A Ubiquitin Ligase of Symbiosis Receptor Kinase Involved in Nodule Organogenesis

Songli Yuan, Hui Zhu, Honglan Gou, Weiwei Fu, Lijing Liu, Tao Chen, Danxia Ke, Heng Kang, Qi Xie, Zonglie Hong, and Zhongming Zhang*

State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China (S.Y., H.Z., H.G., W.F., T.C., D.K., H.K., Z.Z.); State Key Laboratory of Plant Genomics, National Center for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Datun Road, Beijing 100101, China (L.L., Q.X.); and Department of Plant, Soil, and Entomological Sciences and Program of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, Idaho 83844 (Z.H.)

The symbiosis receptor kinase (SymRK) is required for morphological changes of legume root hairs triggered by rhizobial infection. How protein turnover of SymRK is regulated and how the nodulation factor signals are transduced downstream of SymRK are not known. In this report, a SymRK-interacting E3 ubiquitin ligase (SIE3) was shown to bind and ubiquitinate SymRK. The SIE3-SymRK interaction and the ubiquitination of SymRK were shown to occur in vitro and in planta. SIE3 represents a new class of plant-specific E3 ligases that contain a unique pattern of the conserved CTLH (for C-terminal to LisH), CRA (for CT11-RanBPM), and RING (for Really Interesting New Gene) domains. Expression of SIE3 was detected in all tested tissues of Lotus japonicus plants, and its transcript level in roots was enhanced by rhizobial infection. The SIE3 protein was localized to multiple subcellular locations including the nuclei and plasma membrane, where the SIE3-SymRK interaction took place. Overexpression of SIE3 promoted nodulation in transgenic hairy roots, whereas downregulation of SIE3 transcripts by RNA interference inhibited infection thread development and nodule organogenesis. These results suggest that SIE3 represents a new class of E3 ubiquitin ligase, acts as a regulator of SymRK, and is involved in rhizobial infection and nodulation in L. japonicus.

The formation of root nodules is initiated in response to rhizobia-derived nodulation factors (NFs), a group of lipochitooligosaccharides with N-linked acyl groups and other host-specific decorations. NFs can trigger a series of host responses, including root hair deformation, cellular alkalization, membrane potential depolarization, ion flux changes, early nodulin gene expression, and nodule primordial formation (Ehrhardt et al., 1996; Felle et al., 1999; D’Haese and Holsters, 2002; Wais et al., 2002). These responses allow the rhizobia to enter the root epidermal cells through infection threads (ITs). Concomitant with the rhizobial infection, NFs stimulate distant cells of the root pericycle layer to undergo cytoskeletal rearrangement and develop nodule primordia (Timmers et al., 1999). Rhizobia are released from the end of ITs into the cytoplasm of root cortical cells, where they are enclosed by the symbiosome membrane and reduce the atmospheric nitrogen into organic nitrogen compounds that are exported to the host in exchange for carbohydrates.

A series of plant genes involved in perception of rhizobial NF signals have recently been identified in Lotus japonicus, Medicago truncatula, and other legumes (Catoira et al., 2000; Wais et al., 2000; Miwa et al., 2006; Madsen et al., 2010; Oldroyd et al., 2011). NFs are perceived by two receptor kinases, nodulation factor receptor1 (NFR1) and NFR5, which belong to the Lysin-motif receptor-like kinases (RLKs; Madsen et al., 2003; Radutoiu et al., 2003, 2007). Recognition of NFs by their receptors leads to activation of the common symbiosum pathway, which is required for establishing symbiotic relationships with rhizobia and nutrition-improving mycorrhizal fungi (Kistner and Parniske, 2002; Banba et al., 2008).

The symbiosis receptor kinase (SymRK) does not bind NFs directly, but acts in a key step downstream of NFR1/NFR5. The SymRK gene is indispensable for root endosymbiosis with rhizobia and arbuscular mycorrhizal fungi in legumes, and may function at the junction
of rhizobial and fungal signaling cascades (Endre et al., 2002; Stracke et al., 2002). The SymRK protein contains a signal peptide sequence for protein sorting, an extracellular region for interacting with unidentified signal molecules, a transmembrane segment for anchoring to the plasma membrane, and an intracellular protein kinase domain (Endre et al., 2002; Stracke et al., 2002; Mitra et al., 2004; Capoen et al., 2005).

Both the SymRK extracellular region and the kinase domain are important for nodulation in L. japonicus. A recent report has identified a mutation in the Gly-Asp-Pro-Cys motif of the SymRK extracellular region that blocks the IT formation in root hairs (Kosuta et al., 2011). Previously, the SymRK kinase domain has been shown to interact with 3-hydroxy-3-methylglutaryl CoA reductase1 (HMGR1) from M. truncatula (Kevei et al., 2007), and an ARID-type DNA-binding protein (Zhu et al., 2008). HMGR1 catalyzes the formation of mevalonic acid and is the rate-limiting step of the mevalonic acid pathway, which is required for production of specific isoprenoid compounds such as cytokinins, phytosteroids, and isoprenoid-modified signal proteins. Reducing the level of HMGR1 by inhibitor lavastatin or RNA interference (RNAi) expression results in a drastic decrease in nodulation in M. truncatula (Kevei et al., 2007). SIP1 binds the promoter of the NIN (for nodule inception) gene (Zhu et al., 2008), which is required for rhizobial recognition, IT formation, and nodule primordial initiation (Schauser et al., 1999). These results suggest that SymRK may form protein complexes with key regulatory proteins of downstream cellular responses. Symbiotic Remorin1 (SYMREM1) from M. truncatula has been shown to interact with SymRK, and may act as a scaffold protein for assembly of signaling complexes involved in rhizobial infection (Lefebvre et al., 2010).

