a potential role of CIP73 in root-related processes. However, the expression of CIP73 was very low in nodules as compared with roots. This was in contrast with CCaMK, which showed similar expression in both uninoculated roots and nodules. Interestingly, the expression level was significantly decreased after inoculation with *M. loti* for both CIP73 and CCaMK (Fig. 4A). This implied that CIP73 might be functional downstream of CCaMK and probably in the negative feedback in the NF signal transduction pathway (Oldroyd et al., 2001). RNAi knockdown of CIP73 expression by hairy root transformation in *L. japonicus* led to decreased nodule formation (Fig. 6). These data suggested that CIP73 performed an essential role in nodulation. On the other hand, except for regulating rhizobial and mycorrhizal symbioses, little is known about the biological role of CCaMK in plants. But the preferential expression of CCaMK in developing anthers and root tips (Poovaiah et al., 1999) suggested that CCaMK may play a role in mitosis and meiosis (Yang and Poovaiah, 2003). As an interacting partner, CIP73 may also be involved in these processes in addition to its role in the development of the nodule. We are currently generating stable transformants with the CIP73 RNAi constructs by conventional *A. tumefaciens*-mediated hypocotyl transformation to study the biological functions of CIP73 in more detail.

A thorough search of the available protein databases revealed that CIP73 bears limited resemblance to proteins with well-known functions. Analysis of the CIP73 sequence identified an N-terminal region with substantial homology to the Scythe_N domain belonging to the UBQ superfamily. Scythe protein (also known as Bat3) is an apoptotic regulator that is highly conserved in eukaryotes and contains a ubiquitin-like domain near its N terminus. Scythe binds Reaper, a potent apoptotic inducer, and Scythe/Reaper are thought to signal apoptosis, in part through regulating the folding and activity of apoptotic signaling molecules (Thress et al., 1998; Desmots et al., 2005). However, except for the Scythe_N-like domain, CIP73 contained the downstream sequence with no homology to Scythe. The Scythe_N ubiquitin-like domain indicates that CIP73 is a member of the large ubiquitin family proteins. The ubiquitin family proteins are generally divided into two groups: type I ubiquitin-like modifiers and type II ubiquitin-like domain proteins (UDPs).
CIP73 can be classified into type II UDPs embedding ubiquitin-like domains, since it lacks the type-specific conserved C terminus diglycine motif, which can form conjugates with other proteins. There is emerging evidence indicating an exciting link between the functions of UDPs, proteasome, chaperones, and the apoptotic pathway (Jentsch and Pyrowolakis, 2000). At least three UDPs, the DSK2 relative PLIC2/Chap1 BAG-1, and BAT3/Scythe/BAG6 have shown affinities for chaperones of the Hsp70 family (Kaye et al., 2000; Thress et al., 2001; Sasaki et al., 2008; Corduan et al., 2009). We have identified Hsp70-interacting protein as an interacting partner for CIP73 using Y2H interaction screening (data not shown). Nowadays, there is increasing evidence about the ubiquitin-mediated proteolyis during nodule development (Kondorosi et al., 2005; Kiss et al., 2009; Yano et al., 2009). All of these findings implied that CIP73 might mediate protein folding/degradation pathways in the regulation of nodulation signal transduction cascades.

Although proteins containing the Scythe_N domain are widely present in all sequenced eukaryotic organisms, the overall structure of CIP73 appears to be unique to plants, since genes coding for proteins with similar structures could not be found in other organisms in the databases. Genes homologous to CIP73 are clearly present in the moss Physcomitrella patens and in higher plants like R. communis, P. trichocarpa, Vitis vinifera, Arabidopsis, and rice. But amino acid sequences with high overall similarity to CIP73 were only found in legume plants (Supplemental Fig. S2). The Medicago CIP73 orthologs have overall 72% sequence identity with CIP73 and have at least two isoforms (J.M. Aneé, personal communication). The rice CIP73 orthologs, with the same exon and intron structure to the Medicago ortholog, have only overall 30% sequence identity with CIP73 and also have two isoforms (data not shown).

