

A Novel ARID DNA-Binding Protein Interacts with SymRK and Is Expressed during Early Nodule Development in *Lotus japonicus*¹[C][W][OA]

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During the establishment of symbiosis in legume roots, the rhizobial Nod factor signal is perceived by the host cells via receptor-like kinases, including SymRK. The *NODULE INCEPTION* (*NIN*) gene in *Lotus japonicus* is required for rhizobial entry into root cells and for nodule organogenesis. We describe here a novel DNA-binding protein from *L. japonicus*, referred to as SIP1, because it was identified as a SymRK-interacting protein. SIP1 contains a conserved AT-rich interaction domain (ARID) and represents a unique member of the ARID-containing proteins in plants. The C terminus of SIP1 was found to be responsible for its interaction with the kinase domain of SymRK and for homodimerization in the absence of DNA. SIP1 specifically binds to the promoter of *LjNIN* but not to that of *LjCBP1* (a calcium-binding protein gene), both of which are known to be inducible by Nod factors. SIP1 recognizes two of the three AT-rich domains present in the *NIN* gene promoter. Deletion of one of the AT-rich domains at the *NIN* promoter diminishes the binding of SIP1 to the *NIN* promoter. The protein is localized to the nuclei when expressed as a red fluorescence fusion protein in the onion (*Allium cepa*) epidermal cells. The *SIP1* gene is expressed constitutively in the uninfected roots, and its expression levels are elevated after infection by *Mesorhizobium loti*. It is proposed that SIP1 may be required for the expression of *NIN* and involved in the initial communications between the rhizobia and the host root cells.

Legume plants are capable of acquiring nitrogen from rhizobacteria maintained symbiotically in specialized root organ nodules that form through a complex developmental process involving an exchange of signals between the host root cells and the bacteria. At the beginning of nodule organogenesis, specific flavonoid metabolites released by the legume roots notify soil rhizobia that a suitable host is nearby. Inside the bacteria, the flavonoids are recognized by a receptor protein known as NodD. The binding of NodD to these flavonoids activates the protein and promotes the transcription of other *nod* genes involved in the synthesis and secretion of Nod factors, the rhizobial signaling molecules (Peck et al., 2006). The common feature of all Nod factors is the presence of a chitin

backbone and a fatty acyl tail (Denarie et al., 1996). Rhizobial Nod factors, in turn, are capable of inducing a series of specific responses in the host root cells, including root hair deformation, alkalization of the cytosol, depolarization of the plasma membrane, and calcium influx and spiking (Ehrhardt et al., 1992, 1996; Kurkdjian, 1995; Felle et al., 1995, 1996; Limpens and Bisseling, 2003; Gleason et al., 2006). Root hair deformation and curling are the early morphological changes induced by rhizobia. Purified Nod factors can cause root hair deformation at concentrations as low as 10^{-12} M (Lerouge et al., 1990; Spaink et al., 1991; Sanjuan et al., 1992; Margaert et al., 1993), although in most cases curling is only observed when the bacteria are present (Relic et al., 1993).

The perception and signal transduction of Nod factors in the host cells have been subject to intense molecular and genetic studies in recent years. Rhizobial Nod factors have been shown to be recognized by lysin motif (LysM)-containing receptor-like kinases (RLKs), such as NFR1/NFR5 from *Lotus japonicus* and LYK3 and NFP from *Medicago truncatula* (Madsen et al., 2003; Radutoiu et al., 2003; Limpens et al., 2003; Arrighi et al., 2006). The LysM RLKs are localized to the plasma membrane with the LysM domain protruding to the extracellular space and the kinase domain facing the cytoplasm. The LysM domain interacts with the lipochitin-oligosaccharide backbone of Nod factors, resulting in Nod factor recognition (Steen et al., 2003, 2005; Radutoiu et al., 2007). Using forward genetics and map-based cloning approaches, a series

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of host genes involved in the perception of the Nod factor signals, including *SymRK* (symbiosis RLK), *Castor*, *Pollux*, *Nup133*, *Nup85*, *CCaMK*, and *Cyclops*, have been characterized from the legume *L. japonicus* (Kistner et al., 2005). Mutations in any of these genes result in defects in nodule initiation. Downstream of this pathway, putative transcription factors encoded by *LjNIN*, *LjNSP1*, and *LjNSP2* (Schauser et al., 1999; Heckmann et al., 2006) are also required to initiate nodule organogenesis.

SymRK from *L. japonicus* encodes a protein with three Leu-rich repeat (LRR) domains in the predicted extracellular region. Mutations in the *SymRK* gene abolish the host interaction with rhizobia (Stracke et al., 2002). Its orthologs, *NORK*, *DML2*, and *SYM19*, from *Medicago sativa*, *M. truncatula*, and *Pisum sativum*, respectively, have been cloned (Endre et al., 2002; Stracke et al., 2002). The LRR-RKs represent the largest group of receptor kinases in plants, comprising approximately one-half of the predicted receptor kinases in *Arabidopsis* (*Arabidopsis thaliana*; Shiu et al., 2004). LRR-RKs have been implicated in diverse plant signaling pathways, including the perception of pathogen signals, brassinosteroid hormones, and the CLAVATA peptide hormone (Oldroyd and Dowine, 2004). It has been proposed that the three LRRs in *SymRK* may be involved in protein-protein interactions, protein-ligand interactions, or autophosphorylation-regulated kinase activation (Yoshida and Parniske, 2005). The observation that the *symRK/nork* mutant fails to form arbuscular mycorrhiza suggests that *SymRK/NORK* plays a role in the exchange of signals with both symbiotic bacteria and fungi. Although *SymRK* is known to be essential in the Nod signaling pathway in *L. japonicus*, the biochemical mechanism leading to Nod factor-induced transcriptional activation is obscure. Moreover, how the kinase activity of *SymRK* is regulated remains to be determined. Recently, a *NORK*-interacting partner in *M. truncatula* has been identified as 3-hydroxy-3-methylglutaryl-CoA reductase 1, which may link the Nod signaling pathway with the formation of isoprenoid-derived phytohormones (Kevei et al., 2007).

In this study, we demonstrate that a novel protein, designated as SIP1 for *SymRK*-interacting protein 1, interacts with the kinase domain of *SymRK*. SIP1 is a transcription factor containing an AT-rich interaction domain (ARID) and may participate in Nod factor-induced transcriptional activation of genes required for nodule initiation.

