A *Lotus japonicus* Cochaperone Protein Interacts With the Ubiquitin-Like Domain Protein CIP73 and Plays a Negative Regulatory Role in Nodulation

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The calcium/calmodulin-dependent protein kinase CCaMK forms a complex with its phosphorylation target CIP73 (CCaMK-interacting protein of 73 kDa). In this work, a homolog of the animal HSC/HSP70 interacting protein (HIP) was identified as an interacting partner of CIP73 in *Lotus japonicus*. *L. japonicus* HIP contains all functional domains characteristic of animal HIP proteins. The C-terminal STI1-like domain of *L. japonicus* HIP was found to be necessary and sufficient for interaction with CIP73. The interaction between CIP73 and HIP occurred in both the nuclei and cytoplasm in *Nicotiana benthamiana* leaf cells. The interactions between CIP73 and HIP and between CIP73 and CCaMK could take place simultaneously in the same nuclei. HIP transcripts were detected in all plant tissues tested. As nodule primordia developed into young nodules, the expression of *HIP* was downregulated and the *HIP* transcript level became very low in mature nodules. More nodules were formed in transgenic hairy roots of *L. japonicus* expressing *HIP* RNA interference at 16 days postinoculation as compared with the control hairy roots expressing the empty vector. It appears that HIP may play a role as a negative regulator for nodulation.

Legume plants have evolved with two types of mutualistic plant-microbe interactions, arbuscular mycorrhizal symbiosis (AMS) with fungi and root nodule symbiosis (RNS) with soil rhizobia. AMS and RNS supply the host plants with nitrogen and phosphate nutrients, both having agronomic and ecologic importance. Despite of the difference in fungal and bacterial symbionts, genetic dissections of AMS and RNS using model legumes, including *Lotus japonicus* and *Medicago truncatula*, have revealed that both types of symbioses share overlap components of the signaling pathway, composing the “common symbiosis pathway (CSP)” (Oldroyd 2013). Calcium and calmodulin (CaM)-dependent protein kinases (CCaMK) play a crucial role among the CSP (Lévy et al. 2004).

Ca2+-spiking is one of the earliest cellular responses in both AMS and RNS and consists of periodic increases of calcium concentration in the perinuclear and nuclear regions (Capoen et al. 2011; Harper and Harmon 2005; Miwa et al. 2006). CCaMK acts as the decoder of Ca2+-spiking and has the peptide structure features that are consistent with its biochemical function, including a serine/threonine kinase domain, a CaM-binding domain, and three visinin-like EF-hand motifs that trap Ca2+ ions (Ramachandiran et al. 1997; Takezawa et al. 1996). The N-terminal part of plant CCaMK resembles the animal CaM II, which is able to decode Ca2+-spiking involved in the neuronal signaling pathway (De Koninck and Schulman 1998). CCaMK are highly conserved in plants, including *M. truncatula, L. japonicus,* and *Sesbania rostrata* (Capoen et al. 2009; Godfroy et al. 2006; Lévy et al. 2004; Poovaiah et al. 1999; Tirichine et al. 2006). Rice CCaMK is required for AMS and can also functionally complement legume CCaMK knock-out mutations, indicating an equivalent role of CCaMK homologs in nonlegumes (Chen et al. 2007; Godfroy et al. 2006).

Recent biochemical and genetic studies of plant CCaMK have provided new insight into the molecular mechanism for decoding Ca2+-spiking (Miller et al. 2013). In the absence of Ca2+, CCaMK remains in an autoinhibited state. Binding of Ca2+ to the EF-hand motifs induces autophosphorylation at a conserved threonine residue in the kinase domain (T265 in *L. japonicus* CCaMK) to release autoinhibition, which enables high-affinity CaM binding and, consequently, substrate phosphorylation (Ramachandiran et al. 1997; Sathyarayanan et al. 2001). De-regulation of CCaMK by the point mutation in the autophosphorylation site of the kinase domain or deletion of the C-terminal CaM-binding and EF-hands motifs leads to the development of spontaneous nodules in the absence of rhizobia (Gleason et al. 2006; Tirichine et al. 2006). Further detailed genetic analyses have revealed that the deregulated version of CCaMK*T265D* or *snf1* (CCaMK*T265A*) can rescue Ca2+-spiking-deficient mutants, including *symrk, castor, pollux, nup85*, and *nup133* in RNS and AMS (Hayashi et al. 2010; Madsen et al. 2010). On the other hand, expression of the sole kinase domain fused to a nuclear localization signal, lacking the autoinhibitory domain, has been shown to be sufficient for spontaneous nodulation and AM development but not for rhizobial infection (Shimoda et al. 2012; Takeda et al. 2012). Recently, a new autophosphorylation site has
been identified in the CaM-binding domain of CcaMK, both in *L. japonicus* and *M. truncatula* (Liao et al. 2012; Routray et al. 2013). Phosphorylation of this site negatively affects the interaction of CCaMK with CaM and IPD3 and blocks RNs, AMS, and spontaneous nodulation activity of CCaMK<sup>T271A</sup> in *M. truncatula* (Routray et al. 2013).