MATERIALS AND METHODS

Plant Materials

Seeds of Lotus japonicus MG-20 were surface sterilized in 75% ethanol for 2 min and followed by 8 min in 2% sodium hypochlorite and six washes with sterile water. The seeds were left to germinate for 48 h at 22°C on sterile water-soaked filter paper in petri dishes in the dark. Seedlings were subsequently planted in pots with sterile sand supplemented with nitrogen-free Fahraeus medium. The seeds were grown in a growth chamber maintained at 22°C with a 16/8-h day/night cycle. Five-day-old seedlings were inoculated with approximately 107 colony-forming units mL−1 Mesorhizobium loti NZP2037 or MAFF303099. Various organs were collected from plants 2, 4, 6, 8, and 12 d after rhizobial inoculation using the TRIzol reagent (Invitrogen). Total RNA (20 μg) was reverse transcribed into single-stranded cDNAs using oligo(dT) as the primer. The cDNAs were size fractionated using BD Matchmaker Library Construction and Screening Kits (BD Biosciences-Clontech).

Y2H Library Construction

Total RNA was isolated from the equally mixed roots collected 2, 4, 6, 8, and 12 d after rhizobial inoculation using the TRIZol reagent (Invitrogen). Total RNA (2 μg) was reverse transcribed into single-stranded cDNAs using oligo(dT) as the primer. The cDNAs were size fractionated using BD Matchmaker Library Construction and Screening Kits (BD Biosciences-Clontech). cDNA fragments longer than 500 bp were cotransformed with linearized pGADT7-Rec into yeast AH109 cells. The transformants were selected on SD-Leu medium according to the manufacturer’s instruction. The transformation efficiency was approximately 2 × 106 colony-forming units per 3 μg of pGADT7-Rec.

Y2H Library Screening

For the Y2H screenings, bait constructs were cloned into the vector pGBK7. The partial cDNA corresponding to the CCAK (GenBank accession no. AM220793) kinase domain was fused in-frame with the GAL4 BD into the pGBK7 vector. Bait constructs were transformed into yeast strain Y187 by the lithium acetate method. Screening of interaction clones was carried out via mating according to the manufacturer’s instructions (Clontech). A total of 10 million transformants from the cDNA library were screened for growth on the stringent SD-Leu-Trp-His-Ade dropout medium. Positive clones were confirmed by assaying for another yeast reporter, LacZ+, via β-galactosidase assays. To validate observed interactions, prey plasmids were rescued, analyzed by restriction digestion, and transformed again into AH109. Yeast cells with preys were mated one-on-one in parallel against the yeast Y187 expressing the target baits or the negative control plasmid pGBK7-Lam (Clontech).

β-Galactosidase Assay

Yeast cells grown in liquid SD selection medium were pelleted and washed twice with Z-buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, and 1.0 mM MgSO4, pH 7.0). The cells were resuspended in 100 μL of Z-buffer and permeabilized by three freeze-thaw cycles with liquid nitrogen and a 37°C water bath. Cell extracts were added to 0.7 mL of Z-buffer containing 50 mM β-mercaptoethanol and 160 μL of O-nitrophenyl β-d-galactopyranoside (4 mg mL−1 in Z-buffer). After incubation at 30°C for 30 min or until the yellow color appeared, the reaction was terminated by the addition of 0.4 mL of 1.0 mM Na2CO3. The reaction mixture was centrifuged for 10 min at 13,000 rpm to remove cell debris. β-Galactosidase activity in the supernatant was measured at an optical density at 420 nm and expressed in Miller units.

Purification of CCAK and CIP73 Protein

The full-length and truncated CCAK cDNA fragments were PCR amplified and inserted in-frame at the SmaI/XhoI sites of pGEX-6P1 vector (Amersham Pharmacia Biotech) for GST fusion. The full-length CCAK cDNA fragment was inserted into the XhoI/Hind sites of pMAL-C2X vector (New England Biolabs) for maltose-binding protein (MBP) fusion. The two truncated CIP73 cDNA fragments (1–413 and 414–691) were PCR amplified and cloned into pET-28a vector (Novagen) using EcoRI and SalI sites. For protein expression, Escherichia coli BL21-Codon Plus (DE3)-RIL (Stratagene) harboring the plasmids were induced with 0.3 mM isopropyl-1-thio-β-d-galactopyranoside in Luria-Bertani broth for 5 h at 22°C. The GST fusion proteins were purified using glutathione-Sepharose 4B columns (GE Healthcare) using standard procedures. The MBP fusion proteins were purified using amylose resin (New England Biolabs). The His-tagged proteins were purified using nickel-agarose beads (Qiagen) under native conditions according to the manufacturer’s instructions and eluted with a buffer solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, and 200 mM imidazole, pH 8.0). Purified proteins were desalted by dialysis in phosphate-buffered saline and concentrated with polyethylene glycol-8000 powder.