In this study, we identified a SymRK-interacting E3 ligase (SIE3) as a regulator of rhizobial infection and nodulation in L. japonicus. SIE3 was shown to bind and use SymRK as a substrate for ubiquitination in planta. Alteration of SIE3 expression levels in transgenic hairy roots via overexpression and RNAi exhibited drastic effects on rhizobial infection and nodulation. Thus, SIE3 appears to represent a new class of plant-specific E3 ligases that are involved in symbiotic nitrogen fixation in legumes.

RESULTS

SIE3 Is a Novel E3 Ubiquitin Ligase

In search of interacting proteins of SymRK, we took the two-hybrid approach and screened an L. japonicus complementary DNA (cDNA) library constructed in a yeast (Saccharomyces cerevisiae) expression vector (Zhu et al., 2008). Several positive clones were isolated and shown to contain cDNA inserts representing the same gene. Because none of them appeared to contain a full-length coding region, we searched the Lotus database and found two expressed sequence tag clones (LjG0027263 and LjG0032470). We designed primers according to the reconstituted full-length coding region and amplified its cDNA using RNA isolated from L. japonicus roots inoculated with Mesorhizobium loti. The 1,161-bp coding region encodes 387 amino acids, and its sequence has been deposited to GenBank (JQ009197).

The deduced peptide, containing a CTLH (for C-terminal to LisH) motif, a CRA (for CT11-RanBPM) domain, and a RING (for Really Interesting New Gene) finger domain (Fig. 1A), represents a novel class of the RING-type E3 ubiquitin ligase family. This protein was designated as SIE3 for SymRK-interacting E3 ubiquitin ligase. E3 proteins containing the CTLH, CRA, and RING domains are conserved in higher plants with no known biological function (Fig. 1B; Supplemental Fig. S1). Homology modeling revealed that SIE3 may exist as a reverse parallel homodimer (Supplemental Fig. S2A). In yeast cells, SIE3 interacted with itself (Supplemental Fig. S2B), which is consistent with the homodimer model. The C terminus of SIE3 contains a RING finger domain, encompassing eight highly conserved Cys/Ser/His residues that may coordinate two Zn$^{2+}$ ions (Fig. 1C). The first Zn$^{2+}$ ion is coordinated by four Cys/Ser residues at positions 1, 2, 5, and 6, and the second Zn$^{2+}$ by Cys/His residues at positions 3, 4, 7, and 8 (Lorick et al., 1999; Fang and Weissman, 2004). In SIE3 and some of its plant homologs, the Cys residues at positions 2 and 6 are replaced with Ser, Leu, or Thr (Fig. 1C), which disables the coordination of the first Zn$^{2+}$ ion. The four residues coordinating the second Zn$^{2+}$ ion are highly conserved in the plant proteins (Fig. 1C). Despite the substitutions, the three-dimensional model of the degenerated RING finger domain in SIE3 still resembles that of the canonical RING (Supplemental Fig. S2C).

Dissection of Peptide Domains Involved in SIE3-SymRK Interaction in Yeast

To dissect domains required for the interaction between SIE3 and SymRK, deletions of both SymRK and SIE3 cDNAs were made. The clone (pSIE3-DACTLH) isolated from the initial library screen contained the CRA and RING domains but lacked the CTLH domain, suggesting that the CTLH domain is not essential for the interaction (Fig. 2A). When the RING domain was removed (SIE3-ARING; Fig. 2A), the interaction became very weak, suggesting that RING is important for the interaction. Experiments using SymRK deletions showed that both the extracellular region (SymRK-EC) and the PK domain of SymRK (SymRK-PK) have very weak interaction with SIE3 (Fig. 2B). Because of the weak interaction between SymRK and SIE3, we asked if SIE3 also interacts with other RLKs such as NFR1, NFR5, and L. japonicus His kinase1 (LHK1). Our results showed that SIE3 does not interact with the PK domain of NFR1, NFR5, and
LHK1, or with the extracellular region of LHK1 (Fig. 2C). Thus, we conclude that SIE3 can specifically interact with SymRK in yeast cells, and the full-length SIE3 can also interact with both SymRK-EC and SymRK-PK in yeast cells.

