**Lotus japonicus** Clathrin Heavy Chain1 Is Associated with Rho-Like GTPase ROP6 and Involved in Nodule Formation[^OPEN]

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Mechanisms underlying nodulation factor signaling downstream of the nodulation factor receptors (NFRs) have not been fully characterized. In this study, clathrin heavy chain1 (CHC1) was shown to interact with the Rho-Like GTPase ROP6, an interaction partner of NFR5 in *Lotus japonicus*. The CHC1 gene was found to be expressed constitutively in all plant tissues and induced in *Mesorhizobium loti*-infected root hairs and nodule primordia. When expressed in leaves of *Nicotiana benthamiana*, CHC1 and ROP6 were colocalized at the cell circumference and within cytoplasmic punctate structures. In *M. loti*-infected root hairs, the CHC protein was detected in cytoplasmic punctate structures near the infection pocket along the infection thread membrane and the plasma membrane of the host cells. Transgenic plants expressing the CHC1-Hub domain, a dominant negative effector of clathrin-mediated endocytosis, were found to suppress early nodulation gene expression and impair *M. loti* infection, resulting in reduced nodulation. Treatment with tyrphostin A23, an inhibitor of clathrin-mediated endocytosis of plasma membrane cargoes, had a similar effect on down-regulation of early nodulation genes. These findings show an important role of clathrin in the leguminous symbiosis with rhizobia.

The establishment of symbiotic relationships requires a complex molecular dialog between legumes and nitrogen-fixing rhizobia. Flavonoids released by legume roots trigger the synthesis and secretion of rhizobial nodulation factors (NFs), which are recognized by legume roots to activate a symbiosis signaling pathway that allows the rhizobia to enter root hairs and pass through the plant-derived infection threads (ITs). Eventually, rhizobia are released from ITs into symbiosomes of infected cells within the highly specialized plant organ, the root nodule. NF perception by root cells is mediated by two lysin motif-family receptor kinases, nodulation factor receptor1 (NFR1) and NFR5 in *Lotus japonicus* and Nod Factor Perception and Lysin motif domain-containing receptor-like kinase3 (LYK3) in *Medicago truncatula* (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Smit et al., 2007). NFR1 and NFR5 are required for the earliest physiological and cell responses to NFs, such as membrane depolarization, Ca²⁺ oscillations, and root hair deformation. Downstream of NFR1/NFR5 receptors, a series of genes has been identified to be required for successful nodulation. These genes play roles in NF signal transduction, transcription regulation, cytoskeleton rearrangement, cell wall degradation, and hormone homeostasis (Desbrosses and Stougaard, 2011; Oldroyd et al., 2011; Oldroyd, 2013). Two recent reports have shown that NFR1 and NFR5 interact with each other at the plasma membrane (Madsen et al., 2011) and that the two receptors could directly bind NFs at nanomolar concentrations, which is consistent with the concentration range of NFs required for the activation of symbiotic signaling responses (Broghammer et al., 2012). These studies have led to an NF ligand recognition model, which is also supported by the altered specificity of NF recognition in host plants through coexpression of *L. japonicus* NFR1 and NFR5 in *M. truncatula* (Radutoiu et al., 2007).

Endocytosis, a well-studied process in animal cells, is essential for regulation of protein and lipid compositions of the plasma membrane and removal of cargoes from the extracellular space. Endocytosis also occurs in plant cells, regulating plant growth and development as well as membrane cargoes, had a similar effect on down-regulation of early nodulation genes. These findings show an important role of clathrin in the leguminous symbiosis with rhizobia.

[^OPEN]: Articles can be viewed without a subscription.

[^1]: This work was supported by the National Basic Research Program of China (973 Program grant no. 2010CB126502), the National Natural Science Foundation of China (grant nos. 31170224, 30900096, and 31370278), the National Doctoral Fund of the Ministry of Education (grant no. 20130146130003), and the State Key Laboratory of Agricultural Microbiology (grant no. AMLKF200804).

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as hormonal signaling and communication with the environment (Dhonukshe et al., 2007; Robert et al., 2010; Chen et al., 2011; Sharfman et al., 2011; Adam et al., 2012). As in animal cells, clathrin-mediated endocytosis in plants takes place through clathrin-coated vesicles, which can be observed in highly active plant cells, such as the growing pollen tube and root hairs (Dhonukshe et al., 2007). Clathrin-coated vesicles are formed at the plasma membrane, where specific types of cargoes are recognized and packaged for internalization. The clathrin triskelion is composed of three clathrin H chains (CHCs) each binding to a single clathrin light chain (CLC) in the Hub domain. Clathrin triskelia can further interact, forming a polyhedral lattice that surrounds the vesicle (McMahon and Boucrot, 2011). The Hub domain comprises about 600 amino acid residues at the C terminus and is involved in mediating CHC self-assembly and association with CLCs (Liu et al., 1995; Ybe et al., 1999). Thus, overexpression of the Hub domain can have an inhibitory effect on clathrin-mediated endocytosis through competitive binding with the light chains or forming improper clathrin triskelia (Pérez-Gómez and Moore, 2007).

Endocytosis has been suggested to play a role in endosymbiosis between legumes and rhizobia. Electron microscopy studies have revealed the occurrence of numerous coated pits as well as smooth and coated vesicles along the IT membrane (Robertson and Lyttleton, 1982). NFs have been detected inside the root hairs, along ITs, and in the cytoplasm of infected root cells, suggesting that some aspects of the NF signaling pathway may proceed through an endocytosis process (Philip-Hollingsworth et al., 1997; Timmers et al., 1998). The M. truncatula flotillin-like proteins, FLOT2 and FLOT4, have been localized in the membrane microdomain and found to be required for IT initiation and nodule development (Haney and Long, 2010). Flotillins belong to a family of lipid raft-associated integral membrane proteins and are involved in a clathrin-independent endocytic pathway in mammalian cells (Glebov et al., 2006). This further highlights the importance of endocytic pathways in the evolution of IT-mediated entry of symbiotic bacteria. However, direct evidence for the regulation of the Rhizobium-legume symbiosis by clathrin-mediated endocytosis has not been reported.

We have previously identified the small Rho-like GTPase6 (ROP6) as an interacting protein of the NFR NFR5 (Ke et al., 2012). ROP GTPases in plants are known for their roles in the regulation of the cytoskeleton and vesicular trafficking (Nagawa et al., 2010). Here, we report CHC1 as an interactor of ROP6 and propose that clathrin-mediated endocytosis participates in the early stages of the Rhizobium-legume symbiosis.