Work aimed at screening targets of CCaMK has led to identification of CYCLOPS/IPD3 and CIP73, both serving as phosphorylation substrates of CCaMK (Kang et al. 2011; Messinese et al. 2007; Yano et al. 2008). CYCLOPS/IPD3 is a coiled-coil nuclear protein, while CIP73 is a Scythe-N domain-containing, ubiquitin-like protein with unknown functions. The *cyclops* mutants can form nodule primordia but are impaired in rhizobial infection and mycorrhization (Yano et al. 2008). The *ipd3* mutants of two *Medicago* ecotypes, Jemalong and R108, are somewhat different in phenotype. Although both ecotypes can develop uninucleate nodules, Jemalong but not R108 forms infection threads but fails to develop symbiosomes and does not express symbiosomal membrane marker SYMREM1 (Horváth et al. 2011; Lefebvre et al. 2010; Ovchinikova et al. 2011). RNA interference (RNAi)-mediated knockdown of CIP73 expression leads to impaired RNs (Kang et al. 2011). However, how CYCLOPS and CIP73 regulate the function of CCaMK at the biochemical and molecular levels remains unknown.

Animal HSC/HSP70-interacting protein (HIP) interacts with the ATPase domain of heat shock cognate 70 (HSC70) and is part of the progesterone receptor-chaperone complex (Höhfeld et al. 1995; Prapapanich et al. 1996b; Smith 1993). In this work, we report the identification of a homolog of eukaryotic HIP as an interacting partner of CIP73 in *L. japonicus*. *L. japonicus* HIP was colocalized with CcaMK and CIP73 in the nuclei. RNAi-mediated knockdown of HIP expression in *L. japonicus* hairy roots led to increased nodule formation. These results demonstrate a role of *L. japonicus* HIP as a negative regulator of nodulation in legume plants.

**RESULTS**

**Identification of HIP as an interacting partner of CIP73.**

CIP73 has previously been identified as a CcaMK-interacting protein (Kang et al. 2011). In this work, we used the yeast two-hybrid system to identify potential interacting partners of CIP73. When fused to the Gal4 DNA binding domain, the full-length CIP73 caused autoactivation of the Gal4 system and could not be used to screen the library. The C-terminus of CIP73 (414 to 691) did not cause this autoactivation system and could not be used to screen the library. The C-terminus of CIP73 (CIP73-C) interacted with both the full-length HIP and the C-terminal Sti1-like domain (HIP-C) but not with the N-terminus of HIP (HIP-N) (Fig. 2A), indicating that the C-terminal Sti1-like domain of HIP is necessary and sufficient for interaction with CIP73. Because the full-length CIP73 fused with the Gal4 DNA-binding domain led to autoactivation in the yeast system, we switched the Gal4 activation domain to the full-length CIP73 and the binding domain to HIP. The result showed that yeast colonies harboring this combination were able to grow on the selection medium lacking His and Ade (SD-Trp-Leu-His-Ade) and exhibited significant β-galactosidase activities on medium containing X-Gal (Fig. 2B). These results suggest that both CIP73-C and full-length CIP73 are able to interact with HIP.

The interaction between CIP73 and HIP was further confirmed by an in vitro protein pull-down assay. The full-length HIP and its deletion fragments HIP-N and HIP-C were expressed as chitin-binding domain (CBD) fusion proteins. CIP73 was expressed as 6xHis tagged recombinant protein in *Escherichia coli* cells. HIP and its deletion fragments were absorbed on chitin beads and were used to incubate with purified soluble CIP73. After washing with buffer, proteins retained on the beads were eluted and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The presence of CIP73 on the beads was detected by immunoblotting with anti-His tag antibody. Only the full-length HIP and its C-terminal HIP-C could pull down CIP73, confirming that the C-terminal domain of HIP is responsible for interacting with CIP73 (Fig. 2C).

**Interaction between HIP and CIP73 in planta.**

We used bimolecular fluorescence complementation (BiFC) assay to test whether the interaction between CIP73 and HIP took place in plant cells. Proteins fused to the C- or N-terminal yellow fluorescence protein (YFPc and YFPn, respectively) were coexpressed in *Nicotiana benthamiana* epidermal leaf cells. The expression of recombinant proteins in *N. benthamiana* leaves was confirmed by immunoblot analysis using anti-hemagglutinin (HA) and anti-c-myc monoclonal antibodies (Fig. 3G). Coexpression of YFPc-CIP73 and HIP-YFPn led to strong yellow fluorescence signals in both the cytoplasm and nucleus (Fig. 3A), suggesting a positive interaction between CIP73 and HIP in plant cells. The interaction was further confirmed when the split YFP tags were switched between CIP73 and HIP.
although the fluorescence intensity was weaker (Fig. 3B). Because the N-terminal HIP (HIPN) did not interact with CIP73 in yeast cells and in protein pull-down assays (Fig. 2B and C), the combinations of YFPC-CIP73 with HIPN-YFPN (Fig. 3C) and YFPC-HIPN with CIP73-YFPN (Fig. 3D) were used as negative controls and no significant fluorescence signals were detected.

**Homodimerization of HIP.**

To test if HIP formed homodimers in planta, full-length HIP or its N-terminus (HIPN) was fused with the split YFP tags and was expressed in *N. benthamiana* leaf cells. Coexpression of YFPC-HIP and HIP_YFPN (Fig. 3E) or YFPC-HIPN and HIPN_YFPN (Fig. 3F) resulted in significant fluorescence signals in both the cytoplasm and nucleus, suggesting the formation of a homodimer via the N-terminus of HIP protein. This observation is consistent with the previous report on the homodimerization of rat HIP (Irmer and Hohfeld 1997).