Interaction of SIE3 with SymRK in planta

We took the bimolecular fluorescence complementation (BiFC) approach to investigate if the SIE3-SymRK interaction took place in tobacco leaf cells coexpressing recombinant SIE3 and SymRK proteins. SymRK was fused to the split cyan fluorescent protein (CFP) C terminus (SymRK:SCC), while SIE3 was attached to the split CFP N terminus (SIE3:SCN). Interaction of the two proteins would reconstitute the cyan fluorescent chromophore, allowing for detection of cyan fluorescence (Waadt et al., 2008). Leaf epidermal cells were analyzed 2 d after infiltration with Agrobacterium tumefaciens harboring the constructs. Strong cyan fluorescence was observed in the plasma membrane of leaf cells expressing the two proteins (Fig. 3A), suggesting an interaction at the plasma membrane. The fluorescence was as strong as the positive control, which coexpressed Arabidopsis (Arabidopsis thaliana) calcineurin B-like (CBL) and CIPK24 (for CBL-interacting protein kinase24; Waadt et al., 2008). Leaf epidermal cells were analyzed 2 d after infiltration with Agrobacterium tumefaciens harboring the constructs. Strong cyan fluorescence was observed in the plasma membrane of leaf cells expressing the two proteins (Fig. 3A), suggesting an interaction at the plasma membrane. The fluorescence was as strong as the positive control, which coexpressed Arabidopsis (Arabidopsis thaliana) calcineurin B-like (CBL) and CIPK24 (for CBL-interacting protein kinase24; Waadt et al., 2008).

Co-IP of SIE3 with SymRK

The interaction was further confirmed by coimmunoprecipitation (Co-IP). SIE3 was tagged with GFP, whereas SymRK was expressed as a Myc-tagged protein in N. benthamiana leaves. Coexpression of the two recombinant proteins was achieved by infiltration of a mixture of two A. tumefaciens strains (1:1) that contained pSIE3-GFP and pSymRK-myc, respectively. As controls, a mixture of A. tumefaciens strains (1:1) containing pSymRK-myc and pBA-myc, respectively, or containing pSIE3-GFP and pBA-myc, respectively, was used for infiltration. Three days after infiltration, the expression of the recombinant proteins in leaves was confirmed by immunoblot analysis using anti-hemagglutinin (HA) and anti-FLAG monoclonal antibodies (Fig. 3B). These results showed that SIE3 interacts with SymRK at the plasma membrane in plant cells. The interactions between the full-length SIE3 and SymRK-EC or SymRK-PK were further confirmed by the BiFC approach (Supplemental Fig. S3).
that the SIE3-SymRK interaction takes place in plant cells.

**Self-Ubiquitination of SIE3**

The RING domain of SIE3 belongs to the degenerated C3HC4-type variant (Fig. 1C). It is known that a canonical Cys and His pattern in a RING domain is not critical for the activity of E3 ubiquitin ligases. We first tested if SIE3 had an enzyme activity for self-ubiquitination, a hallmark property of E3 ubiquitin ligases. His-SIE3 was expressed in *Escherichia coli* and purified using nickel beads. Self-ubiquitination of His-SIE3 was assayed in the presence of wheat (*Triticum aestivum*) E1 activating enzyme, human (*Homo sapiens*) E2 enzyme, and *Arabidopsis thaliana* E3 activating enzyme. The reaction was initiated by the addition of SIE3 or SymRK and continued for 1 hour at room temperature. The reaction was then stopped by the addition of SDS-sample buffer and heated to 95°C for 5 minutes. The samples were then loaded onto a 15% polyacrylamide gel and electrophoresed. The gels were then stained with Coomassie blue and exposed to autoradiography. The results showed that SIE3 and SymRK were both self-ubiquitinated. SIE3 was shown to have an enzyme activity for self-ubiquitination, a hallmark property of E3 ubiquitin ligases. In conclusion, SIE3 and SymRK are both self-ubiquitinated in planta.

**Figure 2.** Interaction of SIE3 with SymRK in yeast cells. A, Functional domains and deletion constructs of SIE3 and SymRK. SIE3-ACTLH represents the shortest coding region of clones isolated from the library screen. SIE3-ΔRING lacks the RING domain. SymRK contains a signal peptide (SP), a Malec-like motif, a tetrapeptide Gly-Asp-Pro-Cys motif, three Leu-reach repeats (LRR), a transmembrane domain (TM), and PK. Extracellular domains without the signal peptide. B, Interaction between SIE3 and SymRK in yeast cells. Proteins were fused with the G4 DNA binding domain (BD) in pGAD7T7 or with its activation domain (AD) in pGAD7T7. Yeast cells harboring the constructs were maintained on SD/-Trp-Leu medium (SD-2) and selected for protein-protein interaction on SD/-Trp-Leu-His-Ade (SD-4). The interaction between murine tumor-suppressor p53 and simian virus 40 large T-antigen (p53/SV40) and between human lamin C (Lam) and SV40 (pGAD7T7-Lam and pGAD7T7-SV40) served as a positive control. The split CFP fragments were used in negative controls. Bars = 50 μm (Top) row, 25 μm (Bottom). C, Immunoblot analysis of proteins expressed in *N. benthamiana* leaves. Anti-ΔHA and anti-FLAG antibodies detected the HA and FLAG tags present in the SCC and SCX domains, respectively. Antitubulin antibody was used for protein loading references. Asterisk (*) demarks a nonspecific band.