RESULTS
Identification of CHC1 as an Interactor of ROP6 in the Yeast Two-Hybrid System

Using ROP6 as bait to screen an L. japonicus root complementary DNA (cDNA) library for interaction proteins, we isolated four independent clones, which all were derived from the same gene encoding the CHC1 in L. japonicus (Fig. 1B). The isolated cDNA clones encode a C-terminal region of CHC1 consisting of only the last one and one-half of the clathrin heavy chain repeat (CHCR). LjCHC1 is present on chromosome 2 (chr2.CM0002.400.r2.m), and there is a paralogous gene on chromosome 6 (chr6.CM0302.70.r2.m) designated as LjCHC2. The
full-length \textit{CHC1} cDNA contains a 5,103-bp open reading frame encoding a peptide of 1,700 amino acids with a molecular mass of 192.4 kD. Like other CHCs, \textit{CHC1} has multiple subdomains starting with an N-terminal domain and followed by linker, distal leg, knee, proximal leg, and trimerization domains (Ybe et al., 1999). The N-terminal domain folds into a seven-bladed \(\beta\)-propeller structure. The other domains form a superhelix of short \(\alpha\)-helices composed of the smaller structural module CHCRs (Smith and Pearse, 1999). Seven CHCRs are presented in both \textit{CHC1} and \textit{CHC2} (Fig. 1A). \textit{CHC} proteins are highly conserved among plant species (see sequence information in Supplemental Table S1), having an amino acid identity of over 90%. Two clades of \textit{CHC} proteins can be clearly distinguished in legume plants, and only one clade can be distinguished in nonlegumes (Fig. 1C).

\subsection*{Requirement of CHCR7 for the Interaction with ROP6}

The four initial \textit{CHC1} clones isolated from the library screen contained only a partial CHCR6, a complete CHCR7 motif, and a C-terminal tail (Fig. 1B). To test if other CHCRs might also mediate the interaction with ROP6, we performed interaction tests in the yeast \textit{(Saccharomyces cerevisiae)} two-hybrid system. A series of truncated proteins of \textit{CHC1} fused to the Gal4 activation domain (AD) were expressed in yeast cells. These proteins were tested for the interaction with ROP6 fused to the Gal4 DNA binding domain (BD; Fig. 2A). The interactions were quantitatively measured by the \(\beta\)-galactosidase activity assay. The results showed that the only clone that interacted with ROP6 was \textit{CHC1}-8, which contained a complete CHCR7 and a C-terminal tail (Fig. 2A), suggesting that other CHCR motifs are not required for the interaction with ROP6.

To assess the specificity of the \textit{CHC1}/ROP6 interaction, we used other known small GTPases from \textit{L. japonicus} (\textit{LjRAC1}, \textit{LjRAC2}, \textit{LjRAB8A}, and \textit{LjRAB8E}; Supplemental Fig. S1; Borg et al., 1997) to replace ROP6. The results showed that the interaction between RAC1 and \textit{CHC1} is very weak (Fig. 2B), suggesting that other CHCR motifs are not required for the interaction with RAC1.

\subsection*{Interaction between CHC1 and ROP6 in Vitro and in Planta}

The interaction between \textit{CHC1} and ROP6 was confirmed by in vitro protein pull-down and coimmunoprecipitation assays. \textit{CHC1}-8 was expressed as a recombinant protein fused to the C terminus of maltose binding protein (MBP) and was pulled down on amylose resins. Purified His-tagged ROP6 protein was then incubated with MBP-\textit{CHC1}-8 absorbed on amylose resins. MBP alone was used as a negative control. As shown in Figure 2C, ROP6 was pulled down on the MBP-\textit{CHC1}-8 resins, suggesting a protein-protein interaction between \textit{CHC1} and ROP6.

For comparison of \textit{CHC1} and \textit{CHC2}, we also expressed hemagglutinin (HA)-tagged \textit{CHC2}-8 that contained a similar peptide region of \textit{CHC2} as in \textit{CHC1}-8. For coimmunoprecipitation assays, HA-tagged \textit{CHC1}-8 and \textit{CHC2}-8 were coexpressed with myc-tagged ROP6 in \textit{Nicotiana benthamiana} leaves. Proteins precipitated with the myc antibody were detected using horseradish peroxidase (HRP)-conjugated anti-HA antibody. The results showed that both \textit{CHC1} and \textit{CHC2} interact with ROP6 (Fig. 2D). No interactions were detected in the controls that coexpressed either \textit{CHC1} and the empty vector or the empty vector and ROP6.

\subsection*{Competition of CLC with ROP6 for Binding of CHC1}

The CHC-Hub domain (corresponding to residues 1,088–1,700 in \textit{CHC1}) can self-trimerize and bind CLC (Fig. 1A). Deletion mutagenesis of the Hub fragment revealed that the shortest region for CLC binding is the residues 1,213 to 1,522 of bovine CHC (Liu et al., 1995), corresponding to residues 1,227 to 1,537 of \textit{LjCHC1} (Fig. 1A). This CLC binding site overlaps with the interacting region of \textit{CHC1} with ROP6. We asked if CLC had a competitive effect on the interaction of \textit{CHC1} with ROP6. To test the possible effect of light-chain binding to the interaction of \textit{CHC1}/ROP6, we cloned \textit{LjCLC1} and \textit{LjCLC2}, which were derived from the gene loci \textit{LjT24M21.40.r2.a} and chr3.CM0160.80.r2.m., respectively. The results showed that both CLCs interacted with \textit{CHC1}-8 (Fig. 3A; Supplemental Fig. S2C). Because CLC1 interacted with \textit{CHC1} more intensively, it was used further for testing competitive binding of \textit{CHC1}-8 with ROP6 in vitro. For this, ROP6 protein was first pulled down on amylose resins containing MBP-\textit{CHC1}-8. The resulting MBP-\textit{CHC1}-8-ROP6 complex was incubated with different concentrations of CLC1. When the concentration of CLC1 was increased, the amount of ROP6 on the resins was reduced, suggesting that CLC1 proteins may compete for binding to \textit{CHC1}.

\subsection*{Colocalization of CHC1 and ROP6}

We coexpressed \textit{Discosoma} sp. red fluorescent protein (DsRED)-tagged ROP6 with GFP-tagged \textit{CHC1} in

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N. benthamiana leaf cells. ROP6-DsRED was colocalized with CHC1-GFP at the cell circumference and within punctate structures adjacent to the plasma membrane (Fig. 4B). We then replaced CHC1-GFP with a truncated CHC1-8-GFP, which presented a stronger fluorescence signal. When ROP6-DsRED was coexpressed with CHC1-8-GFP, ROP6 could better colocalize with CHC1-8 (Fig. 4C). Fusion proteins expressed in N. benthamiana leaves were detected by western-blot analysis (Supplemental Fig. S3B).