**Subcellular localization of HIP.**

From the BiFC result, HIP appeared to distribute between the cytoplasm and nucleus when coexpressed with CIP73 (Fig. 3A and B) or expressed alone (Fig. 3E and F), which is different from the cytoplasmic localization pattern of rat HIP (Hohfeld et al. 1995). We examined the subcellular localization of *L. japonicus* HIP in *N. benthamiana* leaf epidermal cells expressing HIP-GFP under the control of the Cauliflower mosaic virus (CaMV) 35S promoter (35S::HIP-GFP). GFP-tagged HIP was localized in both the cytoplasm and nucleus (Fig. 4B), consistent with the BiFC analysis (Fig. 3). Similar subcellular localization patterns (Fig. 4D) were also observed when HIP-GFP was expressed in *N. benthamiana* leaf cells under the control of the *L. japonicus* HIP native promoter.

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**Fig. 1.** HSC/HSP70 interacting protein (HIP) homologs in eukaryotes. **A**, Schematic illustrations of functional domains of HIP protein. HIP contains 418 amino acid residues with an N-terminal dimerization domain, an acidic domain, three tetratricopeptide repeats (TPR), a highly charged region, a series of degenerate glycine-glycine-methionine-proline (GGMP) repeats, and a C-terminal Sti1-like region similar to the Sti1/HOP/p60 protein. **B**, A phylogenetic tree of HIP homologous proteins from animals, plants, and protists. Note that in the plant kingdom, brassicas and legumes are divergent from other dicots. Dots highlight LjHIP, which is the subject of this work, and HsST13/P48, which has been characterized in animals with regard to its biological function and could be useful to future work to understand the function of HIP proteins in plants. The scale bar is an indicator of genetic distance based on branch length.
HIP pro:HIP-GFP. Because free GFP cleaved from the fusion product might also result in the cytoplasmic and nuclear localization patterns, we examined the HIP-GFP fusion protein product using immunoblot with anti-GFP antibody. The HIP-GFP fusion protein was found to be intact (Supplementary Fig. 4).

To determine the subcellular localization of HIP in L. japonicus roots, we generated transgenic hairy roots expressing HIP pro: HIP-GFP or 35S::HIP-GFP (Fig. 4E). Similar to the observation in N. benthamiana leaf cells, fluorescent signals of HIP-GFP were found in both the cytoplasm and nuclei in the hairy roots of L. japonicus (Fig. 4E). The fluorescent signal of HIP pro: HIP-GFP was weaker than that of 35S::HIP-GFP (data not shown). The subcellular localization pattern of HIP-GFP was not altered three days after inoculation with Mesorhizobium loti (Supplementary Fig. 5).

Colocalization of CIP73 with HIP and CCaMK in the nuclei.

CIP73 and CCaMK were shown to localize in the nucleus (Kang et al. 2011; Takeda et al. 2012), while HIP was localized in both the nucleus and cytoplasm (Fig. 4). Therefore, we tested whether CIP73, HIP, and CCaMK would colocalize in N. benthamiana leaf cells. We first tested subcellular localization of each protein fused with GFP or mCherry under the control of the 35S promoter. The fluorescent signal of CIP73-GFP and CIP73-mCherry was found in the nucleus (Supplementary Fig. 3A and B). As a control, CYCLOPS, an interacting partner of CCaMK, exhibited similar subcellular localization to CIP73. Interestingly, CCaMK showed a low level of cytoplasmic fluorescence in addition to the strong nuclear signal. To avoid any potential undesirable effect of protein overexpression driven by the 35S promoter, we also investigated the subcellular distribution of CCaMK under the CCaMK native promoter. Similar subcellular localization patterns of CCaMK were observed when the CCaMK native promoter was used.

When coexpressed in N. benthamiana leaf cells, CIP73-GFP and CCaMK-mCherry showed excellent colocalization in the nuclei (Fig. 5A). CIP73-GFP and HIP-mCherry were found to colocalize in the nuclei (Fig. 5B). Both CCaMK-mCherry and HIP-mCherry exhibited a slightly diminished signal of cytoplasmic localization.

![Fig. 2. Interaction of HSC/HSP70 interacting protein (HIP) with CIP73. A, Schematic illustrations of CIP73 and HIP deletion constructs. The C-terminal calcium and calmodulin-dependent protein kinase (CCaMK)-binding domain of CIP73 (aa 414 to 691) was used as bait for isolation of HIP from the two-hybrid library. B, Dissection of functional domains of HIP required for interacting with CIP73. Yeast colonies containing the bait and prey constructs were selected on SD/-Trp-Leu medium, while colonies with positive protein-protein interactions were tested on SD/-Trp-Leu-His-Ade medium. The interaction strength was measured through the β-galactosidase activity on plates containing X-Gal (80 µg ml⁻¹). The combination of BD-53/AD-SV40 was used as a positive control and BD-Lam/AD-SV40 as a negative control (Clontech). Note that the Sti1-like domain (HIPC) of HIP was necessary and sufficient for interaction with CIP73. BD, Gal4 DNA binding domain; AD, Gal4 activation domain. C, Protein pull-down assay for the interaction between CIP73 and HIP. His-tagged CIP73 was incubated with the immobilized chitin-binding domain (CBD)-HIP, CBD-HIP truncated proteins, or CBD alone. After washing, the proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were visualized by staining with Coomassie brilliant blue R250 (top). A similar gel was used for immunoblot with anti-His-tag antibody (bottom). Lane 1 is a positive control that contained a diluted sample of purified His-CIP73 protein. The positions of CBD and CBD-tagged HIP proteins on the Coomassie-stained gels are indicated by arrows.](image-url)
when coexpressed with CIP73-GFP (Fig. 5A and B). Meanwhile, CYCLOPS-GFP also colocalized with CCaMK-mCherry in the nucleus. **Concurrence of interactions of CIP73/CCaMK and CIP73/HIP in the same cell.** We took advantage of a multicolor BiFC system (Waadt et al. 2008) to investigate whether the interactions between CIP73 and CCaMK and between CIP73 and HIP could take place simultaneously in the same cell. For this observation, three Agrobacterium tumefaciens strains, each harboring an expression construct, were mixed and used to coinfiltrate N. benthamiana leaf cells. CIP73