**Figure 3.** Interaction of SIE3 with SymRK in planta. A, BiFC assay. *N. benthamiana* leaves were cotransfected with *A. tumefaciens* carrying constructs for expression of full-length SIE3 and SymRK that were tagged with SCX and SCC, respectively. The interaction between SIE3 and SymRK reconstituted the CFP and allowed fluorescence detection by a confocal microscope. Coexpression of *Arabidopsis* CIPK24 and CBL served as a positive control. The split CFP fragments were used in negative controls. Bars = 50 μm (Top) row, 25 μm (Bottom). B, Immunoblot analysis of proteins expressed in *N. benthamiana* leaves. Anti-ΔHA and anti-FLAG antibodies detected the HA and FLAG tags present in the SCC and SCX domains, respectively. Antitubulin antibody was used for protein loading references. C, Co-IP of SIE3 with SymRK. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells harboring pSIE3-GFP and pMyc-SymRK (Lane 1), pSIE3-GFP and pBA-myc (Lane 2), and pMyc-SymRK and pBA-myc (Lane 3). Leaf extracts were incubated first with anti-GFP antibody and then with immobilized Protein G-beads. Proteins bound to the beads were separated on SDS-PAGE and immunoblotted with anti-Myc antibody. The positions of IgG H chain, Myc-SymRK, polyubiquitinated Myc-SymRK, and protein mass markers are indicated. Asterisk (*) demarks a nonspecific band.
E2 (UBCh5b) conjugating enzyme, and Arabidopsis ubiquitin. SIE3 was converted to a mixture of high-Mr ubiquitinated protein products (Fig. 4A, lane 3), suggesting that SIE3 is capable of undergoing self-ubiquitination.

Ubiquitination of SymRK by SIE3 Ligase

Myc-SymRK and SIE3-GFP were coexpressed in N. benthamiana leaves. Anti-Myc antibody was used for Co-IP, and either anti-Ub (for ubiquitin) or anti-Myc was used for immunoblotting. When Myc-SymRK was expressed alone, Myc-SymRK and its ubiquitinated products that were likely to be generated by endogenous E3 enzymes were detected (Fig. 4B, lane 2). When SIE3 was coexpressed with SymRK, Myc-SymRK and its polyubiquitinated products were detected (Fig. 4B, lane 1). The intensity of SymRK-related bands were much stronger in the leaf cells coexpressing SymRK and SIE3 (Fig. 4B, lane 1) than those expressing SymRK alone (Fig. 4B, lane 2). These results showed that SIE3 could promote in vivo ubiquitination of SymRK. Using anti-Ub antibody, we were able to confirm that the high molecular mass smear ladders (Fig. 4B, lane 1) and the degraded Myc-SymRK (Fig. 4B lane 2) bands were indeed ubiquitinated (Fig. 4B, right).

SIE3 Gene Expression

Real-time quantitative reverse transcription (qRT)-PCR results showed that the SIE3 mRNA was present in all tested tissues of L. japonicus plants (Fig. 5A). M. loti-inoculated roots with developing nodules were found to have the highest expression level of SIE3 mRNA. The lowest expression was found in mature nodules. During the nodulation period, the expression of SIE3 was elevated 8 d after rhizobial infection and continued to stay at high levels 10 d post inoculation (Fig. 5B). These observations were consistent with the proposed role of SIE3 in rhizobial entry and nodule organogenesis (see below).

Subcellular Localization

GFP-SIE3 was expressed in transgenic onion (Allium cepa) epidermal cells by infiltration with Agrobacterium rhizogenes. Subcellular localization of GFP-SIE3 was observed using a confocal microscope. GFP alone served as a control. As expected, GFP was found in both the cytoplasm and nuclei (Fig. 5C, top). The green fluorescence of GFP-SIE3 was concentrated to the nuclei and plasma membrane, and could also be found in the cytoplasm and extracellular space in plasmolyzed cells induced by 4% (w/v) NaCl (Fig. 5C, bottom). When expressed in transgenic hairy roots of L. japonicus and transgenic N. benthamiana leaves, a strong fluorescent signal was found in the nuclei and plasma membrane, and a weak fluorescent signal was also observed in the cytoplasm (Fig. 5, D and E). Comparing the subcellular localization data (Fig. 5, C–E) with the BiFC results of interaction with SymRK (Fig. 3A; Supplemental Fig. S3), we conclude that the

Figure 4. Ubiquitination of SymRK by SIE3. A, Self-ubiquitination of SIE3 in vitro. His-SIE3 was incubated with wheat E1, human E2 (UBCh5b), and Arabidopsis ubiquitin. Reaction products were immunoblotted using anti-Ub antibody. The positions of Ub, monoubiquitinated E2 (Ub-E2) and polyubiquitinated SIE3 (PUb-SIE3) are indicated. Protein mass markers are shown in kD. B, Ubiquitination of SymRK by SIE3 in N. benthamiana leaf cells. Leaf extracts expressing pSIE3-GFP and pMyc-SymRK (Lane 1), pMyc-SymRK and pBA-myc (Lane 2), and pMyc-GFP and pBA-myc (Lane 3) were immunoprecipitated with anti-Myc antibody. The immunocomplex was absorbed to Protein G beads and resolved on SDS-PAGE. Anti-Myc and anti-Ub antibodies were used to detect Myc-tagged proteins and ubiquitinated products. Protein mass markers are shown in kD. The positions of IgG H chain, Myc-SymRK, ubiquitinated SymRK (Ub-SymRK), and degraded SymRK are indicated.
SIE3-SymRK interaction occurs at both sides of the plasma membrane, although SIE3 may have additional functions in the nuclei.