**Figure 2.** Interaction between CHC1 and ROP6. A, Dissection of functional domains of CHC1 required for interaction with ROP6. A series of truncated CHC1 proteins was tested for interaction with ROP6. The interaction strength was assessed by the β-galactosidase activity either on plates containing X-gal (80 μg mL⁻¹) or by quantification assay. Values represent means ± s.e. of interaction strength of three individual colonies of each interaction combination. Combinations of p53/SV40 and lam/SV40 served as positive and negative controls, respectively. B, Specificity of the interaction between CHC1 and ROP6. Homologs of ROP6 from L. japonicus were tested for potential interactions with CHC1-8. LjRAC1 showed weak interaction with ROP6. C, In vitro protein-protein interaction assay between CHC1-8 and ROP6. The positions of His-ROP6, MBP, and MBP-CHC1-8 are indicated. Proteins retained on the affinity resins were separated on SDS-PAGE and immunoblotted with HRP-conjugated anti-MBP antibody (top) or anti-His antibody (bottom). D, Coimmunoprecipitation of CHC1-8 and ROP6. Myc-tagged ROP6 and HA-tagged CHC1-8 or CHC2-8 proteins were coexpressed in N. benthamiana leaf epidermal cells. The combinations of ROP6 with the empty HA tag and CHC1-8 with empty myc tag served as negative controls. The input samples were probed with anti-HA antibody (bottom). Antimyc antibody was used for immunoprecipitation. The protein products were analyzed on western blots (WBs) using HRP-conjugated antimyc antibody (top) or anti-HA antibody (middle). SD/-2, SD/-Trp/-Leu; SD/-4, SD/-Trp/Leu/-His/-Ade.
Subcellular Localization of CHC1 in *Mesorhizobium loti*-Infected Cells

Early electron microscopy studies have shown the presence of vesicles along the ITs in infected root hairs (Robertson and Lyttleton, 1982). We performed in situ immunolocalization using CHC antibody that reacted with both LjCHC1 and LjCHC2 by western-blot analysis (Supplemental Fig. S3C). CHC proteins were found at the cytosolic punctate structures. They presumably corresponded to the endocytic vesicles and endosomes, which were present abundantly in the infection pocket (IP; Fig. 5B) and surrounding the ITs (Fig. 5, E and F). The immunofluorescence signal was stronger in the infected root hairs than in noninfected root hairs, indicating an elevated level of CHC proteins in infected root hairs (Fig. 5F). This is consistent with the enhanced gene expression of CHC1 as indicated by the promoter-GUS assay (Fig. 6L; Supplemental Fig. S4I). The control analyzed in the absence of the primary antibody showed no fluorescence signals (Fig. 5A). These data suggest a role of clathrin in the early symbiotic signal exchange during the initiation and development of ITs. We also expressed 35S:CHC1-GFP in transgenic *L. japonicus* hairy roots, which revealed a localization pattern similar to that observed by immunolocalization (Fig. 5D).

Temporal and Spatial Expression of *LjCHC1*

We extracted RNA from various plant organs, including roots infected with *M. loti*, and measured the expression levels of *LjCHC1* by quantitative reverse transcription (qRT)-PCR. *CHC1* was found to be constitutively and highly expressed in roots, stems, leaves, flower, and nodules, suggesting a housekeeping role of this gene in plant growth and development (Fig. 6A). After inoculation with *M. loti*, the expression levels of *CHC1* in roots were not altered significantly (Fig. 6B). Some specific expression patterns that are closely related to symbiotic interactions cannot be found by testing RNA levels of whole-root tissues. Thus, we generated stable transgenic *L. japonicus* lines expressing the reporter GUS gene under the control of the native CHC1 promoter. The expression of CHC1 was high in stigmas, stamens, and pods (Supplemental Fig. S4), implying an important role of CHC1 in plant reproduction. In uninoculated roots, CHC1 was uniformly expressed in roots and root hairs (Fig. 6I). After inoculation with *M. loti*, CHC1 was highly expressed in the root hairs and epidermal cells surrounding the infection sites (Fig. 6, E and L–N; Supplemental Fig. S4). During nodule development, CHC1 was highly expressed in dividing cortical cells and the vascular system of the root (Fig. 6E; Supplemental Fig. S4). It is notable that the expression of CHC1 was highly enhanced in dividing cortical cells before rhizobia entered these cells (Fig. 6, O–Q). In fully developed nodules, CHC1 expression was reduced and restricted to the vascular tissues and uninfected cortical cells (Fig. 6, H, J, and K).

Impaired Nodule Development by Expression of CHC1-Hub in Stable Transgenic Plants

Seven stable transgenic *L. japonicus* plants expressing the CHC1-Hub domain under the enhanced *Cauliflower mosaic virus* (CaMV) 35S promoter were generated and characterized. Plants transformed with the pCAMBIA1301 empty vector served as a control. All T1 transgenic lines exhibited impaired reproductive capacity (Supplemental Fig. S5). This is consistent with the proposed role of clathrin-mediated endocytosis in pollen tube growth and embryogenesis (Zhao et al., 2010; Kitakura et al., 2011; Kim et al., 2013). As a result, very limited amounts of transgenic seeds could be obtained from these transgenic plants. The expression of CHC1-Hub in transgenic plants was confirmed by reverse transcription-PCR (Supplemental Fig. S6D). Two independent T1 transgenic lines, CHC1-Hub lines 1 and 2, and 10 to 20 plants were chosen for characterization of the root and nodule development. Root length was reduced by approximately 50% in CHC1-Hub lines compared with the control 3 d after seedlings were transplanted to vermiculite before rhizobial inoculation (Supplemental Fig. S6, A and B). The root hairs in CHC1-Hub lines were nearly 2-fold longer than those of the control (Supplemental Fig. S6C). The nodule number of CHC1-Hub lines was significantly lower than that of the control at both 10 (*n* = 10, 11, 10, and 12 for control lines 1 and 2 and Hub lines 1 and 2, respectively) and 20 (*n* = 24,
Reduction in Rhizobial Infection Events by Expression of CHC1-Hub in Stable Transgenic Plants

We also tested whether decreased nodulation was a result of defective rhizobial infection in CHC1-Hub transgenic lines. Ten individual plants of each line were inoculated with rhizobia constitutively expressing GFP and observed 7 days after inoculation. The results showed that, although rhizobia could normally infect root hairs of CHC1-Hub lines, the numbers of ITs and bumps per plant were significantly reduced (Fig. 7D). Additional examination on the frequencies of infection initiation and densities of IPs in the CHC1-Hub line 1 revealed a similar defect in rhizobial infection in this Hub line (Supplemental Fig. S7B). Because the roots of CHC1-Hub transgenic lines were shorter than the control expressing the empty vector, we compared the numbers of IPs, ITs, and bumps per centimeter of roots. The result showed that the densities of IPs, ITs, and bumps were also significantly reduced by CHC1-Hub expression (Fig. 7E; Supplemental Fig. S7C), suggesting that the impaired symbiosis phenotype was not caused directly by the reduced root length of CHC1-Hub lines. In conclusion, expression of CHC1-Hub had deleterious effects on not only root development but also, rhizobial infection and IT growth, which ultimately led to reduction in the numbers of ITs and nodules per plant.