![Fig. 3. Interaction between CIP73 and HSC/HSP70 interacting protein (HIP) and homodimerization of HIP in planta. A to D, Interaction between CIP73 and HIP in planta. CIP73 and HIP were expressed in Nicotiana benthamiana leaves as fusion proteins with the C-terminal yellow fluorescent protein (YFP) and N-terminal YFP, respectively. Coexpression of YFPC-CIP73 and HIP-YFPN led to strong fluorescence signals in both the cytoplasm and nucleus (A). The interaction was further confirmed by switching the split YFP tags between CIP73 and HIP (B). Coexpression of YFPC-CIP73 and HIPN-YFPN (C) and coexpression of YFPC-HIP and CIP73-YFPN (D) served as negative controls. E to F, Homodimerization of HIP in planta. Full-length HIP or its N-terminal dimerization domain (HIPN) was fused with the split YFP tags. Coexpression of YFPC-HIP and HIP-YFPN (E) and YFPC-HIPN with HIPN-YFPN (F) resulted in strong fluorescence signals in both the cytoplasm and nucleus. Images were taken 3 days postinfiltration with Agrobacterium cells harboring appropriate expression plasmids. G, Immunoblot analysis of proteins expressed in N. benthamiana leaves. Anti-hemagglutinin (HA) and anti-c-myc antibodies detected the HA and c-myc tags present in the YFPC and YFPN domains, respectively. Anti-tubulin antibody was used for protein loading references. Lane 1, leaf extract expressing YFPC-CIP73 and HIPN-YFPN; Lane 2, leaf extract expressing YFPC-CIP73 and HIP-YFPN; Lane 3, control leaf. Scale bars represent 20 μm.**

![Fig. 4. Subcellular localization of HSC/HSP70 interacting protein (HIP) in Nicotiana benthamiana and Lotus japonicus cells. A to D, Localization of HIP in N. benthamiana leaf cells. HIP-green fluorescent protein (GFP) was expressed under the control of either the Cauliflower mosaic virus 35S promoter (B) or the HIP native promoter (D). GFP alone served as a control (A and C). E, Localization of GFP-tagged HIP under the control of the 35S promoter in L. japonicus hairy roots. The hairy roots were treated with 4% NaCl for 5 min before imaging. GFP alone served as a control. Green fluorescence (left) and brightfield images of the same cells were superimposed to produce the overlay images (right). Scale bars represent 20 μm.]
was fused with the C-terminus of cyan fluorescent protein (CFP-CIP73), CCaMK was fused with the N-terminus of CFP (CCaMK-CFPN), and HIP with the N-terminus of Venus (HIP-VenusN). Three days after infiltration, leaf cells were subjected to confocal laser-scanning microscopic examination. The interaction between CIP73 and HIP was detected as green fluorescence signal and the interaction between CIP73 and CCaMK was observed in red fluorescence (Fig. 5C). Both green and red fluorescence signals were detected in the nuclei of the same cells (Fig. 5C). Identical results were obtained when the split fluorescent tags were exchanged between CCaMK and HIP (Fig. 5D). These results suggest that both interactions between CIP73 and HIP and between CIP73 and CCaMK could concur in the same nucleus and a protein complex consisting of the three partners may exist in the nuclei.

**Downregulated expression of HIP during nodulation.**

To determine the temporal and spatial expression of the HIP gene, we examined the transcriptional expression patterns from the *Lotus japonicus* Gene Expression Atlas (LjGEA) (Verdier et al. 2013). HIP cDNA sequence identified two probe sets (chr4.CM0126.62 at and chr4.CM0126.62.1 at) on the Affymetrix *Lotus* Gene Chip. Analysis of the microarray data revealed that HIP is widely expressed in various tissues, including roots, nodules, stems, leaves, petiole, flowers, pods, and seeds. To confirm the HIP gene expression data, we took a real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) approach to measure the transcript levels of HIP in different tissues and nodulation stages. HIP transcripts were detected at higher levels in both roots and shoots but at a very low level in nodules (Fig. 6B). After inoculation with *M. loti*, the transcript level of HIP was slightly elevated in roots 1 day postinoculation (dpi) and soon returned to its original level at 2 dpi. The expression level then dropped drastically between 8 and 25 dpi (Fig. 6A), indicating that HIP expression is down-regulated during nodule development and nodule senescence.