Promotion of Nodulation by SIE3 Overexpression

SIE3 was expressed under the control of the maize (Zea mays) ubiquitin promoter (SIE3OX) in transgenic hairy roots of L. japonicus (Kumagai and Kouchi, 2003; Chen et al., 2012). The phenotypes of nodulation were scored 4 weeks after inoculation with M. loti strain MAFF303099, which expresses β-galactosidase (lacZ) as a constitutive marker for the presence of rhizobial cells (Tansengco et al., 2003). Real-time qRT-PCR analysis indicated that the expression level of the SIE3 transgene was increased 10-fold in the control roots harvested 5 h after mock treatment with water. Nodule numbers per root system were increased from 7.6 in the control to 13.3 in SIE3OX hairy roots in experiment I, and from 4.0 in the control to 10.2 in SIE3OX hairy roots in experiment II (Supplemental Table S1; Supplemental Fig. S5A). These results suggest that SIE3 plays an important role as a positive regulator of nodule initiation in L. japonicus.

Suppression of Rhizobial Infection by SIE3 RNAi

An SIE3-specific RNAi construct was prepared to target a 210-bp fragment containing the 5′-untranslated region and a short sequence of the coding region. Control hairy roots were generated using cloning vector pCAMBIA1301-35S-int-T7. Transgenic hairy roots were inoculated with M. loti strain MAFF303099 for nodulation. Real-time qRT-PCR analysis showed that the SIE3 transcript was reduced to approximately 50% in RNAi hairy roots as compared with that in the control. Nodule phenotypes were scored 4 weeks after rhizobial inoculation. As shown in Figure 7A and Supplemental Table S2, nodule numbers in SIE3 RNAi hairy roots...
were significantly lower than those in the control in all three large-scale experiments. Analysis of the distribution of nodule numbers per plant revealed that 40% to 70% of SIE3 RNAi hairy roots developed only one or no nodule. In contrast, 90% of the control hairy roots produced more than six nodules (Supplemental Fig. S5B). These data suggest that SIE3 plays a positive regulator role in nodulation in *L. japonicus*, which is consistent with the conclusion drawn from the analysis of SIE3OX hairy roots.

To investigate if nodulin gene expression was affected by SIE3 RNAi, we examined the expression levels of *NIN* and *ENOD40-2*, two early nodulin genes implicated in the processes of rhizobial entrance, nodule initiation, and subsequent organogenesis (Schauer et al., 1999; Kumagai et al., 2006), as well as the expression of *Lb* (for *leghemoglobin*), a typical nodulin gene required for nitrogen fixation (Ott et al., 2005). Real-time PCR analysis showed that the expression levels of the two early nodulin genes (*NIN* and *ENOD40-2*), but not *Lb*, were reduced drastically in the SIE3 RNAi hairy roots, as compared with those in the control hairy roots (Fig. 7C; Supplemental Fig. S6). Thus, the reduction in nodulation in SIE3 RNAi hairy roots could be a result of disruption in the processes of nodule development.

**DISCUSSION**

SymRK is believed to function in both NF perception and downstream signal transduction, and is required for the earliest detectable root hair responses (Endre et al., 2002; Stracke et al., 2002). However, little is known about how SymRK transduces the NF signal to downstream components. In this report, we describe a novel E3 ubiquitin ligase, SIE3, which mediates ubiquitination of SymRK. Our results demonstrate a pivotal role of SIE3 in the early events of rhizobial infection. Taken together, we have identified an E3 ligase of SymRK that acts as a key regulator of nodule development.

**A Novel RING Finger E3 Ubiquitin Ligase**

SIE3 is related to and distinct from human RMD5 and yeast GID2. It is not closely related to any previously characterized RING finger E3 ligases, such as the cullin Ring-based ligases (CRLS) from higher plants (Petroski and Deshaies, 2005; Deshaies and Joazeiro, 2009). It defines a novel class of RING domain E3 ligases, and will be referred to hereafter as the SIE3-related ligases. First, this family of ligases contains a characteristic arrangement of three conserved domains,
the CTLH, CRA, and a degenerate RING finger. Although the function of the CTLH motif remains unknown, the CRA and RING domains have been implicated in protein-protein interactions. Yeast GID2 E3 ligase does not contain the CRA domain. Second, SIE3-related ligases have a characteristic pattern of substitutions in the RING domain, where Cys residues at positions 2 and 6 are replaced with Ser or Thr (S/T), resulting in the coordination of only one zinc ion. This type of RING belongs to the RING-S/T variant and consists of C-x_2-S/L-x_1-C-x-H-x_2-C-x_2-S/T-x_13-C-x_2-C, where x could be any amino acid. In contrast, the canonical C_3HC_4 RING (C-x_2-C-x_3-C-x_13-H-x_2-C-x_2-C-x_4-C-x_7-C) is capable of coordinating with two zinc ions (Lorick et al., 1999; Fang and Weissman, 2004; Deshaies and Joazeiro, 2009). Human RMD5 has an even more degenerated RING finger, which may not bind zinc at all and may be involved only in protein-protein interactions (Santt et al., 2008). Third, SIE3-related ligases are conserved in plants (Fig. 1B;
Ubiquitination of SymRK by SIE3