Reduction in Nodule Numbers by Expression of CHC1-Hub and CHC1 RNA interference in Transgenic Hairy Roots

To avoid the lethal effect of CHC1-Hub expression on reproduction of transgenic plants, we generated a chimeric plant with transformed roots attached to an untransformed shoot (Kumagai and Kouchi, 2003; Limpens et al., 2004). CHC1-Hub was expressed under the enhanced CaMV 35S promoter in transgenic L. japonicus hairy roots. After hairy roots were generated, a root tip of 2 to 3 mm was excised for GUS staining. Two GUS-positive hairy roots were preserved on each plant, and all of the GUS-negative hairy roots were removed. Nodule numbers were recorded 3 weeks after the transgenic

Figure 4. Subcellular colocalization of CHC1 and ROP6 in N. benthamiana cells. Agrobacterium tumefaciens cells harboring appropriate plasmids were used to infect N. benthamiana leaf cells. The GFP (left) and DsRED (center) fluorescence images were merged. Colocalization signals are shown in yellow (right). A, Nuclear and cytoplasmic localizations of free enhanced GFP (eGFP) and DsRED expressed from the control vector. B and C, Colocalization at the cell circumference (arrows) and within punctate structures (arrowheads) of CHC1-GFP and ROP6-DsRED (B) or CHC1-8-GFP and ROP6-DsRED (C). Bars = 50 μm.

Figure 5. Subcellular localization of CHC1 in M. loti-infected cells. A, B, E, and F, Roots of L. japonicus were inoculated with M. loti constitutively expressing GFP (green channel). Seven dpi, the roots were collected and reacted with anti-CHC antibody followed by detection using second antibody labeled with Cy3 (red channel). The root sample that was analyzed in the absence of the primary antibody served as the negative control (A). Cytoplasmic punctate structures were found in the IP (white arrow in B), surrounding the IT, and at the plasma membrane of the infected root hair (arrowheads in E). ITs filled with GFP-labeled rhizobial cells (green) are indicated by arrows (E and F). C and D, Green fluorescence was found in the nucleus and cytoplasm of the root hair expressing free enhanced GFP (35S: CHC1-GFP) (D). Bars = 10 μm.
plants were transplanted to the nitrogen-free growth soil and inoculated with *M. loti*. The nodule number in hairy roots expressing CHC1-Hub (n = 35) was reduced by about 50% compared with the control hairy roots expressing the empty vector (n = 41; Fig. 7H). We also examined the suppressive effect of CHC1 expression on nodulation by expressing two RNAi constructs targeting the 5′-untranslated region (UTR) region (RNAi-1) and the 3′-UTR region (RNAi-2) of the CHC1 mRNA. Hairy roots from four individual transgenic plants expressing empty or RNAi vectors were randomly selected for total RNA isolation and qRT-PCR assay. The mRNA levels of CHC1 in transgenic hairy roots were reduced by over 60% as measured by qRT-PCR, whereas CHC2 mRNA was not affected by the expression of CHC1 RNAi (Fig. 7, J and K). The results showed similar reduction in nodule numbers by approximately 60% in CHC1-RNAi-1 (n = 28) and CHC1-RNAi-2 (n = 26) roots (Fig. 7I). The observation of

Figure 6. Temporal and spatial expression of *LjCHC1*. A, qRT-PCR assay showing constitutive expression of CHC1 in various tissues of the plant. Total RNA was extracted from roots (Rs), stems (Ss), leaves (Ls), flowers (Fs), nodules (Ns), and pods (Ps) of wild-type *L. japonicus* plants. RNA was also isolated from roots inoculated 6 h (or 0.3 d) and 1, 3, 5, and 7 dpi with *M. loti*. Roots before inoculation (0 dpi) served as a control. Relative expression levels (rel. expression) of CHC1 were measured by qRT-PCR. Means ± se of three biological repeats are presented. B, qRT-PCR assay showed no induced expression of CHC1 in roots after *M. loti* inoculation. C to H, Histochemical GUS staining of stable transgenic plants expressing *LjCHC1pro*::GUS. Plant tissues were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid for less than 4 h to avoid nonspecific staining. Constitutive and high expression of CHC1 was observed in roots (C) and root hairs (I). High levels of GUS staining were observed in the cortical region of roots (D), nodule primordia (G), and developing nodules (H): 3, 5, and 9 dpi. Magnified longitudinal section of the boxed region in D showing expression of CHC1 in root hairs of the rhizobial infected region (E). CHC1 expression was also elevated in the dividing cortical cells that eventually develop to nodule primordia (E). Magnified longitudinal section of the circled region in D shows high expression of CHC1 in the lateral root primordia (F). J and K, In mature nodules, the expression of CHC1 was restricted to the vascular tissues (arrowhead). L to N, CHC1 was expressed in a root hair cell where an IT was developed (L). The presence of ITs was indicated by GFP, which was expressed constitutively by an *M. loti* strain MAFF303099 (M). Superimposed image (N) of L and M. O to Q, CHC1 was expressed in dividing cortical cells before rhizobial cells were released from the ITs (O). The ITs were visualized by the GFP fluorescence (P). Superimposed image (Q) of O and P. Bars = 10 mm (in C, D, G, and H) and 1 mm (in E, F, J, K, N, and Q. 


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the reduction in nodule number by expression of CHC1-Hub and CHC1-RNAi could be reproduced in repetitive experiments, avoiding the variation of the hairy root system.

Suppression of Early Nodulation Genes by Expression of CHC1-Hub

We further tested whether the expression of early nodulation genes, including NODULE INCEPTION (NIN), nodulation pectate lyase (NPL) nuclear factor-YA1 (NF-YA1), and NF-YB1, was affected in CHC1-Hub transgenic lines. Symbiosis Receptor-like Kinase (SymRK) gene and NFR5 are known to be expressed constitutively during the nodulation process (Stracke et al., 2002) and were used as controls in this work. NIN is a transcription regulator that acts early in rhizobial infection and nodule initiation (Schauser et al., 1999). NPL is a nodulation pectate lyase that is required for rhizobia to penetrate the cell wall and initiate formation of ITs (Xie et al., 2012).
NF-YA1 and NF-YB1 are two subunits of the NF-Y, which are expressed in root nodule primordia and play an important role in cortical cell division (Soyano et al., 2013). Total RNA was extracted from roots 2.5 dpi, and qRT-PCR was performed to measure the expression levels of each gene. The results showed that all of the selected genes, except SymRK and NFR5 (Fig. 8, A and B), were induced by rhizobial inoculation as expected (Fig. 8, C–F). The expression levels of NIN, NPL, NF-YA1, and NF-YB1 were suppressed by CHC1-Hub expression (Fig. 8, C–F), which was consistent with the results that CHC1-Hub impaired IT initiation and nodulation. We also used tyrphostin A23 (A23), an inhibitor of cargo recruitment into clathrin-coated vesicles from the plasma membrane (Dhonukshe et al., 2007; Irani and Russinova, 2009), to test if clathrin-mediated endocytosis played a role in the induction of early nodulation genes. Tyrphostin A51 (A51), a biologically inactive structural analog, was used as a negative control. Pretreatment of roots with the inhibitor reduced the induction of early nodulation genes in response to a treatment with NF (Fig. 8, G–I) but did not influence the background expression of these genes in roots without NF treatment (Supplemental Fig. S8). At high concentration (30 μM) of A23, the NF-triggered up-regulation of early nodulation genes was blocked. These results indicate the NF-triggered gene induction is dependent on clathrin-mediated endocytosis of plasma membrane proteins.