RESULTS

Characterization of a *SymRK*-Associated Protein

The *SymRK* peptide contains three LRRs, a transmembrane domain, and an intracellular kinase domain. The structure features suggest potential roles of *SymRK* in the perception of extracellular signals

and transduction of the signals through the intracellular kinase domain (Stracke et al., 2002). In an attempt to identify *SymRK*-associated proteins, we used the kinase domain of *SymRK* as a bait of the yeast two-hybrid system and screened a *Lotus* cDNA library constructed in the prey vector pGADT7-Rec2. Approximately five million yeast *Saccharomyces cerevisiae* colonies expressing the cDNA library were assayed for their ability to grow on selective synthetic dextrose medium (SD/-Leu-Trp-His-Ade). The bait plasmids were isolated from positive colonies and reintroduced back to yeast cells containing the prey plasmid. Colonies that failed to grow in the second round of testing were considered as false positives. After eliminating false positives, several clones were identified as potential interaction partners of *SymRK*-protein kinase (PK). One positive cDNA was isolated from three independent yeast colonies. It encoded a novel protein designated SIP1 (Fig. 1A; Supplemental Fig. S1).

The full-length *SIP1* cDNA (GenBank accession no. EU559710) contained an open reading frame of 1,224 nucleotides encoding a peptide of 408 amino acid residues with a predicted molecular mass of 45.7 kD. Analysis of the peptide sequence revealed the presence of a conserved ARID (Fig. 1B) that has been implicated in sequence-specific DNA binding (Gregory et al., 1996). ARID-containing proteins appear to play important roles in diverse biological functions, including cell proliferation and differentiation, and organ development (Herrscher et al., 1995; Gregory et al., 1996; Kortschak et al., 2000). The mouse BRIGHT (B-cell regulator of immunoglobulin H transcription) protein and the fruit fly DRI (*Drosophila* dead ringer) are two well-characterized ARID-containing transcription factors (Herrscher et al., 1995; Gregory et al., 1996; Valentine et al., 1998; Iwahara et al., 2002; Wilsker et al., 2005). The predicted three-dimensional model of the ARID domain of SIP1 consists of eight α -helices, two β -strands, and four structure-undefined loops (Fig. 1B). The structural features of this 91-residue motif were identical to those found in animal ARID-containing proteins such as DRI (Fig. 1B; Iwahara and Clubb, 1999) and indicated that SIP1 might bind to the AT-rich region of plant promoters.

ARID-containing proteins are widely present in plant genomes. Ten such proteins have been found in *Arabidopsis* (Fig. 1C) and can be grouped into four subfamilies, designated high-mobility group (HMG), EGL-27 and MTA1 homology 2 (ELM2), plant homeodomain (PHD), and heat stress protein 20-like (Hsp20)-related proteins (Fig. 1D). Animal proteins containing HMG and ELM2 motifs have been implicated in DNA binding and transcription regulation (Ding et al., 2003; Stros et al., 2007). PHD-containing proteins are known to be involved in binding to methylated histone H3 (Ramon-Maiques et al., 2007). Hsp20-like proteins, also known as α -crystallin domain-containing proteins, are believed to function in protecting other proteins from denaturation by heat

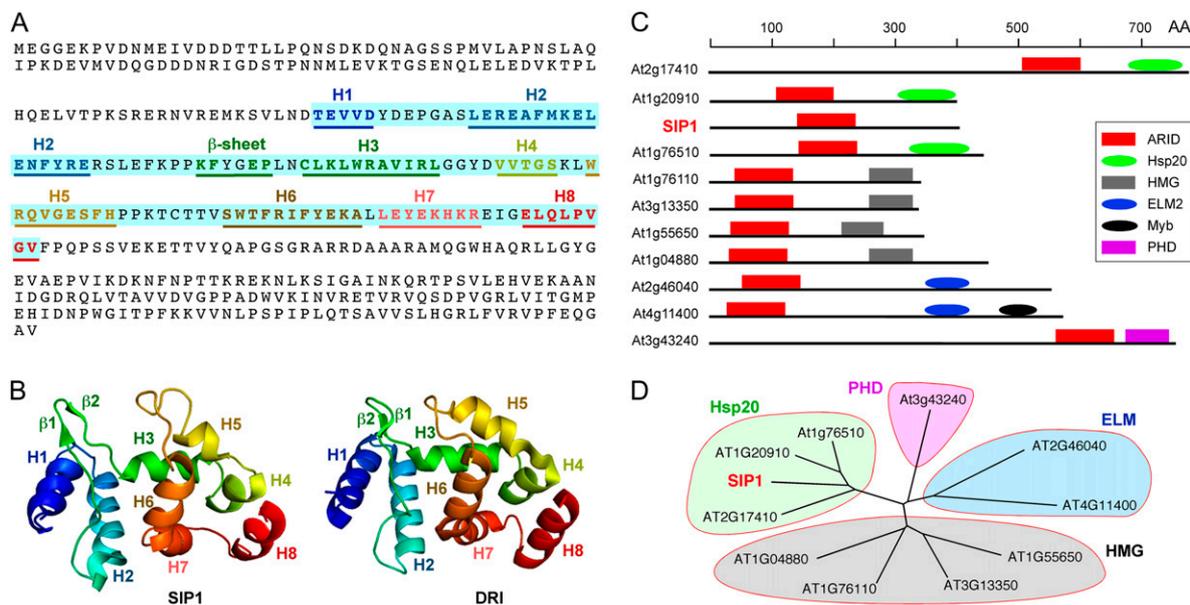


Figure 1. SIP1 is a novel member of the ARID family of DNA-binding proteins in plants. A, The deduced amino acid sequence of SIP1 contains 408 amino acid residues with a calculated molecular mass of 45.7 kD. The ARID region is shaded in light blue. The conserved α -helices (H1–H8) and β -sheet (β 1 and β 2 strands) are underlined. The colors of the residues in these regions match those shown in B. B, Comparison of the ribbon models of ARIDs present in plant SIP1 (residues 126–217) and animal DRI (residues 265–388; Iwahara and Clubb, 1999) proteins shows that both are able to form eight α -helices (H1–H8) and two β -strands (β 1 and β 2). The models were produced using PyMOL (v0.99) software (<http://www.delsci.com/rel/099/>). C, Schematic representation of functional motifs present in SIP1 and its homologs in Arabidopsis. Contrary to the *Lotus* SIP1, all Arabidopsis homologs contain other motifs in addition to ARID, suggesting that SIP1 may have a legume-specific function. References: Hsp20, Scharf et al. (2001); HMG (domain implicated in DNA binding), Stros et al. (2007); ELM2 (domain implicated in transcription regulation), Ding et al. (2003); PHD (involved in binding modules of methylated histone H3), Ramon-Maiques et al. (2007). Myb, Myb DNA-binding domain. D, Phylogenetic tree of SIP1 and its Arabidopsis homologs. SIP1 may have evolved from one of the two ARID subfamilies containing Hsp20-like domains.