To analyze the spatial expression pattern of HIP at the cellular level, the HIP gene promoter was used to drive the expression of β-glucuronidase (GUS) reporter in transgenic hairy roots of *L. japonicus*. In noninoculated hairy roots, GUS activity was mainly detected in the root tip, vascular bundles, and

**Fig. 5.** Co-localization of CIP73, HSC/HSP70 interacting protein (HIP), and calcium and calmodulin-dependent protein kinase (CCaMK) and multicolor bimolecular fluorescence complementation (BiFC) analysis of subcellular sites of interactions. A, Coexpression of CIP73-GFP and CCaMK-mCherry in *Nicotiana benthamiana* leaf cells. CCaMK-mCherry (red) colocalized with CIP73-GFP (green) in the nuclei as shown by the yellow color in the overlay image. B, Coexpression of CIP73-GFP and HIP-mCherry in *N. benthamiana* leaf cells. HIP-mCherry (red) colocalized with CIP73-GFP (green) in the nuclei as shown by the yellow color in the overlay image. C and D, Multicolor BiFC analysis of concurrence of the interactions between CIP73 and HIP and between CIP73 and CCaMK in the same *N. benthamiana* leaf cells. CIP73 was expressed as a fusion protein with the C-terminal cyan fluorescent protein (CFP). HIP and CCaMK were fused with the N-terminal CFP and Venus, respectively. The interaction between CC-CIP73 and HIP-VN resulted in green fluorescence, whereas the interaction between CC-CIP73 and CCaMK-CN led to red fluorescence (C). Identical results were obtained when the fluorescent tags were exchanged between CCaMK and HIP (D). Fluorescent images of *N. benthamiana* epidermal leaf cells were taken using confocal laser scanning microscope 3 days postinfiltration with *Agrobacterium* cells harboring appropriate constructs. Overlay images were generated by superimposing green and red images. CC, C-terminal CFP; CN, N-terminal CFP; VN, N-terminal Venus. Scale bars represent 20 µm.
After inoculation with *M. loti*, the HIP promoter activity was observed at the nodule primordia (Fig. 6C and D). After inoculation with *M. loti*, the HIP promoter activity was observed at the nodule primordia (Fig. 6C and D). After inoculation with *M. loti*, the HIP promoter activity was observed at the nodule primordia (Fig. 6E) and was down-regulated drastically in fully matured nodules (Fig. 6F and G).

HIP plays a negative role during early nodulation.
To investigate the possible role of HIP in nodulation, we attempted to identify mutants of *L. japonicus* HIP from the LORE1 retrotransposon insertion population (Fukai et al. 2012; Urbański et al. 2012). One mutant line, 30002690, was identified with an insertion in the first intron of the HIP gene (Supplementary Fig. 7A and B). We determined the HIP mRNA levels in this mutant and did not find any difference in the HIP mRNA level between the mutant and the wild-type control. Apparently, the first intron containing the inserted retrotransposon was spliced out with no effect on the HIP mRNA level. We then examined the role of HIP in nodulation, using an RNAi approach in transgenic hairy roots. Two HIP-specific RNAi constructs were prepared to knockdown HIP expression by targeting the 438-bp 5' region (RNAi-1) and the 299-bp 3' region (RNAi-2) of the HIP gene transcript. We generated transgenic hairy roots expressing the two HIP RNAi constructs, and the empty vector was used as a control. The level of HIP transcripts in transgenic hairy roots was reduced to 22% in HIP RNAi-1 and 51% in HIP RNAi-2, on average, as compared with the control hairy roots (Fig. 7A). Transgenic hairy roots were inoculated with *M. loti*, and nodulation phenotypes were examined. At 16 dpi, the average number of nodules formed on control hairy roots was 4.5 (n = 62), while that on RNAi-1 and RNAi-2 hairy roots was increased to 7.6 (n = 63) and 6.1 (n = 65), respectively (Fig. 7B). This increase in nodule number by HIP RNAi expression was statistically significant (P < 0.05, t test). However, the difference was no longer significant at 32 dpi (Fig. 7B), indicating that RNAi-mediated knockdown of HIP expression could only promote nodulation at the early stage. The increased nodulation on RNAi hairy roots at 16 DPI suggests that HIP may play a negative regulatory role in the early stages of nodulation.

DISCUSSION
CCaMK is a Ca^{2+}-spiking decoder and a central regulator for both RNS and AMS. It has long been thought that NSP1, NSP2, and other transcription factors may act as direct targets of symbiosis signals downstream of CcaMK, in order to activate symbiosis-specific gene expression. However, no direct evidence has been presented to support this hypothesis. No transcription factor has been identified to be a direct phosphorylation target of...
CCaMK. On the other hand, two novel proteins with unknown functions, CYCLOPS and CIP73, have been found to be direct phosphorylation targets of CCaMK (Kang et al. 2011; Yano et al. 2008). Moreover, the molecular mechanisms underlying the regulation of CCaMK and signal relay between CCaMK, CYCLOPS, and CIP73 have yet to be elucidated. In this report, we identified HIP as an interacting partner of CIP73 and show that CIP73 may serve as a scaffold that brings HIP and CCaMK together to form a protein complex in the nuclei.

HIP is a conserved protein in eukaryotes.