Phenotypes of che1Δ2 Heterozygous Plants

Arabidopsis (Arabidopsis thaliana) contains two CHC genes that are partially redundant, and the double-mutant che1/che2 showed gametophytic lethality (Kitakura et al., 2011). We obtained two L. japonicus mutant lines, P0665 and 30013963, both of which contained a transposon insertion in the exon of CHC1 (Supplemental Fig. S9; Fukai et al., 2012; Urbaris et al., 2012). Heterozygous che1Δ2 plants exhibited severe defects in reproduction. The pods of P0665 che1Δ2 mutant plants were shorter and contained fewer seeds than those of the wild-type lines (Supplemental Fig. S8C). This reproductive phenotype was similar to that of CHC1-Hub transgenic lines (Supplemental Fig. S5). No homozygous seedlings were identified from 96 progenies of P0665 che1Δ2 mutant plants, implying lethality in gametogenesis or seed development. Although the che1Δ2 heterozygous plants exhibited severe defects in reproduction, no vegetative growth and altered nodulation phenotype were observed for the che1Δ2 heterozygous plants (data not shown).

DISCUSSION

LjCHC1 Is a Conserved Protein with a Unique Role in Symbiosis

Clathrin-mediated endocytosis is a very well-conserved process in animal and plant cells and involves highly homologous protein components, including CHC, CLC, adaptor protein complex2, and the eight-core component protein complex, TPLATE (Pérez-Gómez and Moore, 2007; Kitakura et al., 2011; Di Rubbo et al., 2013; Kim et al., 2013; Wang et al., 2013a, Gadeyne et al., 2014). CHCs are highly conserved (>90% similarity) in the plant kingdom. Phylogenetic analysis showed the existence of two conserved clades of CHCs in legume plants but only one in nonlegumes (Fig. 1C). It is possible that paralogs of CHCs in legume plants may partially evolve to carry out a unique feature of leguminosae (for instance, symbiotic relationships with rhizobia). LjCHC1 and LjCHC2 share 93% identity in the CHC1-8/CHC2-8 region. ROP6 was found to interact with both CHC1 and CHC2 in plant cells as shown in the coimmunoprecipitation assay, suggesting that there may be functional redundancy between CHC1 and CHC2 in association with ROP6. The peptide region responsible for the interaction with ROP6 was mapped to the C terminus of LjCHC1 containing a CHCR7 and the C-terminal tail (Fig. 2A). It has been shown that the N-terminal domain of CHC is a seven-blade β-propeller and critical for the interaction with multiple adaptor molecules (Smith and Pearse, 1999). The N-terminal domain is oriented toward the plasma membrane in coated pits and responsible for adaptor-receptor recruitment to the coated pits. However, ROP6 interacts with the C-terminal region, which overlaps with the CLC binding site (Figs. 2A and 3A; Supplemental Fig. S2A). The competition analysis in vitro showed that CLC1 competitively inhibits the interaction between ROP6 and CHC1 by direct interaction with CHC1 (Fig. 3B). CLC has been shown to be required in vivo for efficient trimerization and clathrin assembly (Huang et al., 1997). In addition, CHC1 did not interact directly with NFR5 in yeast cells (Fig. 2A; Supplemental Fig. S2A). We also examined the formation of a possible heterotrimeric complex of NFR5, ROP6, and CHC1 at the plasma membrane. These three proteins were coexpressed in N. benthamiana cells, and coimmunoprecipitation assays were performed. Preliminary data on the coimmunoprecipitation assays did not suggest the formation of such a trimeric protein complex (data not shown). Therefore, ROP6 probably does not participate in the assembly of the clathrin lattice complex that is destined to generate clathrin-mediated endocytosis. Instead, ROP6 may play a role in recruiting CHC1 monomer from the cytoplasm to the plasma membrane, thereby promoting endocytosis of plasma membrane proteins. ROP6 is ultimately removed from the clathrin complex through competition of the binding site with CLC1. This hypothesis is also partially supported by the colocalization analysis of ROP6 and CHC1 that showed the colocalization of the two proteins at the cell circumference as well as the punctate structures in the cytoplasm or the vicinity of the plasma membrane (Fig. 4, B and C). ROP proteins are Rho-like GTPases that control multiple developmental processes in plants (Li et al., 2001). They have previously been reported as regulators for clathrin-dependent endocytosis of auxin efflux carriers PIN-FORMED (PIN) proteins at the plasma membrane in Arabidopsis (Chen et al., 2012; Nagawa et al., 2012). The signaling module auxin-AUXIN BINDING
PROTEIN1-ROP6/ROP-interactive CRIB motif-containing protein1-clathrin-PIN1/PIN2 has been proposed as a shared component of the feedback regulation of auxin transport during both root and aerial development. Whether ROPs regulate clathrin-mediated endocytosis of PINs through direct interaction with CHC in Arabidopsis has not been tested. The nodule sizes were larger in CHC1-Hub plants than in the control (Fig. 7G), which could be simply a consequence of the reduced number of nodules in CHC1-Hub plants. Another possible explanation is that the auxin pathway may be altered in the nodules (Suzaki et al., 2012, 2013), because the redistribution of the auxin efflux carrier PINs is known to be mediated by clathrin (Dhonukshe et al., 2007).

**LjCHC1 Is Required for Reproduction and Symbiosis**

Clathrin has been known to be required for plant growth, development, and reproduction (Zhao et al., 2010; Kitakura et al., 2011; Kim et al., 2013). The chc1-1 heterozygous mutant lines characterized in this work exhibited normal plant growth and development but had defects in reproduction. No homozygous seeds could be obtained, suggesting that CHC1 is required for plant reproduction.
reproduction. Stable transgenic plants expressing CHC1-Hub, a dominant negative form that has widely been used to interfere with clathrin-mediate endocytosis, also exhibited similar reproductive defects as well as other nonsymbiotic phenotypes (Supplemental Figs. S5 and S6). Promoter-GUS analysis of CHC1proGUS (Supplemental Fig. S4, A–F) also supported the observation of requirements of CHC1 in plant growth and reproduction. In addition to the conserved nonsymbiotic phenotypes, plants expressing CHC1-Hub also triggered prominent symbiotic defects in nodulation. The CHC1-Hub plants formed fewer IPs, IIs, and nodules (Fig. 7; Suppemental Fig. S7). This conclusion is also supported by the high expression of the CHC1 gene in M. loti-infected root hairs and the in situ immunolocalization of CHC in IPs and surrounding IIs (Figs. 5 and 6L; Supplemental Fig. S4I).