(Scharf et al., 2001). All of these proteins contain at least another motif in addition to ARID. However, there was no other recognizable motif except ARID in the *Lotus* SIP1. Phylogenetic tree analysis (Fig. 1D) revealed that SIP1 is closely related to the Hsp20 subfamily of ARID proteins in Arabidopsis. The Hsp20 subfamily is characterized by the presence of an Hsp20-like domain in the C terminus of the protein. The C terminus of SIP1 does not contain the Hsp20-like domain, but has instead evolved to perform a new function, i.e. interaction with SymRK (see below). Taken together, our data suggest that SIP1 may have evolved from the Hsp20 subfamily of ARID-containing proteins during evolution and acquired the ability to act as a transcriptional regulator involved in the Nod factor signaling in *L. japonicus*.

Interaction between SymRK and SIP1

To determine which domain of SIP1 is responsible for its interaction with SymRK-PK, we constructed a series of SIP1 deletions in pGADT7 (Fig. 2A). The N-terminal half of SIP1 (SIP1N) containing the N terminus and the ARID domain was not found to interact with SymRK-PK. The ARID domain alone, SIP1A, also did not interact with SymRK-PK. However, yeast

colonies expressing the C terminus of SIP1, SIP1C, were able to grow on the selection medium lacking His (SD/-Leu-Trp-His) and exhibited significant β -galactosidase activities (Fig. 2C), suggesting that the C-terminal 184 amino acid residues of SIP1 are critical for its interaction with SymRK. This interaction was further confirmed using an in vitro protein-protein interaction assay (Fig. 2D). For this assay, SIP1 and its deletion fragments were expressed as His- or chitin-binding domain (CBD)-tagged recombinant proteins and immobilized to nickel beads or chitin beads. After incubation of the beads with purified SymRK-PK, followed by washing with buffer, proteins retained to the beads were eluted in SDS sample buffer and resolved on SDS-PAGE. The presence of SymRK on the beads was detected by immunoblotting with the anti-SymRK antibody. As shown in Figure 2D, only the full-length SIP1 and SIP1C could pull down SymRK-PK, confirming that the C terminus of SIP1 was responsible for its interaction with SymRK.

SIP1 Binds AT-Rich Double-Stranded DNA

The ARID motif of SIP1 contains a noncanonical helix-turn-helix motif (helices H5 and H6) for potential DNA binding. To test this DNA-binding activity,

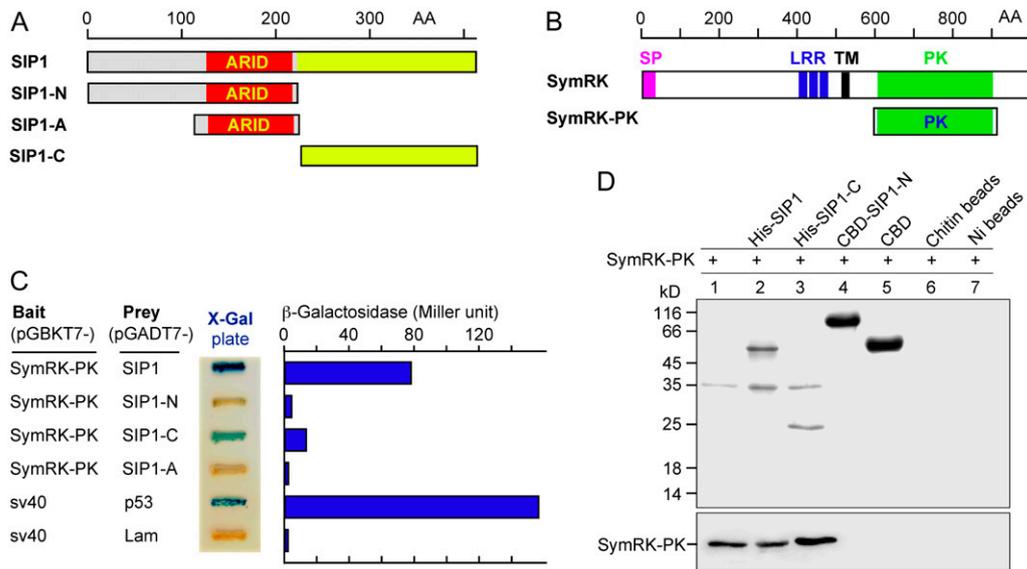


Figure 2. Interaction of SIP1 with SymRK. A and B, Schematic illustrations of SIP1 (A) and SymRK deletions (B). SP, Signal peptide sequence; TM, transmembrane domain. C, Yeast two-hybrid assays of the interactions of SymRK-PK with SIP1 and its deletions. SymRK-PK was expressed as a fusion protein with the GAL4 DNA-binding domain in the bait vector (pGBKT7). SIP1 and its deletions were expressed from the prey vector (pGADT7). Yeast AH109 cells harboring the two plasmids were streaked on the SD/-Leu-Trp plate containing X-Gal (80 μ g/mL; X-Gal plate) and were cultured in SD/-Leu-Trp broth for β -galactosidase activity assays. The combination of pGBKT7-53/pGADT7-SV40 was used as a positive control and pGBKT7-Lam/pGADT7-SV40 as a negative control (CLONTECH). D, In vitro pull-down assay for the interaction between SymRK-PK and SIP1. His-tagged SIP1 bound to nickel beads was mixed with purified SymRK-PK protein. After washing, the proteins pulled down by the nickel beads were separated on SDS-PAGE (top). A similar gel was used for western blot using anti-SymRK antibodies (bottom). [See online article for color version of this figure.]

we purified His-tagged SIP1 and performed electrophoretic mobility shift assays (EMSA) with a 32 P-end-labeled double-stranded DNA trimer of NP₃ or TTA₉, the consensus binding sites of the *Drosophila* home-domain protein Engrailed (Gregory et al., 1996). The results showed that SIP1 bound both NP₃ and TTA₉ (Fig. 3A) and could be competed out by 10-fold excess of unlabeled NP₃ oligonucleotide trimer (Fig. 3A, lane 5).

We further examined whether SIP1 contains a transcription activation domain (AD). We constructed plasmids that would express the GAL4 DNA-binding domain fused with SIP1 or its deletion fragments. The plasmids were transferred into the yeast strain AH109 that expressed *ADE2*, *HIS3*, *lacZ*, and *MEL1* reporter constructs under GAL4-responsive promoters (CLONTECH). If SIP1 contained an AD, the SIP1 fusion protein should bind to the GAL4-responsive promoters and drive the expression of the *ADE2*, *HIS3*, *lacZ*, and *MEL1* reporters. A known transcription activator, NSP1 (Smit et al., 2005; Heckmann et al., 2006), was used as a positive control and proved able to allow yeast colonies to grow on SD/-Trp-His and SD/-Trp-Ade media (Fig. 3B). However, as shown in Figure 3B, colonies expressing SIP1 and its deletion fragments could not grow on these media, indicating that SIP1 did not contain a transcription AD (Fig. 3B). It would be interesting to test if SIP1 forms hetero-

oligomers with other proteins that contain an activator or repressor domain.