In Vitro Protein-Protein Interaction

To assay the interaction between CCAK and CIP73, GST-tagged CCAK or GST alone was bound to glutathione-Sepharose 4B beads. The beads were incubated with 2 μg of purified His-CIP73 protein in 1 mL of interaction buffer (20 mM Tris-HCl, 100 mM KCl, 2 mM MgCl2, and 5% glycerol, pH 8.0) in the presence of 0.5 mM CaCl2 or 2.5 mM EGTA. The retained proteins were eluted by boiling for 5 min in 1× SDS sample buffer (2% SDS, 29.1 mmol L−1 Tris, pH 6.8, 10% glycerol, and 0.01% bromphenol blue). Samples were analyzed by 12% SDS-PAGE followed by immunoblotting with the anti-His tag antibody or visualized by staining with Coomassie Brilliant Blue R250.
In Vitro Kinase Assay

A full-length cDNA of CaM at position 5'-CGACATCCTGAGCTGATGAC-3' for L. japonicus was amplified by RT-PCR using the primers 5'-GAGGTAGGTTACCTGTCTCTC-3' and 5'-CGGAATTCCTTACCTGACCGCTACGAC-3'. The amplified fragment was cloned into the NdeI/EcoRI site of pET28a vector and expressed as a 739 terminal 6x His-tagged fusion protein in E. coli strain BL21. Expression protein was affinity purified via nickel-agarose beads (Qiagen) under native conditions and eluted with a buffer solution (50 mM Tris-HCl, 200 mM NaCl, and 200 mM imidazole, pH 8.0). Purified protein was desalted and concentrated by Microcon Ultrade YM-3 centrifugal filter devices (Millipore). Protein concentrations were determined by SDS-PAGE using bovine serum albumin as a standard (Fermentas). The kinase assays were performed at 25°C for 30 min in 40 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 10 μM ATP, and 10 μCl of [γ-32P]ATP. Either 4 μg EDTA or 0.5 mM Ca²⁺ with or without 0.5 μg of purified CaM was added as indicated. Each reaction was carried out by using approximately 1 μg of MBP-CaM protein and 2 μg of substrate. Kinase reactions were stopped by mixing with SDS-PAGE sample buffer and boiling for 5 min. Samples were separated by 12% SDS-PAGE, and the gel was subsequently stained by Coomassie Brilliant Blue R250. Signal was scanned with a Fujiﬁlm FLA-5000 phosphorimager.

BiFC Analysis

The full-length cDNA of CcaMK was PCR amplified and cloned into the SpeI/XhoI site of pSPCYCe-R vector (Waszl et al., 2008) to obtain CcaMK-SCFP3A fusion. Full-length cDNA of CIP73 and CYCLOPS without stop codon were cloned into the BamHI/SalI site or the SalI/Smal site of pSCYNe or pVYNe, respectively, to obtain CIP73-SCFP3A, or CYCLOPS-SCFP3A. The constructs were transfected into Arabidopsis thaliana strain CV3101/PM96 by electroporation for future N. benthamiana transformation. The Arabidopsis were harvested by centrifugation and were resuspended in agaroinfusion buffer (10 mM MES, pH 5.7, 10 mM MgCl₂, and 150 μM acetoxyserine). The strains were mixed to a final optical density at 600 nm of 0.5 for each Agrobacterium and incubated on the bench for 2 to 4 h at room temperature. The bacterial mixture was infiltrated into the top leaves of 6-week-old N. benthamiana with a 1- to 2-mL syringe. The fluorescence was assayed from 3 d to 5 d after infiltration using a Zeiss LSM510 laser-scanning microscope with CFP (excitation wavelength, 405 nm; emission wavelength, 488 nm; emission wavelength, 515 nm) for SCFP3A/SCFP3A complexes and GFP (excitation wavelength, 488 nm; emission wavelength, 515 nm) for SCFP3A/Venus complexes.

Expression Analysis

The expression profile of the CIP73 gene was analyzed by semiquantitative RT-PCR in leaves, shoots, nodules, and roots of L. japonicus MG-20 following inoculation with M. loti MAF630399. Roots were collected at each time point, and total RNA was isolated using the TRZol reagent (Invirotreat) and treated with DNase (Promega) to eliminate genomic DNA contamination. The amount of total RNA was normalized by measuring the RNA concentration at 260 nm, and 1 μg of total RNA was added to individual tubes to transcribe first-strand cDNA using oligo(dT) primer. PCR was carried out on an iCycler Thermal Cycler system (Bio-Rad; 5-min soak at 95°C to initiate cDNA synthesis) using the primers 5'-GACTGCTTTAATTAATCCAC-3' and 5'-GACTAGTCTTCCATCTTTGGCC-3'. The amplified fragment was cloned into the Ncol/XbaI site of pCAMBA1302 vector for the selection of transformed hairy roots, the hygromycin resistance gene in pCAMBA1302 was replaced by the GUS gene. The construct was transferred into Agrobacterium rhizogenes LBA1334 by electroporation. The transformed hairy roots selected by GUS staining were used to examine using the Zeiss LSM510 confocal microscope.