The expression of early nodulation genes, including NIN, NPL, NF-YA1, and NF-YB1, was found to be suppressed in the CHC1-Hub plants (Fig. 8, C–F), indicating a crucial role of CHC1 in the NF signaling pathway and nodule development. A role of clathrin-mediated endocytosis in induction of NF-responsive genes is also directly supported by the chemical inhibition assay (Fig. 8, G and H). Tyrophostins are Tyr analogs developed as inhibitors of Tyr kinases. Tyrophostin A23 but not other tyrophostins, such as A51, is able to inhibit the clathrin-mediated pathway in plants. Therefore, A51 is usually used as a negative control for the treatment of A23. However, it increased the expression of NIN and NPL upon inoculation with NF (Fig. 8, G and H). The results could be reproduced by several experimental replicates, suggesting a potential effect of A51 on plant cells that had not been found before. It also cannot be entirely excluded that A23 may also affect other processes. However, the suppressed expression of NF-responsive genes (NIN and NPL) together with the CHC1-Hub effects on IT initiation implicate the involvement of clathrin-mediated endocytosis in the leguminous symbiosis with rhizobia.

Internalization of Plasma Membrane Proteins in Plants

Endocytosis is an essential process by which eukaryotic cells internalize exogenous material or signaling molecules at the cell surface. In plant cells, clathrin-dependent endocytosis constitutes the predominant pathway for constitutive internalization of plasma membrane proteins (Dhonukshe et al., 2007). Endocytosis-mediated signaling of receptors has been described for several plant receptors, including BRASSINOSTEROID INSENSITIVE1 (Kinoshita et al., 2005), FLAGELLIN-SENSING2 (Chinchilla et al., 2007), CLAVATA1 (Ohyama et al., 2009), and the fungal elicitor ethylene-inducing xylanase receptor2 (Ron and Avni, 2004; Bar and Avni, 2009). A potential link between NFR5 and clathrin implies the possibility of endocytosis of NFRs. Similar implication was also reported in the vesicle-like localization of LYK3 (an ortholog of NFR1) protein (Moling et al., 2014). However, both need to be shown in additional research.

MATERIALS AND METHODS

Plant Materials and Bacterial Strains

Plants of Lotus japonicus (Handberg and Stougaard, 1992; Kawaguchi et al., 2005) ‘Miyakojima MG-20’ used for nodulation assays were grown in a mixed soil medium containing perlite and vermiculite at a 1:1 volume ratio supplied with a one-half-strength Broughton and Dilworth (B&D) nitrogen-free medium in growth cabinets with a 16-h-light/8-h-dark cycle at 22°C ± 1°C. L. japonicus ecotype Gifu-129 mutant seeds P0665 and 30013963 were provided by the Japanese National BioResource Project (http://www.legumebase.brc.miyazaki-u.ac.jp/) and the Centre for Carbohydrate Recognition and Signaling (http://users-mb.au.dk/prmgp/), respectively. Plants of Nicotiana benthamiana were grown in nutrient solution under a 16-h-light/8-h-dark cycle at 26°C. Plants were inoculated with Mesorhizobium loti strain MFe83099, which constitutively expresses GFP.

Plant Transformation and N. benthamiana Leaf Infiltration

Hairy root transformation with Agrobacterium rhizogenes strain LBA1334 was performed as described previously (Wang et al., 2013b). The hygromycin gene in vector pCAMBIA1302 was substituted by the GUS gene, generating pCAMBIA1302GUS. The GUS marker in vectors pCAMBIA1301 and pCAMBIA1302GUS was used for selection of transgenic hairy roots. Stable transformation of L. japonicus was carried using hypocotyls and cotyledons as the explants materials. In brief, explants excised from L. japonicus MG20 seedlings were infected with Agrobacterium tumefaciens strain EHA105 harboring proper constructs. Generated calli were screened for hygromycin resistance, and the regenerated plants (T0) were grown to harvest the T1 seeds. More than 10 independent T1 transgenic lines were generated, and plants with identical phenotypes were selected and propagated further. T1 seedlings produced genetic segregation, and a small piece of cotyledon from every seedling was removed for GUS staining. GUS-positive seedlings were transplanted and used for subsequent investigation on IT formation, nodulation, and gene regulation.

For infiltration of N. benthamiana leaves, fresh A. tumefaciens strain EHA105 carrying proper constructs was grown in Luria-Bertani medium containing 10 mM MES-KOH (pH 6.0) and 40 μM acetoxy- safranin overnight until optical density at 600 nm ≥ 1.0. A. tumefaciens cells were collected and suspended in infiltration buffer (10 mM MgCl2, 10 mM MES-KOH, pH 6.0, and 200 μM acetoxy- safranin). The cells were incubated at room temperature for 4 h before infiltration. For coexpression of proteins, equal amounts of two or three A. tumefaciens strains harboring proper constructs were mixed and used for infiltration. Leaves were observed 36 to 48 h after infiltration using a confocal laser-scanning microscope.

Yeast Two-Hybrid Screening

The full-length cDNA of ROP6 (GenBank accession no. ADY166610.1) was amplified by PCR and inserted into the EoR1/Sul1 sites of the vector pGBK7 for expressing the bait protein GAL4-DNA binding domain (BD):ROP6. Screening of interaction clones was carried out according to the procedures described previously (Zhu et al., 2008). To test protein-protein interactions in yeast (Saccharomyces cerevisiae) cells, a BD construct plasmid and an AD construct plasmid were transformed to yeast strains Y187 and YAH109, respectively. After mating of the two yeast strains, colonies grown on SD/-Trp/-Leu plates were transferred to SD/-Trp/-His/-Ade/5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (X-gal; SD/-4/X-gal) and SD/-Trp/-Leu/X-gal (SD/-2/X-gal) plates for confirmation of protein-protein interactions. The screened and selected colonies were grown on YPM agar plates for confirmation of protein-protein interactions. The strength of interactions was evaluated in three colonies of each interaction group by measuring β-galactosidase activity and presented as the average values of three replicates.