SIP1 Binds Specifically to the *NIN* Promoter

The expression of the *NIN* (nodule inception) and *CBP1* (calcium-binding protein 1) genes is induced by Nod factors (Schauser et al., 1999; Webb et al., 2000; Radutoiu et al., 2003). To test if SIP1 plays a role in the induction of *NIN* and *CBP1* by Nod factors, we cloned the promoter regions of both genes and assayed for their affinity with SIP1 in the yeast one-hybrid system. The 500-bp *NIN* promoter (Borisov et al., 2003) and the 482-bp *CBP1* promoter (Webb et al., 2000) were inserted in front of the *HIS3* reporter, generating *NIN*_{pro}::*HIS3* and *CBP1*_{pro}::*HIS3* constructs. SIP1 was expressed as a fusion protein with the GAL4 AD. This plasmid (pGAD-SIP1), along with one of the reporter constructs, was cotransformed into yeast Y187 cells. If SIP1 could bind to the promoter, the GAL4 AD would drive the expression of the *HIS3* reporter, and the yeast cells would grow on SD/-Trp-Leu-His plates supplemented with 3-amino-1,2,4-triazole (3-AT). The results showed that the cotransformants of pGAD-SIP1 and *NIN*_{pro}::*HIS3* (Fig. 4C, colony 3) were indeed able to grow on the selective plates, whereas the cotransformants of pGAD-SIP1 and *CBP1*_{pro}::*HIS3* (Fig. 4C, colony 5) did not grow, indicating that SIP1

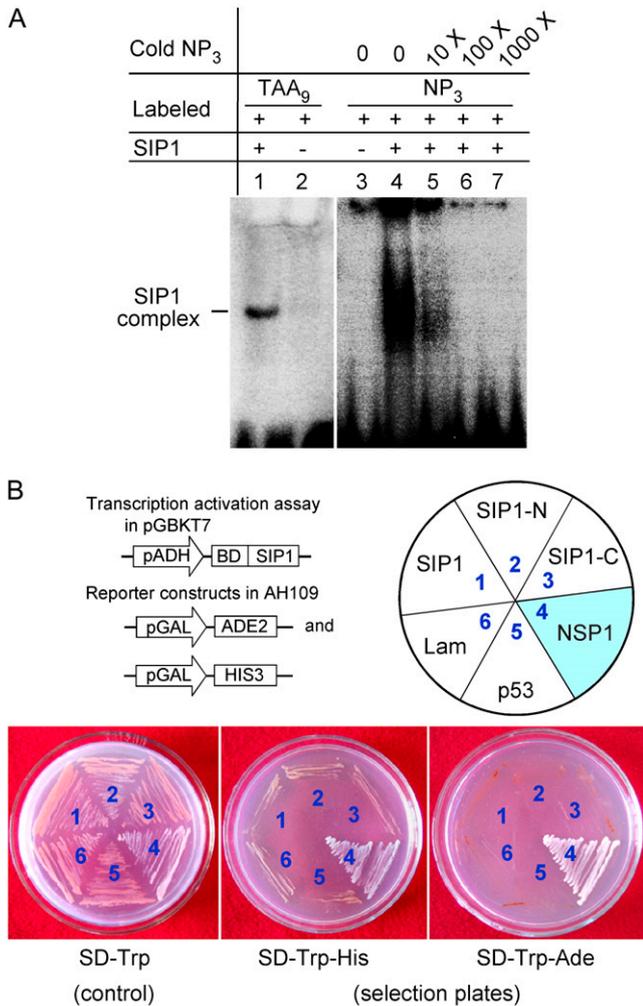


Figure 3. DNA binding of SIP1. A, EMSA was performed using purified SIP1 and ³²P-end-labeled oligonucleotide probes (TTA₉ and NP₃). NP₃: (TCAATTAATGA)₃; (TTA)₉: (TTATTATTA)₃. B, Transcription activation assay of SIP1 was performed in the yeast strain AH109, which contains the *HIS3* and *ADE2* reporter genes under distinct GAL4-responsive promoter elements. Plasmids each expressing a fusion protein of the GAL4 DNA-binding domain (BD) with SIP1 or its deletion fragments were transformed into AH109. The yeast cells were streaked on SD/-Trp (control medium), SD/-Trp-His, and SD/-Trp-Ade (selection media). Yeast cells harboring pGBKT7-lam or pGBKT7-p53 (CLONTECH) served as negative controls, whereas NSP1 (Smit et al., 2005) was used as a positive control. [See online article for color version of this figure.]

specifically binds to the *NIN* promoter but not the *CBP1* promoter.

A search of the promoter region (about 4 kb upstream of the first ATG) of the *NIN* gene identified three potential AT-rich motifs, located in the regions from -2,299 to -2,287, from -393 to -367, and from -69 to -59 bp, respectively (Borisov et al., 2003; Fig. 4A). To determine whether SIP1 binds to these sequences in vitro, we synthesized three oligonucleotides corresponding to Oligo1 to Oligo3 (Fig. 4A) and performed EMSA assays using purified SIP1. As shown

in Figure 4B, SIP1 specifically recognized and bound to Oligo1 and Oligo3 but not Oligo2.

Because the 500-bp *NIN* promoter fragment used in the yeast one-hybrid assay (Fig. 4A) did not contain Oligo1 (Fig. 4C), we hypothesized that Oligo3 should be the AT-rich site for SIP1 binding. We further reasoned that deletion of the Oligo3 site from the *NIN* promoter should diminish the ability of the yeast cells to grow on SD-His+3-AT medium. We removed a 70-bp fragment (-1 to -69 bp) from the *NIN* promoter and showed that the Δ *NIN* promoter lost the binding site for SIP1 and yeast cells were no longer able to grow on the selection medium (Fig. 4C, colony 4). Taken together, our data demonstrate that SIP1 specifically recognizes the Oligo3 AT-rich site of the *NIN* promoter and may be required for the Nod factor-induced *NIN* gene expression.

SIP1 Dimerization

The mouse BRIGHT protein forms a tetramer that binds to DNA (Herrscher et al., 1995) but exists as a dimer in the absence of DNA (Nixon et al., 2004). To determine whether SIP1 dimerizes in the absence of DNA, we coexpressed two SIP1 fusion proteins, one with the GAL4 DNA-binding domain (SIP1-BD) and the other with the GAL4 AD (SIP1-AD) in the yeast two-hybrid system. The N and C termini and the ARID domain of SIP1 were also fused with the GAL4 AD, generating SIP1N-AD, SIP1C-AD, and SIP1A-AD. As shown in Figure 5A, full-length SIP1-AD interacted with SIP1-BD, suggesting that SIP1 dimerizes. The interactions were also detected when the C terminus of SIP1 was used instead (SIP1C with SIP1; SIP1C with SIP1C; Fig. 5A), suggesting that the C-terminal 184 residues are responsible for SIP1 dimerization in the absence of DNA. In contrast, the N terminus (223 residues) of SIP1, which contains the ARID domain, was not required for SIP1 dimerization.