HIP homologs are widely present in eukaryotes, including protists, animals, and plants (Fig. 1B) but are absent in prokaryotes, suggesting that HIP proteins may play roles that are unique to eukaryotic cells. Animal HIP has been shown to facilitate the chaperone function of HSC/HSP70 in protein folding and repair and in controlling the assembly of steroid receptors and the activity of regulators of cell proliferation and apoptosis (Caruso and Reiners 2006; Li et al. 2013; Nollen et al. 2000). Although the bioinformatics data of an Arabidopsis HIP homolog has been reported (Webb et al. 2001), the biological function of HIP proteins in plants have not been characterized. In this work, HIP was linked to the symbiosis signaling pathway via interaction with CIP73. Contrary to HIP, both CIP73 and CCaMK proteins appear to be unique to plants, although the Scythe_N ubiquitin-like domain in CIP73 and the N-terminal protein kinase domain and CaM-binding domain in CCaMK share significant homology with animal proteins (Kang et al. 2011; Harper et al. 2004).

HIP is present in both the cytoplasm and nucleus.

In animal cells, HIP was first identified as a cytoplasmic homo-oligomeric protein (Höhfeld et al. 1995). Further studies on HIP have demonstrated that animal HIP is the 48-kDa component (p48) of the progesterone receptor-chaperone complexes and may shuttle between the cytoplasm and nucleus (Prapapanich et al. 1996b). Consistent with this, our results showed that L. japonicus HIP is distributed in both the cytoplasm and nucleus (Fig. 4) and may interact with CIP73 in both subcellular locations (Fig. 3). Previous studies have shown that DMI3/CCaMK is localized exclusively in the nucleus of root-hair cells and epidermal root cells in M. truncatula (Kaló et al. 2005; Smit et al. 2005). However, our data showed that L. japonicus CCaMK is localized in both the cytoplasm and nucleus. Expression of the kinase domain of L. japonicus CCaMK in N. benthamiana leaf epidermal cells has been shown to target the protein to both the cytoplasm and nucleus (Takeda et al. 2012). Recombinant rice CCaMK has also been shown to distribute in the nucleus, cytoplasm, and plasma membrane when expressed in rice protoplasts (Shi et al. 2012). The occurrence and function of CCaMK in the cytoplasm and nucleus warrant further investigation in future studies.

A negative regulator of nodulation.

Animal HIP is also known as ST13 (suppression of tumorigenicity 13), a downregulated protein in colorectal cancers (Shi et al. 2007). Short hairpin RNA–mediated downregulation of ST13 has been found to significantly increase cell proliferation and tumorigenicity (Bai et al. 2012). The striking protein similarity of L. japonicus HIP to ST13 supports the idea that L. japonicus HIP may play a similar role in regulation of cell proliferation in plants. Consistent with this notion, the expression of L. japonicus HIP was found to be downregulated during the nodulation process and in mature nodules (Fig. 6). RNAi-mediated knockdown of L. japonicus HIP expression in transgenic hairy roots led to increased nodule formation at early stages of nodulation (Fig. 7A). Total numbers of nodules per plant on transgenic hairy roots expressing the empty vector (control), HIP RNAi-1, and HIP RNAi-2 constructs, and relative HIP transcript levels were quantified by qRT-PCR using Ubiquitin gene expression as an internal control. Hairy roots expressing the empty vector served as a control. Error bars indicate ± standard deviation for six independent transformants per line and three technical replicates. B, Total numbers of nodules per plant on transgenic hairy roots expressing the empty vector (control), HIP RNAi-1, and HIP RNAi-2 at 16 and 32 days postinoculation (DPI), respectively. Asterisks above the bars indicate statistically significant differences (P < 0.05, t test) between the control and HIP RNAi-1 or RNAi-2. Note that the difference was no longer significant at 32 days postinoculation. Error bars represent the standard deviation.
nodule formation (Fig. 7). These data suggest that *L. japonicus* HIP may play a negative regulatory role in nodulation by serving as a repressor of cell proliferation similar to the role of ST13 in cancer cells.

**Search for other cochaperones for HIP.**

*L. japonicus* HIP protein contains multiple functional domains, including a conserved N-terminal dimerization domain, a central TPR domain flanked by highly charged regions, a stretch of degenerate GGMP repeats, and a C-terminal domain similar to the ST11/HOP/p60 protein (Irmer and Höflund 1997; Prapapanich et al. 1996a). Compared with the animal HIPs, plant HIPs have an expanded region of GGMP repeats. The animal HIP has been well characterized with regard to interaction with HSC/HSP70 and other cochaperones (Demand et al. 1998; Höflund et al. 1995), but whether *L. japonicus* HIP interacts with HSC/HSP70 and other cochaperones is not known. The TPR domain and its flanking charged regions of animal HIP proteins are known to be required for binding with the ATPase domain of HSC/HSP70 (Liu et al. 1999). It remains to be tested whether the N-terminal TPR domain and GGMP motifs of *L. japonicus* HIP interact with *L. japonicus* HSC/HSP70. Our results show that the C-terminal ST11-like domain of *L. japonicus* HIP is necessary and sufficient for the interaction with CIP73 (Fig. 2). The yeast ortholog of animal ST11 is co-chaperone HOP/p60, which mediates organization of HSP70 and HSP90 in multichaperone heterocomplexes (Carrigan et al. 2004; Smith et al. 1993). The TPR domain is also present in yeast HOP/p60 and animal HSP90-binding immunoophilins (Owens-Grillo et al. 1996; Smith et al. 1993). HSC/HSP70 represents a large molecular chaperone family composed of both the constitutively expressed 70-kDa heat-shock cognate protein HSC70 and the heat-inducible HSP70 (Boorstein et al. 1994). HSC/HSP70 proteins are ubiquitous and conserved in both animals and plants. Members of the HSC/HSP70 family in plants have been found in multiple subcellular compartments, including the nucleus, cytoplasm, mitochondria, endoplasmic reticulum, chloroplast, and peroxisome (Boston et al. 1996; Miernyk 1999), but their functions are not well-known.