Plasmid Construction

To produce fusion proteins with the GAL4-DNA BD, cDNAs encoding ROP6, CLC1, CLC2, RAC1, RAC2, RABA8, RABB1, CHC1-7, CHC1-8, CHC1-9, and NFR5-PK were amplified by PCR. The amplified fragments were cloned into the proper sites of pCAGI7. To produce fusion proteins with the GAL4 AD, cDNAs encoding the CHC1 truncated forms (from CHC1-1 to CHC1-9), ROP6, CLC1, CLC2, RAC1, RAC2, RABA8, RABB1, and NFR5-PK were amplified and cloned into the proper sites of pCAGI7. For coimmunoprecipitation analysis, the ROP6 cDNA without the stop codon was amplified and cloned into the Sul1/Smal
site of pVNYE for expressing ROP6-(myc)-VN. The truncated CHC1-8 (1,419-1,700 amino acids) and CHC2-8 (1,419-2,102 amino acids) fragments were amplified and cloned into the SalI/SmaI site of pSCEY-C for expressing SCCR-HA/CHC1-8 and SCCR-HA/CHC2-8. These constructs were introduced to A. tumefaciens EHA105 for transformation. For in vitro interaction and competition assays, the CHC1-8 cDNA fragment was introduced into the EcoRI/SalI site of pMAL-c2x for expression of MBP-CHC1-8. The ROP6 and CLC1 cDNAs were amplified and inserted into EcoRI/NotI site of pET28a for expression of His-tagged ROP6 and CLC1 recombinant proteins. For gene knockdown analyses, a 157-bp 5′-UTR (RNAi-1) and a 125-bp 3′-UTR (RNAi-2) fragments of CHC1 were amplified from L. japonicus total cDNA. The forward primers contained PstI/Smal sites, and the reverse primers had SalI/BamHI sites. Amplified products were digested and ligated into the SalI/BamHI site and then, the PstI/XbaI site of pCAMBIA1301-S35-int/77. The resulting constructs containing a sense and an antisense CHC1 mRNA interrupted by an intron were driven by the CaMV 35S promoter. For competition of the dominant negative form of CHC1, the cDNA encoding CHC1-Hub was placed behind the enhanced CaMV 35S promoter. The whole expression unit was then cloned into the HindIII site of pCAMBIA1301, which contained both Gus and hygromycin selection markers for A. rhizogenes-mediated hairy root transformation and A. tumefaciens-mediated stable transformation. For subcellular localization, the full-length CHC1 coding sequence was obtained by primers that corresponded to the 5′- and 3′-UTRs, which were unique between CHC1 and CHC2. Using the PCR products as the template, the full-length CHC1 was reamplified and cloned to the Ncol/SphI site of pCAMBIA1302GUS. For colocalization study, the ROP6 cDNA was cloned into the NcoI/SphI site of pC310GUS-DaRed, and the CHC1-8 fragment was cloned into the Ncol/SphI site of pC3102. For promoter-GUS reporter analysis, a 2 kb LCHC1 promoter fragment was amplified from MC20 genomic DNA and cloned to pMD18T (Takara). The fragment between the SalI and SmaI sites was then inserted upstream of the Gus gene in the binary vector pCAMBIA1391Z. All constructs together with primers, restriction enzyme cleavage sites, and short descriptions are listed in Supplemental Table S2.

In Vitro Protein-Protein Interactions

To assay the interactions of CHC1 with ROP6 and CLC1 in vitro, MBP-tagged CHC1-8 was absorbed to amylose resins and then incubated with 20 μg of purified ROP6 or CLC1 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% [v/v] glycerol, pH 7.4) on ice for 1 h with gentle shaking. The suspension (100 μL) was mixed with SDS loading buffer and used as the input sample. After discarding the supernatant, amylose resins were washed 10 times each with 1 mL of interaction buffer. Retained proteins were eluted in 1× SDS loading buffer and separated on an SDS-PAGE gel. Subsequent immunoblotting of pulsed and unbound proteins was carried out by HRP-conjugated anti-MBP and anti-Myco-His antibodies. These experiments were performed at least three times with identical or similar results. His-tagged ROP6 and CLC1 were purified using nickel-agarose beads (Qiagen).

Combinoprecipitation Analysis

Myetagged ROP6 and HA-tagged CHC1-8 or CHC2-8 were expressed from bimolecular fluorescence complementation vectors pVNYE and pSCEY-C, the former containing an myc tag and the latter having an HA tag (Wada et al., 2008). ROP6 protein was coexpressed with CHC1-8 or CHC2-8 in N. benthamiana leaves through coinfiltration of two A. tumefaciens strains EHA105 harboring proper constructs. Three days after infiltration, N. benthamiana leaves were powdered in liquid nitrogen, and proteins were extracted by a 1:1 g mL⁻¹ volume ratio of leaf to homogenization buffer. The homogenization buffer contained 50 μL HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% [w/v] Suc, and 1.5% [v/v] Triton X-100 with 1% protease inhibitor cocktail (Roche) and 2 mM phenylmethylsulfonyl fluoride (Sigma) added before use. Samples were incubated on ice for 20 min and centrifuged at 13,000g at 4°C two times for 20 min each. The supernatant (600 μL) was first subjected to preclearing by incubation for 30 min at 4°C with gentle rotation with 30 μL of protein A/G magnetic beads (I00277; GenScript), which were prewashed three times with washing buffer containing 50 μL HEPES, pH 7.5, 10 mM EDTA, 150 mM NaCl, and 0.5% (v/v) Triton X-100. After magnetic separation, 30 μL of eluate was mixed with SDS loading buffer and used as the input sample control. For immunoprecipitation, anti-His antibody (I100) was added to the eluate and incubated for 2 h at 4°C with gentle rotation. After that, 40 μL of washed protein A/G magnetic beads were added to the eluate and incubated for 2 h at 4°C with gentle rotation followed by magnetic separation. The unbound extract was discarded, and the beads were washed three times with washing buffer. The beads were resuspended in 100 μL of 1× SDS loading buffer and boiled for 10 min at 100°C to dissociate the immunocomplexes from the beads followed by magnetic separation of the beads. The resulting eluate was used for protein separation by SDS-PAGE and western-blot analysis using HRP-conjugated antimyc and anti-HA antibodies (Promoter Biotechnology Ltd.).

In Vitro Protein Competition Analysis

MBP-CHC1-8 bound to amylose resins was incubated with 20 μg of purified His-ROP6 protein in 1 mL of interaction buffer with gentle shaking for 1 h. After washing three times, six aliquots of amylose resins containing the MBP-CHC1-8/ROP6 complex were incubated with different concentrations (0, 20, 40, 80, 160, and 320 μg) of CLC1 protein in 1 mL of interaction buffer for 1 h. After washing seven times, retained proteins on amylose resins were eluted in SDS loading buffer and separated on an SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue, and a similar gel with identical input was used for western-blot analysis using an HRP-conjugated antityplo-His antibody. In the in vitro protein competition analysis was performed three times with identical results.