To further confirm this interaction, we immobilized glutathione S-transferase (GST)-SIP1, CBD-SIP1N, and CBD-SIP1C fusion proteins on GST beads or chitin beads and incubated the beads with His-tagged SIP1, SIP1N, and SIP1C, respectively. After washing, the proteins retained on the beads were subjected to immunoblotting with the anti-His-tag antibody. As shown in Figure 5B, GST-SIP1 and CBD-SIP1C could pull down SIP1 (lanes 1 and 3), whereas CBD-SIP1N could not (lane 2). Moreover, CBD-SIP1C was able to pull down SIP1C (lane 6), suggesting the C termini are sufficient to form dimers.

Induction of SIP1 Gene Expression by Rhizobial Infection

SymRK is expressed constitutively in the roots of *Lotus*, and its mRNA level does not change upon the treatment with Nod factors for 24 and 48 h or after the inoculation with *Mesorhizobium loti* (Stracke et al., 2002). The *NIN* gene, on the other hand, is not expressed in the uninfected roots and is induced by treatments with

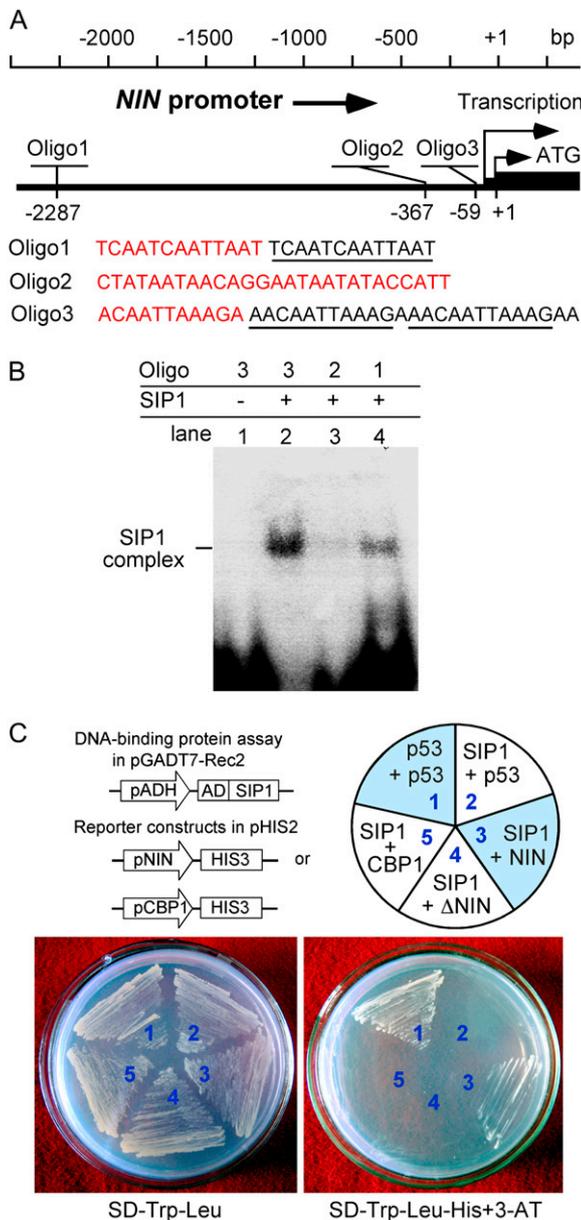


Figure 4. Binding of SIP1 to the *NIN* promoter. **A**, A search of the 4-kb 5' region of the *NIN* gene identified three AT-rich motifs. Three oligos used in this experiment correspond to the DNA sequences of the *NIN* promoter from $-2,287$ to $-2,299$ for Oligo1, -367 to -393 for Oligo2, and -59 to -69 for Oligo3. Red letters correspond to the DNA sequences of the *NIN* promoter, whereas the underlined black letters indicate a repeat (Oligo1) or double repeats (Oligo3). **B**, EMSA was performed using purified SIP1 and 32 P-end-labeled Oligos. The components of the binding reactions are indicated at the top of the lanes. **C**, In vivo assay of SIP1 binding to the *NIN* promoter. SIP1 was expressed as a fusion protein with the GAL4 AD (pGADT7-SIP1) in yeast Y187 cells harboring the reporter construct *NIN*_{pro}::HIS3, Δ *NIN*_{pro}::HIS3, or *CBP1*_{pro}::HIS3, which expresses the *HIS3* reporter gene under the promoter of the *NIN* or *CBP1* gene. The *NIN* promoter is a 500-bp fragment (-1 to -500 bp), whereas the Δ *NIN* promoter contains a 430-bp fragment (-70 to -500 bp) lacking the Oligo3 AT-rich site. The *CBP1* promoter (462 bp) was amplified by PCR (Webb et al., 2000). Yeast cells were examined for growth on the SD/-Trp-Leu (control) and SD/-Trp-Leu-His plates in the presence of

Nod factors or rhizobial infection (Schauser et al., 1999; Radutoiu et al., 2003). Using quantitative PCR, we examined the *SIP1* and *NIN* mRNA levels in different tissues of *L. japonicus*. *SIP1* mRNA was expressed constitutively in leaves and control (uninoculated) roots (Fig. 6A). In the young roots (2 d old), *SIP1* expression levels were low but increased to a steady state after 6 to 8 d. In the stem, its expression levels were relatively low but detectable. In contrast, *NIN* expression levels were relatively low in stems, leaves, and control roots (Fig. 6B).

We then focused on the expression levels of *SIP1* and *NIN* in roots after infection with *M. loti*. When inoculated with *M. loti*, an induction of *SIP1* mRNA was observed as early as 5 h post inoculation (hpi). In 24 hpi, the expression levels dropped down to a steady level, which was slightly higher than that observed in the control roots. This expression pattern was distinct from that of *NIN* (Fig. 6B), which exhibited significant induction 5 h after rhizobial inoculation and maintained a high expression level in inoculated roots. It is important to note that the timing of the *SIP1* induction (5 hpi) correlates well with that of *NIN*. After the initial induction, *NIN* continued to be expressed at relatively high levels. In conclusion, the *SIP1* gene is expressed constitutively in roots and leaves, and its expression levels are elevated in roots transiently (5 hpi–1 d postinoculation [dpi]) after rhizobial infection. It may play a role in the induction of the *NIN* gene during the process of rhizobial entry and nodule organogenesis.