Whether *L. japonicus* HIP interacts with HSC/HSP70 and other cochaperones in *L. japonicus*, as in animals, remains to be investigated.

In conclusion, the identification of HIP as an interacting partner of CIP73 revealed possible involvement of HIP in CaMK-mediated C2⁺ decoding and symbiosis signaling during nodulation development. CIP73 may act as a scaffold for the formation of a HIP-CIP73-CaMK protein complex that may exert its biochemical function in the nuclei. As a negative regulator of nodulation, HIP may prove to be an important player in regulating cell proliferation required for nodule organogenesis.

**MATERIALS AND METHODS**

**Plant materials and growth conditions.**

Seeds of *L. japonicus* MG-20 Miyakojima were surface-sterilized in 75% ethanol for 2 min and, then, in 2% sodium hypochlorite for 8 min, followed by washing seven times 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 filled with sand and vermiculite (1:1 vol/vol) supplemented with half-strength nitrogen-free Broughton & Dilworth medium and were grown in a growth chamber maintained at 22 ± 1°C with a 16-h-day and 8-h-night cycle. Five-day-old seedlings were inoculated with *Mesorhizobium loti* MAFF303099. *Nicotiana benthamiana* plants were grown in nutrient soil in growth chambers with a 16-h-light and 8-h-dark cycle at 26°C for about 1 to 1.5 months before infiltration with *Agrobacterium tumefaciens*. After infiltration, plants were maintained under the same growth conditions.

**Yeast two-hybrid screening.**

A cDNA fragment encoding the C-terminal region of CIP73 (414 to 691 amino acids), which did not have autoactivation in yeast cells, was fused in-frame with the GAL4 DNA-binding domain in pGBKKT7 vector. Bait constructs were transformed into yeast strain Y187 by the lithium acetate method. Screening of a *L. japonicus* root cDNA library constructed in prey vector (Zhu et al. 2008) was carried out via mating, according to the manufacturer’s instructions (Clontech). For testing protein-protein interactions, a bait construct plasmid and a prey construct plasmid were transformed into yeast strains SD/-Trp/-Leu plates were transferred to SD/-Trp/-Leu-X-gal and SD/-Trp/-Leu-Gal-S plates for evaluation of protein-protein interactions.

**Phylogenetic analysis and sequence alignment.**

HIP proteins were aligned using ClustalX version 1.83 (Kim and Joo 2010) and an unrooted tree was generated with the neighbor-joining method (Saitou and Nei 1987) and bootstrap analysis (1000 replicates). The tree was analyzed and displayed using MEGA software version 5 (Tamura et al. 2011).

**In vitro protein-protein interaction.**

To assay the interaction between CIP73 and HIP in vitro, CBD-tagged HIP was bound to chitin beads (New England Biolabs). The beads were incubated with 2 µg of purified His-CIP73 protein in 1 ml of interaction buffer (20 mM Tris–HCl, 100 mM NaCl, 50 mM KCl, 0.5 mM EDTA, 1 mM MgCl₂, and 5% glycerol, pH 8.0). The reaction was incubated for 1 h on ice with gentle shaking. The chitin beads were collected and were washed five times in 1 ml of interaction buffer. The retained proteins were eluted by boiling for 5 min in 1 x SDS sample buffer and were separated by SDS-PAGE. Subsequent immunoblotting of pulled-down proteins was carried out with the anti-His-tag antibody or visualized by staining with Coomassie brilliant blue. His-tagged CIP73 were purified using nickelagarose beads (Qiagen).

**Tobacco leaf infiltration and multicolor BiFC analysis.**

The full-length coding region of CIP73 was PCR-amplified and was cloned into the SpeI/SalI site of pSCYCE-R vector (Waadt et al. 2008) to obtain the CIP73-CC fusion. The full-length coding regions of HIP and CaMK without the stop codon were cloned into the BamHI/Smal site or the SpeI/Smal sites of pSCYNE, respectively, to obtain the HIP-VN and CaMK-CN or HIP-CN and CaMK-VN fusions. The constructs were transferred into *A. tumefaciens* GV3101/PMP90 or EHA105 by electroporation. Fresh *Agrobacterium* cells containing proper plasmids were grown in Luria Bertani (LB) medium at 28°C in a shaker for 24 h. The cells were transferred to fresh LB medium (1:100 ratio, vol/vol) containing 10 mM (N-morpholine)-ethanesulfonic acid (MES) (pH 5.6) and 40 µM acetylsyringone (AS). After 16 h of growth at 28°C, the bacterial cells were spun down gently at 5,000 × g for 5 min. The pellet was resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES-KOH, 200 µM AS) at a final optical density at 600 nm (OD₆₀₀) of 1.5. For *Agrobacterium* sp. strain p19, a final OD₆₀₀ of 1.0 was used instead. The cells were incubated on bench for 2 to 4 h at room temperature before infiltration. For co-infiltration, an equal volume of different *Agrobacterium* strains carrying plasmids was mixed together prior to infiltration. The bacteria mixture was infiltrated into the top leaves of 6-week-old *Nicotiana benthamiana* with a 1- to 2-ml syringe. Fluorescence of
leaf cells was assayed 2 to 3 days after infiltration using an Olympus FV1000 laser scanning microscope.