Construction of the RNAi Plasmid

A 225-bp fragment of the 3'-untranslated region with a short part of the coding region of CIP73 (RNAi-1) and a 261-bp fragment of the 3'-untranslated region (RNAi-2) were amplified by RT-PCR using the following primers: 5'-TGCGTTATATAAATATCCAC-3', containing a Smal or Pst site at the 5' end, and 5'-GATCCCTTACCTGACCGCTACGAC-3', containing a BamHII or SalI site at the 5' end for RNAi-1; and 5'-GACTAGTCTTCCATCTTTGGCC-3', containing a Smal or Pst site at the 5' end, and 5'-CTCCTGTTGAAACGATGC3', containing a BamHII or SalI site at the 5' end for RNAi-2. The amplification products were digested with Smal-BamHII or Pst-Sal and ligated into the pCAMBA1301-35S-int-T7 plasmid vector (a gift from Dr. D. Luo), in which the sense and antisense CIP73 RNA sequences were located in tandem with the Arabidopsis (Arabidopsis thaliana) actin-11 intron between them, and this intron-containing hairpin RNA construct was placed behind the CaMV 35S promoter. The constructs were transferred into A. rhizogenes LBA1334 by electroporation.

Hairy Root Transformation and Identification

Hairy root transformation of L. japonicus MG20 using A. rhizogenes strain LBA1334 was performed as described previously (Kumagai and Kouchi, 2004) with some minor modifications. In brief, seeds were sterilized and germinated in water for 4 d in the dark at 22°C, followed by 2 d in a 16-h light/8-h dark photoperiod in a growth chamber. The seedlings were cut at the base of the hypocotyl and placed in a petri dish for several minutes. The seedlings were collected and cultured on MS (Sigma) plates with 1.5% Suc and cocultivated for 5 d in a growth chamber. Then, the plants were transferred onto fresh MS medium containing 100 μg/mL kanamycin and grown for 10 d until the hairy roots were developed from the section of hypocotyl. For the selection of transformed hairy roots, transformed roots were immersed in GUS staining solution [100 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, 0.1% N-laurylsarcosine, 10 mM Na₂EDTA, 1 mM K₃Fe(CN)₆, 1 mM K₃Fe(CN)₅, and 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid] and incubated overnight at 37°C in the dark.

Rhizobial and Mycorrhizal Infection Assay

For rhizobial infection assay, transgenic hairy roots were inoculated with M. loti strain MAF630399 and allowed to continue growing with the same medium. For the selection of transformed hairy roots, transformed roots were immersed in GUS staining solution [100 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, 0.1% N-laurylsarcosine, 10 mM Na₂EDTA, 1 mM K₃Fe(CN)₆, 1 mM K₃Fe(CN)₅, and 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid] and incubated overnight at 37°C in the dark.

Subcellular Localization of CIP73 in Onion Epidermal Cells and Hairy Root Cells

The full-length cDNA of CIP73 was cloned into the EcoRI/BamHI site of pMION30060 vector. The resulting construct consisted of CIP73 fused to the C terminus of the GFP under the control of the CaMV 35S promoter. The plasmid was used for transient expression in the onion (Allium cepa) epidermal cells by particle bombardment using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). After incubation for 12 to 24 h at 23°C in the dark, the epidermal cell layers were examined using a confocal laser-scanning microscope (Zeiss LSM510; excitation wavelength, 488 nm by argon laser; emission wavelength, 580 nm).
Providing Dr. Bin Zhao for technical advice on mycorrhizal infection and for kindly providing RNAi vector pCAMBIA1301-35S-int-T7. We are grateful to Dr. Guojiang Wu for providing M. loti MAFF303099 and Dr. D. Luo for providing RNAi vector pCambia1301-35S-int-T7. We thank Dr. Guojiang Wu for providing M. loti MAFF303099 and Dr. D. Luo for technical advice on mycorrhizal infection and for kindly providing G. intraradices inocula. We also thank Yao Hang for advice on transient expression and confocal laser-scanning microscopy.

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