Subcellular Localization of SIP1

To determine the subcellular localization of SIP1, we expressed SIP1 as a fusion protein with the *Discosoma* red fluorescent protein (DsRed) under the control of the cauliflower mosaic virus 35S promoter. The fusion protein was transiently expressed in the onion (*Allium cepa*) epidermal cells via particle bombardment, and its expression was monitored using a confocal laser-scanning microscope. As expected in the control cells expressing DsRed alone, the red fluorescence was detected only in the cytoplasm (Fig. 7, D–F). In the onion epidermal cells expressing the SIP1-DsRed fusion protein, the red fluorescence was concentrated to the nuclei (Fig. 7, A–C). This result is consistent with its potential function in DNA binding and transcription regulation. However, it remains to be determined how SIP1 interacts with SymRK in *Lotus* roots after rhizobial infection.

DISCUSSION

SymRK is a member of a large family of LRR RLKs in plants and is required for the legume root cells to

40 mM 3-AT (selection). Yeast cells harboring p53-HIS2 and pGADT7-53 were used as a positive control, whereas those containing p53-HIS2 and pGADT7-SIP1 served as negative control. [See online article for color version of this figure.]

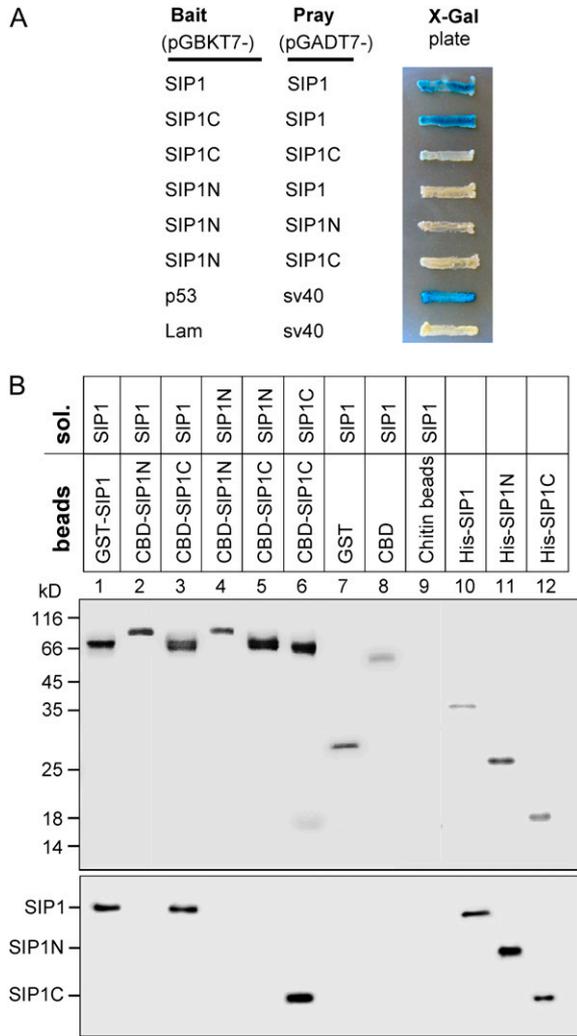


Figure 5. Dimerization of SIP1. A, Yeast AH109 cells were cotransformed with different combinations of the bait and prey constructs containing either the full-length SIP1 or its deletion fragments. The yeast cells were transferred from SD/-Leu-Trp-His-Ade to SD/-Leu-Trp plate containing X-Gal (80 µg/mL). Yeast cells harboring pGBKT7-53/pGADT7-SV40 were used as a positive control, whereas those containing pGBKT7-Lam/pGADT7-SV40 served as a negative control. B, In vitro protein-protein interaction assay. SIP1 and its deletion fragments were expressed as GST- or CBD-tagged proteins and immobilized to glutathione- or chitin beads. SIP1 and its deletion fragments were also expressed and purified as His-tagged proteins, followed by elution from the nickel-column with imidazole. The eluted soluble SIP1 or its deletion fragments were then mixed with glutathione beads or CBD beads containing an immobilized peptide. After washing with buffer, proteins retained to the beads were solubilized in SDS sample buffer and separated on SDS-PAGE (top), and the interacting proteins were detected using anti-His antibodies (bottom). [See online article for color version of this figure.]

perceive the Nod factor signals released by the infecting rhizobia (Yoshida and Parniske, 2005). NIN is a key transcription factor required for rhizobial entry into the root cells and for nodule organogenesis (Schauser et al., 1999; Radutoiu et al., 2003). In this report, we

describe a novel DNA-binding protein, SIP1, which may provide a link between SymRK and NIN. We demonstrate that SIP1 interacts with the intracellular kinase domain of SymRK (Fig. 2) and specifically binds to the Oligo3 AT-rich site of the *NIN* promoter (Fig. 4). Because SIP1 does not contain a transcription AD (Fig. 3), it may form hetero-oligomers with other unidentified proteins that contain an activator or repressor domain. Plu-1, a human ARID-containing protein, also lacks an AD but contains transcriptional repressor properties and forms hetero-oligomers with other transcription factors (Tan et al., 2003). The initial signal communications between the rhizobia and roots involve a number of different molecular exchanges culminating in calcium spiking in the host cells. SIP1, by linking the SymRK receptor kinase to the NIN transcription factor, may play a pivotal role for the successful establishment of rhizobia-legume symbiosis.

SIP1 represents a new member of the conserved ARID family of proteins in plants. The ARID domain was initially identified in the mouse BRIGHT protein

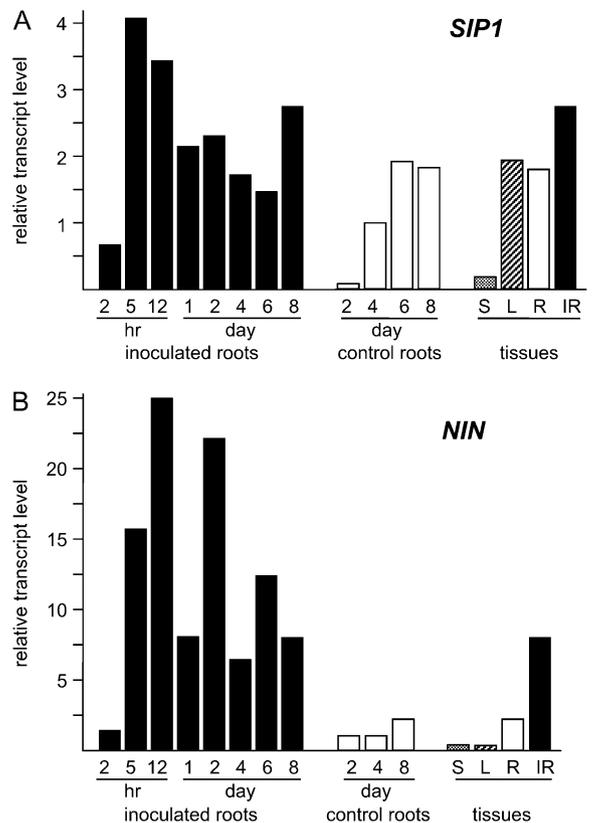
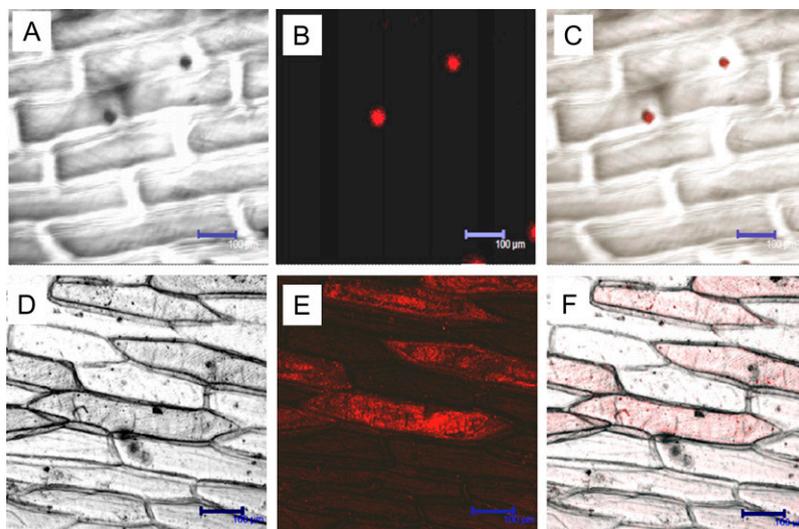