Subcellular localization of CIP73, HIP, and CCAmK.

Two expression vectors, pCAMBIA1301-eGFP and pCAMBIA1301-mCherry, were constructed for subcellular localization and colocalization studies. The cDNA encoding eGFP (enhanced green fluorescent protein) (Clontech) with the napoline synthase terminator was placed behind the enhanced CaMV 35S promoter. The whole expression unit was then cloned into the HindIII/EcoRI site of pCAMBIA1301 to generate pCAMBIA1301-eGFP, which eliminated the multiple cloning sites of pCAMBIA1301 and introduced new multiple cloning sites in front of eGFP. The eGFP coding region was replaced by mCherry coding region to generate pCAMBIA1301-mCherry. For expression of the tagged protein under the control of the HIP gene promoter, the CaMV 35S promoter in the two vectors was replaced by the HIP gene promoter.

The CIP73, HIP, and CCAmK cDNAs without the stop codon were cloned into the corresponding cloning sites of pCAMBIA1301-eGFP or pCAMBIA1301-mCherry to obtain CIP73-eGFP, HIP-eGFP, and CCAmK-eGFP or CIP73-mCherry, HIP-mCherry, and CCAmK-mCherry fusions. These constructs were transfected into Agrobacterium rhizogenes LBA1334 for Lotus japonicus hairy root transformation or A. tumefaciens EHA105 for N. benthamiana transformation. Fluorescence signals of transgenic cells were examined using an Olympus FV1000 laser scanning microscope.

Hairy-root transformation and GUS-staining assays.

Hairy root transformation of L. japonicus MG20 using A. rhizogenes LBA1334 was performed as described previously (Kang et al. 2011). The hygromycin gene in vector pC1302 was substituted by the GUS gene to generate pCAMBIA1302GUS vector. The GUS gene in vectors pCAMBIA1301 and pCAMBIA1302GUS was used for selection of transgenic hairy roots. For selection of transformed hairy roots, a root tip of hairy roots was removed and was incubated 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 K4Fe(CN)6, and 0.5 mg ml−1 5-bromo-4-chloro-3-indolyl-D-glucuronic acid [X-Gluc] [Sigma]) overnight at 37°C in the dark. GUS-positive hairy roots were allowed to continue to grow and were used for further analysis.

Gene expression analysis.

Expression profile of the HIP gene was analyzed by qRT-PCR. Total RNAs were isolated from L. japonicus plants using Trizol reagent (Invitrogen). The amount of total RNA was normalized by measuring the RNA concentration at 260 nm using a NanoDrop 2000 (Thermo) apparatus according to manufacturer’s instructions. The Primerscript RT reagent kit (Takara) was used to eliminate genomic DNA contamination and to synthesize first-strand cDNA. Real-time RT-PCR was performed using the SYBR Select master mix (Clontech) with the napoline synthase terminator was placed behind the enhanced CaMV 35S promoter. The whole expression unit was then cloned into the HindIII/EcoRI site of pCAMBIA1301 to generate pCAMBIA1301-eGFP, which eliminated the multiple cloning sites of pCAMBIA1301 and introduced new multiple cloning sites in front of eGFP. The eGFP coding region was replaced by mCherry coding region to generate pCAMBIA1301-mCherry. For expression of the tagged protein under the control of the HIP gene promoter, the CaMV 35S promoter in the two vectors was replaced by the HIP gene promoter.

For HIP promoter-GUS construction.

For HIP promoter-GUS reporter analysis, a 1.6-kb genomic DNA upstream of the HIP coding region was amplified by PCR using primers 5′-AAGTGCAGCACTTCTCATATTTTAACCG-3′ and 5′-ATCGAGATCTTGGTGAGGAAAGC-3′. The promoter was cloned into the PstI/BamHI site upstream of the GUS gene in the binary vector of pCAMBIA1391Z. Transgenic hairy roots of L. japonicus expressing HIPpromGUS were incubated with X-Gluc solution. GUS activity of hairy roots without rhizobial inoculation served as a control. To make sections, stained nodules were embedded into 3% agarose and were sliced into 200-µm-thick sections using a vibratome (Leica VT1200).

Knockdown of HIP by RNAi.

For HIP knockdown analyses, a 438-bp 5′ region (RNAi-1) and a 299-bp 3′ region (RNAi-2) of the HIP transcript were amplified by PCR from L. japonicus cDNA. The forward primers contained PstI-SmaI sites and the reverse primers had Sali-BamHI sites. Amplified cDNA products were digested and ligated into the Sma/BamHI site and, then, the Pst/SmaI site of pCAMBIA1301-35S-intT7 vector. The resulting construct contained a sense and an antisense HIP cDNA sequence, which was interrupted by the Arabidopsis actin-11 intron. This intron-hairpin RNA construct was placed behind the CaMV 35S promoter. The RNAi binary vector was transferred into A. rhizogenes hairy root transformation. Analysis of root growth and nodulation phenotypes was performed as described previously (Kang et al. 2014).

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LITERATURE CITED


**AUTHOR-RECOMMENDED INTERNET RESOURCES**

The Gene Index Project: compbio.dfci.harvard.edu/tgi
The *Lotus japonicus* Gene Expression Atlas (LjGEA) Project: ljgea.noble.org/v2