In the presence of E1, E2, and ubiquitin, SIE3 exhibited ligase activity for self-ubiquitination in vitro (Fig. 4A). When expressed in plant cells, the level of ubiquitination of SymRK was increased by coexpression of SIE3 (Fig. 4B). There are two main modes of action by which an E3 could exert an effect at the cellular level through interaction with an RLK. First, an E3 could mediate the degradation of an RLK through ubiquitination. Second, the RLK could modulate, via phosphorylation, the enzyme activity of the E3, which in turn regulates the stability of downstream component(s). Although plant E3 ligases have previously been shown to interact with RLKs (Gu et al., 1998; Kim et al., 2003; Wang et al., 2006; Samuel et al., 2008; Mbengue et al., 2010), these E3 ligases belong to the Ankyrin motif-containing proteins and the plant U-box (PUB) enzymes, which differ in protein structure from SIE3-related ligases, and their direct role in RLK ubiquitination has not been shown. In rice (Oryza sativa), the kinase domain of XA21 binds and phosphorylates the E3 ligase XB3 (Wang et al., 2006). In Arabidopsis, the kinase domain of the S-locus receptor kinase bins and phosphorylates the E3 ligase ARC1 (Samuel et al., 2008). Similarly in M. truncatula, the kinase domain of Nod factor receptor LYK3 phosphorylates the E3 ligase PUB1 (Mbengue et al., 2010). Because there has not been evidence for direct ubiquitination of RLKs by these E3 ligases, the possible mode of action for these E3 ligases is the second one; The activity of these E3 ligases is modulated by RLKs, and they then regulate downstream components. For SIE3, both modes of action could apply. Our observation clearly shows that SIE3 could mediate ubiquitination of SymRK. However, whether the SIE3 activity is modulated by SymRK and whether SIE3 regulates the stability of downstream components remains to be investigated.

A Positive Regulator of Nodulation

SymRK is the checkpoint of bacterial entry and essential for nodulation (Stracke et al., 2002; Radutoiu et al., 2003; Yoshida and Parniske, 2005). SIE3 could exert its biological function through ubiquitination of SymRK, and it is possible that ubiquitinated SymRK may allow sustained signal transduction to downstream host responses. In SIE3OX hairy roots, the elevated SIE3 level promoted nodule formation (Supplemental Table S1, Fig. 6). Consistent with this finding, fewer ITs and nodules were developed in hairy roots expressing SIE3RNAi (Supplemental Table S2; Fig. 7, A, B, and D). These data support the notion that both SIE3 and SymRK serve as positive regulators of NF signaling, rhizobial infection, and nodulation. Ubiquitination is a posttranslational modification specific to eukaryotes and has been recognized as a major means in regulation of protein stability. Recently, more and more nonproteolytic functions of ubiquitination have been discovered. It can regulate the transcription activity of transcription factors (Herrera and Triezenberg, 2004), and modulate the enzyme activity and protein subcellular localization (Chen and Sun, 2009; Komander, 2009; Yang et al., 2010). We propose that SIE3 may exert its positive effect on symbiotic signaling via ubiquitination of SymRK. There are at least two possible mechanisms. First, SIE3 may promote SymRK signaling through activating its protein kinase activity. Second, ubiquitination of SymRK by SIE3 may help maintain the protein conformation in the membrane, which could be useful for its biological function. However, the exact mechanism by which SIE3 regulates SymRK remains to be further investigated. SIE3 represents a new class of RING-domain E3 ligases from higher plants. This work has opened a new avenue for future research on ubiquitination and receptor kinases in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of wild-type Lotus japonicus 'MG-20' were surface-sterilized and germinated on agar plates. Seedlings grown in sterilized conditions were used for generation of transgenic hairy roots via Agrobacterium rhizogenes. Wild-type Nicotiana benthamiana plants were grown in a growth chamber at 22°C and 70% relative humidity under a 16-h-light/8-h-dark photoperiod for about 4 weeks before infiltration with Agrobacterium spp. cells. After infiltration, plants were kept under the same growth conditions.

Yeast Two-Hybrid Screen

Screening of interaction clones was performed as described previously (Zhu et al., 2008). A total of 105 transformants were screened for protein-protein interaction on synthetic defined medium SD-/Leu-Trp-His-Ade. Plasmids from positive clones were verified for interaction by retransformation into yeast (Saccharomyces cerevisiae) AH109 cells containing pGBK7/SymRK-EC. Positive colonies were also tested for expression of the lacZ reporter (Chen et al., 2012).