In Situ Immunolocalization

Roots of L. japonicus were fixed in 1% (w/v) freshly depolymerized paraformaldehyde in 1× phosphate-buffered saline (PBS), pH 7.4, for 30 min at 4°C. After washing three times for 5 min each, roots were blocked in 1× PBS containing 3% (w/v) bovine serum albumin and 0.3% [v/v] Triton X-100 for 2 h and then incubated with a 50-fold diluted anti-CHC polyclonal antibody overnight at 4°C in 1× PBS containing 3% (w/v) bovine serum albumin and 0.3% [v/v] Triton X-100. The anti-CHC secondary antibody labeled with cyanine dye (Cy3) was used according to the manufacturer’s instructions (Promoter Biotechnology Ltd.). A control root sample was performed in the absence of the primary antibody. The anti-CHC polyclonal antibody, generated against a conserved peptide (FNELISLMESGICLGERAHMC; Wang et al., 2013a) that is present in ACHC, LCHC1, and LCHC2, was provided by Jianwei Pan.

Analysis of Gene Expression

Total RNAs were isolated from L. japonicus plants using Trizol reagent (Invitrogen). Primerscript RT Reagent Kit (Takara) was used to eliminate genomic contamination of total RNAs and synthesize first strand cDNAs. qRT-PCR was performed using the SYBR Select Master Mix (ABI) reagent. All PCR reactions were performed using an ABI Viia 7 Real-Time PCR System under the standard cycling mode: 2 min at 50°C for uracil-DNA glycosylase activation and 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All of the expression levels were analyzed and normalized using Adenyl pyrophosphatase (ATPase) gene (AW719841), which is constitutively expressed in L. japonicus. For the expression assay of early nodulation genes in transgenic plants, seeds were germinated on one-half-strength Murashige and Skoog (without Suc) plates for 5 d. Then, seedlings were transplanted to soil medium containing perlite and vermiculite at a 1:1 volume for a 4-d growth followed by inoculation with 0.1 mL of 2.5 d. Roots were harvested for total RNA isolation and qRT-PCR analysis.

GUS Staining

Roots were washed with water and incubated in GUS staining solution [100 mM NaClO buffer, pH 7.0, 0.1% [w/v] Triton X-100, 0.1% [w/v] N-laurylsarcosine, 10 mM EDTA, 1 mM K3Fe(CN)6, 1 mM K4Fe(CN)6, and 0.5 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid] for 2 to 4 h at 37°C. For GUS staining of leaf, flower, and pod samples, the tissues were degassed under vacuum for 5 min and then incubated in the GUS solution for 6 h followed by washing with 75% (v/v) ethanol.

Treatment with Endocytosis Inhibitors

Swollen seeds were spread on one-half-strength Murashige and Skoog (without Suc) plates. Plates were placed upside down for 30 to 40 h at 22°C in the dark for hypocotyl elongation (approximately 1 cm). Seeds were then transferred to one-half-strength B&D medium (1.2% [w/v] agar, pH 6.8) for 3 d of growth for root elongation (approximately 3–4 cm) and cotyledon expansion in closed plastic containers vertically placed in a greenhouse. Seedlings of four to six plants for each sample were immersed in 4 mL of liquid one-half-strength...
B&K medium containing various concentrations (10, 20, and 30 μM) of inhibitors (A23 or A51 dissolved in DMSO as stock solutions at concentrations of 10, 20, and 30 μM) for 6 h in the dark in six-well tissue culture dishes. An equal volume of DMSO was used as a solvent control. After that, the diluted (1:100,000) crude extracts of NF were added to the medium to induce gene expression for another 12 h at 22°C in the dark. Plants immersed in one-half-strength B&K medium for 18 h without any treatment served as a negative control (NF−). Crude extracts of NFs (1 mL) were prepared from 1 L of liquid culture of M. loti R7A (OD = 1.0) carrying plasmid pMP2112 as described previously (Cárdenas et al., 1995; Miwa et al., 2006). Treated plants were harvested for total RNA isolation and qRT-PCR analysis. As a control for A23 and A51 without NF treatment, seedlings treated with A23, A51, or DMSO for 6 h and seedlings without any treatments were harvested and used for qRT-PCR analysis.

**Microscopic Analysis**

Microscopic analysis was performed using an Olympus FV1000 confocal laser-scanning fluorescence microscope. For the subcellular localization and colocalization analyses, GFP fluorescence was excited at 488 nm, and the emission was detected at 505 to 535 nm. DsRED and Cy3 fluorescence was excited at 559 nm, and emission wavelengths were detected at 660 to 675 nm for DsRED and 650 to 665 nm for Cy3. Z projections of root hairs were reconstructed using more than 20 images taken at increments of 2 μm.

**Identification of L. japonicus Mutants**

Genomic DNAs were isolated from individual mutant plants and used for PCR amplification of 5 min at 95°C and 35 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C followed by 6 min at 72°C. Primer designs were performed as described previously (Fukai et al., 2012; Ubarski et al., 2012).

**Phylogenetic Analysis**

Conserved motifs of CHC1 were predicted by the domain annotation tool of SWISS-MODEL (http://swissmodel.expasy.org/). Full-length CHC protein sequences were aligned using Clustal X, and the alignment was used to generate the phylogenetic tree using the minimum evolution method of MEGA4.0. Sequence information used for phylogenetic analysis is listed in Supplemental Table S1.

Sequence data from this article can be found under the following GenBank accession numbers: KJ184306 for CHC1, KJ184309 for CHC2, KJ184307 for CLC1, and KJ184308 for CLC2. Other sequences cited in this article have the following accession numbers: UniProtKB O04369.1 for RAC1, UniProtKB Q40220.1 for RAC2, GenBank CAA98172.1 for RABA8, CAA98176.1 for RABA8E, and ADDY16660.1 for ROP6.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Amino acid sequence alignment of ROP6 homologs from L. japonicus.

**Supplemental Figure S2.** Protein-protein interactions in yeast cells and in vitro pull-down assays.

**Supplemental Figure S3.** Western-blot analysis of proteins expressed in yeast cells, N. benthamiana, and L. japonicus.

**Supplemental Figure S4.** Tissue-specific expression of CHC1 by promoter-GUS analysis.

**Supplemental Figure S5.** Impaired seed development in transgenic plants expressing CHC1-Hub.

**Supplemental Figure S6.** Root phenotypes of transgenic plants of L. japonicus expressing CHC1-Hub.

**Supplemental Figure S7.** Effects of CHC1-Hub expression on rhizobial infection in L. japonicus.

**Supplemental Figure S8.** Control samples for A23 and A51 without NF treatment.

**Supplemental Figure S9.** Defects in reproduction in chc1−/− heterozygous plants.

**Supplemental Table S1.** Database accession numbers of plant CHC sequences.

**Supplemental Table S2.** Primers and constructs used in this study.

**ACKNOWLEDGMENTS**

We thank Allen Downie (John Innes Centre) for providing M. loti strain R7A carrying pMP2112, Jianwei Pan (Zhejiang Normal University) for providing anti-CHC polyclonal antibody, and the Japanese National BioResource Project and the Centre for Carbonhydrate Recognition and Signaling for providing for the Lotus Retrotransposon1 chc1-1 (P0665) and chc1-2 (30013963) mutant seeds.

Received December 21, 2014; accepted February 23, 2015; published February 25, 2015.

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