Figure 6. Expression of *SIP1* and *NIN* genes in *L. japonicus*. Roots were harvested 2, 5, and 12 hpi, and 1, 2, 4, 6, and 8 dpi with *M. loti*. Roots treated with water served as the mock control. Total RNA was isolated from stems (S), leaves (L), control roots (R), and *Rhizobium*-inoculated roots (IR). Steady-state transcript levels of *SIP1* (A) and *NIN* (B) were measured by quantitative PCR on a real-time PCR system (Radutoiu et al., 2003). The *ATPase* gene (AW719841) was used as an internal control. Relative values of transcripts normalized to the control roots (4 dpi) are shown.

Figure 7. Subcellular localization of SIP1. SIP1 was expressed as a fusion protein with DsRed under the control of the 35S promoter. The plasmid was delivered to the onion epidermal cells via particle bombardment. The plasmid (35S::DsRed) expressing DsRed alone served as a control. The onion cells were observed with a confocal laser-scanning microscope, and pictures were taken in the light transmission channel (A and D) or using the 583/558-nm filter (B and E). The photos were superimposed using Photoshop software (C and F). Note that DsRed is distributed in the cytosol, whereas SIP1:DsRed goes to the nuclei.



(Herrscher et al., 1995) and *Drosophila* DRI (Gregory et al., 1996). ARID-containing proteins have now been shown to be present in all sequenced eukaryotic organisms, including mammals, insects, plants, nematodes, and yeast (Wilsker et al., 2002). Although ARID is an α -helix-based DNA-binding domain, the structures and functions of ARID-containing proteins are highly diverse (Wilsker et al., 2002). These proteins appear to play vital roles in the regulation of development and tissue-specific gene expression.

The ARID domains, especially their 3-D structures, are conserved between animals and plants (Fig. 1B). However, other parts of the molecules can be very different among ARID-containing proteins in an organism. In *Arabidopsis*, the 10 ARID-containing proteins vary in length from 319 residues in At3g13350 to 786 residues in At2g17410. They can be grouped into four subfamilies on the basis of their phylogenetic relationship and the presence of functional motifs (Fig. 1D). *L. japonicus* SIP1 is closely related to the Hsp20 subfamily in *Arabidopsis*, although it does not contain an Hsp20-like domain. The Hsp20 motif is found in the C termini of the *Arabidopsis* ARID proteins (Fig. 1C). In *L. japonicus* SIP1, the C terminus has evolved into a different domain that is responsible for interacting with SymRK (Fig. 2) and for the homodimerization (Fig. 5). SIP1 represents a new ARID-containing transcription factor in plants and is potentially involved in linking the Nod signal perception to the transcriptional regulation during nodule development in *L. japonicus*.

The ARID domain has not been found in Archaea and eubacteria, but is widespread in protozoa, metazoans, green algae, fungi, plants, and animals. There are 15 ARID proteins in the human genome and two in *S. cerevisiae* (Wilsker et al., 2002, 2005). The four *Arabidopsis* ARID subfamilies (Fig. 1D) do not correlate closely with the seven subfamilies of the human ARID proteins (Wilsker et al., 2005). At3g43240 is a single

member of the *Arabidopsis* subfamily that possesses an ARID and a PHD domain. In humans, the most closely related proteins are the four ARID proteins in the JARID1 subfamily, which contain two or more PHD domains each, in addition to several other functional motifs (Wilsker et al., 2005), and are also twice as large as At3g43240. The remaining three subfamilies of *Arabidopsis* ARID proteins are plant specific, because no known human ARID protein contains an Hsp20, HMG, or ELM2 domain (Wilsker et al., 2002, 2005). This diversity in peptide length and motif arrangements suggests that the specific ARID subfamilies have apparently evolved independently after the divergence of plant and animal lineages.

ARID proteins in higher eukaryotes are involved in differentiation and transcriptional regulation of gene expression (Wang et al., 2007). At least two human ARID proteins are known to be induced during tumorigenesis. Plu-1 (JARID1B) was identified as an up-regulated gene product in human breast cancer and is not expressed in normal adult tissues except testis. RBP1L1 (ARID4B) was identified as a tumor antigen and is highly expressed in all cancer cell lines (Wilsker et al., 2002; Tan et al., 2003). Nodule initiation involves the de-differentiation of the root cortical cells into the meristematic cells and the activation of a series of nodule-specific genes. Our study shows that SIP1 is constitutively expressed in roots, and its expression is highly induced 5 h after rhizobial infection (Fig. 6). Although *L. japonicus* SIP1 is not homologous to the human Plu-1 and RBP1L1 except within the ARID domain, they share some similarity in their function in reprogramming the differentiated cells back to the dividing status, i.e. tumorigenesis in human and nodule organogenesis in the legume.

There are three AT-rich motifs in the promoter of the *NIN* gene, two of which showed affinity with SIP1 in *in vitro* assays (Fig. 4). In the yeast one-hybrid system, the 500-bp promoter region of the *NIN* gene was found

to contain cis-DNA elements for SIP1 binding. The binding of SIP1 to the *NIN* promoter was apparently brought about via the Oligo3 AT-rich motif, which is localized to the promoter region between -59 to -69 bp. Because SIP1 does not have a transcription AD (Fig. 3B), it is likely that SIP1 may function as a DNA sequence-specific binding protein and may form an oligomer with another transcription activator or repressor. We are currently in the process of searching for these potential SIP1-interacting partners and examining the biological functions of SIP1 and its associated proteins in nodule organogenesis in *L. japonicus*. Further analyses of the signaling pathway involving SIP1 may eventually lead to the identification of new molecular events required for the later steps in the symbiotic establishment in legume roots.