Plasmid Construction for Protein-Protein Interactions in Yeast

Full-length cDNA of SIE3 and SIE3RING cDNAs were amplified by PCR and inserted into NdeI/EcoRI sites of pGBK7 and pGAD77; The SymRK-PK and SymRK/Ring cDNAs were amplified by PCR and inserted into NdeI/EcoRI sites of pGBK7 and pGAD77; NFR5-PK cDNAs were amplified by PCR and inserted into EcoRI/XhoI of pGAD77 and EcoRI/Sall of pGBK7; NFR1-PK cDNAs were amplified by PCR and inserted into EcoRI/Sall of pGBK7; LHK1-PK cDNAs were amplified by PCR and inserted into EcoRI/Sall of
pGBK7T; and LH1K-EC cDNAs were amplified by PCR and inserted into BamHI/SalI of pGBK7T. Sequences of primers are provided in Supplemental Table S4.

Real-Time PCR

RNA samples were treated with DNase I (Promega, Tokyo, Japan) and reverse-transcribed using SYBR PrimeScript RT-PCR kit II (Takara) with oligo (dT) as primer. The cDNA from RT of about 300 ng of RNA was used as template for real-time PCR using a Mini Opticon real-time PCR system (LightCycler 480). Primer sets used for real-time PCR were listed in Table 4. Cycling conditions were as follows: preheating at 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 12 s, followed by 72°C for 5 s. Poly-ubiquitin (ATW720576) transcript was used as an internal control. The identity of amplified PCR products was verified by DNA sequencing. Gene expression data were normalized on the basis that the expression levels of ubiquitin were assumed to be the same in all RNA samples.

BiFC Experiments

Full-length cDNA of SymRK, SymRK-EC, and SymRK-PK were cloned into the SpeI/SalI site of pSCYCE-R vector to obtain SymRK-SCC, SymRK-EC-SCC, and SymRK-PK-SCC fusions (Waadt et al., 2008). Full-length cDNA of SIE3 was cloned into the BamHI/XhoI site of pSCYNE vector to obtain SIE3-SCC fusion. Plasmids were introduced into Agrobacterium tumefaciens strain GV3101 cells by electroporation. A. tumefaciens cells containing plasmids were pelleted and resuspended in the infiltration buffer (10 mM MES, pH 5.7, 10 mM MgCl₂, 150 μM acetylserine). A. tumefaciens strains containing different plasmids were mixed to a final optical density at 600 nm of 0.5 for each A. tumefaciens strain. The mixture of A. tumefaciens strains was incubated on bench for 2 to 4 h at room temperature and used for infiltration into the top leaves of 4- to 6-week-old N. benthamiana plants using a 2-ml syringe. Cyan fluorescence was assayed 2 to 5 d after infiltration with A. tumefaciens using a Zeiss LSM510 laser scanning microscope with a CFP filter set (excitation wavelength at 405 nm, emission wavelength at 477 nm). Cyan fluorescence of leaf cells expressing the two proteins (SIE3-SCC and SymRK-EC-SCC or SymRK-PK-SCC) were observed using a confocal microscope (Olympus BX61WI).

Generation of Transgenic Hairy Roots

Wild-type L. japonicus 'MG-20' was used to produce transgenic hairy roots using an A. rhizogenes-mediated procedure as described previously (Kumagai and Kouchi, 2003; Chen et al., 2012; Ke et al., 2012). Briefly, seedlings germinated on sterile agar plates were cut at the base of the hypocotyls and placed onto agar plates containing 250 μM acetosyringone. After 3 to 4 h growth at 28°C, surfactant Silwet L-77 was added at a final concentration of 0.01% (v/v). Onion bulb slices (1.0 cm²) were surface-sterilized in 75% (v/v) ethanol for 1 min and washed with 1× MS liquid medium, followed by treatment with 2% (v/v) sodium hypochlorite for 2 min. After four times of washing with 1× MS liquid medium, the onion slices were treated with 4 μM NaCl for 20 min, and then incubated with the A. rhizogenes cell culture in 0.01% (v/v) Silwet L-77 for 90 min. After removal of the surface liquid, onion slices were placed onto MS medium-agar plates and grown at 26°C for 36 to 48 h in the dark. GFP signals were observed using confocal microscope Zeiss LSM510.

Overexpression of SIE3

The full-length coding sequence of SIE3 was cloned into the NptI/BamHI site of pU1301, generating pMuB/SIE3. A. rhizogenes strain LBA1334 cells carrying pMuB/SIE3 were used to induce hairy root formation in L. japonicus. Nodulation phenotypes of transgenic hairy roots were scored 4 weeks for inoculation with M. loti. Transgenic hairy roots expressing the empty vector (pU1301) were used as a control.

SIE3-Specific RNAi

A 210-bp fragment of the 5' region of SIE3 cDNA containing a short sequence of the coding region was amplified by PCR and cloned into pCAMBIA3001-35S-int-T7, generating pSIE3-RNAi in which the sense and antisense SIE3 RNA sequences would be linked in tandem separated by the Arabidopsis (Arabidopsis thaliana) Actin11 intron. This SIE3-specific intron-hairpin RNA was expressed under the control of the cauliflower mosaic virus 35S promoter. A. rhizogenes LBA1334 cells harboring pSIE3-RNAi was used to induce formation of transgenic hairy roots in L. japonicus. After inoculation with M. loti MAF303099, plants with transgenic hairy roots were grown for 4 weeks and nodulation phenotypes were scored.

Protein Extraction and Immunoblot Analysis

Proteins were extracted from N. benthamiana leaves expressing recombinant proteins using two protein extraction buffers as described in Liu et al., 2010. The denaturing buffer contained 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 4 mM urea, and 1 mM phenylmethylsulfonyl fluoride or protease inhibitor cocktail CompleteMini tablets (Roche). The native extraction buffer 1 contained 50 mM Tris-MES, pH 8.0, 0.5 mM Suc, 1 mM MgCl₂, 10 mM EDTA, 5 mM dithiothreitol, and protease inhibitor cocktail CompleteMini tablets (Roche). The extracts were used for immunoblot analysis and immunoprecipitation. Proteins prepared using the denaturing buffer was subjected to western-blot analysis using the Milepore Chemiluminescent HRP Substrate kit (Milestone). The sources and dilutions of antibodies used in these experiments were as follows: anti-HA antibody (Santa Cruz, 1:500), anti-Myc antibody (Santa Cruz, 1:500), anti-GFP antibody (Clontech, 1:1,000), antiubiquitin monoclonal antibody (obtained from the Xie laboratory, Chinese Academy of Sciences, 1:5,000), and goat antimouse horseradish peroxidase-conjugated antibody (Proteintech, 1:2,500).

Immunoprecipitation

SymRK-Myc protein and the protein coexpressed SymRK-myc and SIE3-GFP extracted using the denaturation extraction buffer and SIE3-GFP protein extracted using the native extraction buffer were used for immunoprecipitation. Corresponding antibodies were added to the extract (10 μg/ml proteins). MG132 was also added at a final concentration of 50 μM to prevent protein degradation. The mixtures were kept at 4°C with gentle shaking for 3 h to overnight. Protein G agarose beads (Milestone) was added to the mixture at 20 μg/ml in order to immobilize the immunocomplex. After gentle shaking at 4°C for 3 h, the agarose beads were recovered by centrifugation at 4,000 rpm for 1 min and washed three times with cold phosphate-buffered saline.
Ubiquitination Assay

For in vitro E3 ubiquitin ligase activity assays, purified His-tagged SIE3 was incubated with wheat (Triticum aestivum) E1 (GenBank ID GI:136632, human (Homo sapiens) E2 (UBCh5b), and Arabidopsis ubiquitin (UBQ14) as described elsewhere (Liu et al., 2010). Reactions were carried out with agitation for 1.5 h at 30°C. Proteins were resolved on SDS-PAGE, followed by immunoblot analysis using anti-ubiquitin monoclonal antibody.

To test if SymRK could serve as a substrate for ubiquitination by SIE3, we expressed Myc-tagged SymRK and GFP-tagged SIE3 in leaves of N. benthamiana plants via infiltration with A. tumefaciens strain EHA105. We performed ubiquitination assays using SymRK as a substrate in vivo systems. For in vivo ubiquitination assays, we transfected plants with pMyc-SymRK alone, or a combination of pMyc-SymRK and pSIE3-GFP. We used pMyc-GFP to replace pMyc-SymRK in the control. Protein extracts from transfected N. benthamiana leaves were immunoprecipitated with anti-Myc antibody. Western blot analysis was carried out using anti-Myc antibody and anti-ubiquitin monoclonal antibody.

Rhizobial Infection Assay

For rhizobial infection assay, transgenic hairy roots were inoculated with M. loti strain MAFF303099, which harbored the lacZ gene as a constitutive marker (Tansengco et al., 2003), and was grown in pots containing sand and vermiculite at a 1:1 volume ratio. Seven days after rhizobial inoculation, transgenic hairy roots were stained for lacZ activity (Tansengco et al., 2003), and was grown in pots containing sand and vermiculite.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Amino acid sequence alignment of SIE3-like ubiquitin ligases from plants.

Supplemental Figure S2. Protein structure and dimerization of L. japonicus SIE3.

Supplemental Figure S3. BiFC assay of interaction of SIE3 with the extra-cellular or intracellular portion of SymRK.

Supplemental Figure S4. Time course of protein expression in N. benthamiana leaves.

Supplemental Figure S5. Nodulation phenotypes of transgenic hairy roots with altered SIE3 transcript levels.

Supplemental Figure S6. Real-time qRT-PCR analysis of gene expression in SIE3 RNAi transgenic hairy roots.

Supplemental Figure S7. Classification of infection threads in transgenic hairy roots.

Supplemental Table S1. Nodulation phenotypes of SIE3 RNAi hairy roots.

Supplemental Table S2. Nodulation phenotypes of SIE3 RNAi hairy roots.

Supplemental Table S3. Phenotypes of rhizobial infection in SIE3 RNAi hairy roots.

Supplemental Table S4. Primers used in this study.

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LITERATURE CITED


A Ubiquitin Ligase of Symbiosis Receptor Kinase

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