MATERIALS AND METHODS

Plant Materials

Seeds of *Lotus japonicus* MG-20 were surface-sterilized in 2% sodium hypochlorite for 8 min and washed seven times with sterile water. The seeds were left to germinate for 48 h at 22°C on sterile water-soaked filter paper in petri dishes in a dark room. Seedlings were subsequently planted in pots on sterile sands supplemented with nitrogen-free Fahraeus medium (Fahraeus, 1957) and were grown in a growth chamber maintained at 22°C with a 16-h-light/8-h-dark cycle. Five-day-old seedlings were inoculated with approximately 10^7 CFU/mL of *Mesorhizobium loti* NZP2037. Roots were collected from plants 2, 4, 6, 8, and 12 d after rhizobial inoculation and were frozen in liquid nitrogen.

cDNA Library Construction

Total RNA was isolated from the equally mixed roots collected 2, 4, 6, 8, and 12 d after rhizobial inoculation using the TRIzol reagent (Invitrogen). Total RNA (2 µg) was reverse transcribed into single-stranded cDNA using oligo(dT) as the primer. The cDNA was size-fractionated using BD Matchmaker library construction and screening kits (BD Biosciences CLONTECH). The cDNA fragments longer than 500 bp were cotransformed with linearized pGADT7-Rec into yeast (*Saccharomyces cerevisiae*) AH109 cells. The transformants were selected on SD/-Leu medium according to the manufacturer's instruction. The transformation efficiency was approximately 2×10^6 CFU/3 µg pGADT7-Rec.

Library Screening

The *SymRK-PK* cDNA (GenBank accession no. AF492655) was amplified by reverse transcription-PCR with the following gene-specific primers: 5'-GGGCATATGATGGAGAGGTACAAAACCTTG-3' and 5'-AAAGAATTCTCTCGGCTGTGGGTGAG-3'. PCR was carried out using ExTaq DNA polymerase (TaKaRa) with an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min. After the last cycle, an extension step for 10 min at 72°C was carried out. Purified PCR products were inserted into the *Nde1-EcoR1* site of pGBKT7 vector. Screening of interaction clones was carried out according to the manufacturer's instructions (CLONTECH). A total of 1×10^7 transformants from the cDNA library were screened for growth on the SD/-Leu-Trp-His-Ade media. Positive clones were verified by retransformation of the rescued plasmid into the AH109 containing the bait plasmid (pGBKT7-SymRK-PK). Plasmid pGBKT7-Lam (CLONTECH) was used as a negative control. Colonies growing on the SD/-Leu-Trp-His-Ade media were transferred to selective media-containing X-Gal (80 µg/mL) or were left on filters as described (Ma et al., 2007). The blue colonies were characterized.

β-Galactosidase Assay

Yeast cells grown in liquid selection media were pelleted and washed twice with Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1.0 mM MgSO₂,

pH 7.0). The cells were resuspended in 300 µL of Z-buffer and permeabilized by two freeze-thaw cycles in liquid nitrogen and 37°C. Cell extracts were added 0.7 mL of Z-buffer containing 50 mM β-mercaptoethanol and 160 µL of *O*-nitrophenyl β-D-galactopyranoside (4 mg/mL) in Z-buffer. After incubation at 30°C for 30 min or until the yellow color appeared, the reaction was terminated by the addition of 0.4 mL of 1.0 M Na₂CO₃. The reaction mixture was centrifuged for 10 min at 14,000 rpm to remove cell debris. β-Galactosidase activity in the supernatant was measured at OD₄₂₀ and expressed in Miller units (Miller, 1972).

Expression and Purification of Fusion Proteins

The full-length cDNA of *SIP1* was inserted at the *Nde1-Sal1* site of pET28 vector (Novagen), generating pET-SIP1. The 3' region of *SIP1* was amplified by PCR using primers 5'-CTTTCTAGACCATATGAAGTCCAGAGAAAGGA-3' and 5'-TTTTGTGACAAGCTTCCAGGCTCCCTG-3'. The fragment was cloned into the *Nde1-Sal1* site of pET28a vector, generating pET-SIP1C. The 5' region of *SIP1* was amplified using primers 5'-CCGGTACCCATATGGAAATTGTGAT-3' and 5'-CGGAAGCTTGTGCTAGACTGTAACTC-3'. The fragment was cloned into the *Nde1-Sal1* site of pTYB12 (New England Biolabs), yielding pTYB-SIP1N. For protein expression, *Escherichia coli* BL21 (Novagen) or ER2566 cells (New England Biolabs) harboring the plasmid were induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside in Luria-Bertani broth for 8 h at 16°C. His-tagged proteins were purified using nickel-agarose beads (Qiagen) according to the manufacturer's instruction. CBD-tagged proteins were purified using chitin beads (New England Biolabs) as described previously (Zhang et al., 2003). Purified proteins were desalted by dialysis in PBS buffer and concentrated with PEG-8000 powder.

In Vitro Protein-Protein Interaction

To assay the interaction between SymRK-PK and SIP1, His-tagged SIP1 and SIP1C were bound to nickel-agarose beads, while CBD-SIP1N was adsorbed on the chitin beads. The beads were incubated with 5 µg of purified SymRK-PK protein in the interaction buffer (20 mM Tris-HCl, 100 mM KCl, 2 mM MgCl₂, 5% glycerol, pH 8.0) for 1 h on ice with gentle shaking. The nickel beads were washed three times with a buffer solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 100 mM imidazole, 5% glycerol, pH 8.0), whereas the chitin beads were washed three times with 1.0 mL of TEG buffer (20 mM Tris-HCl, 1 mM EDTA, 5% glycerol, pH 8.0). The retained proteins were eluted by boiling for 3 min in 1× SDS sample buffer (2% SDS, 29.1 mmol/L Tris, pH 6.8, 10% glycerol, and 0.01% bromophenol blue; Zhang et al., 2003). Samples were analyzed on 10% SDS-PAGE, followed by immunoblotting with the anti-LjSymRK antibody.

To assay SIP1 dimerization, GST-tagged SIP1 was expressed and purified on glutathione-Sepharose 4B (Sigma) as described elsewhere (Zhang et al., 2000). CBD-tagged SIP1N and SIP1C were immobilized on chitin beads. The beads were incubated with purified His-SIP1, His-SIP1N, and His-SIP1C proteins for 1 h on ice with gentle shaking. Following washing, the retained proteins were resolved on 10% PAGE and detected using the anti-His-tag antibody.

EMSA

EMSA of SIP1 was performed on 5% nondenaturing polyacrylamide gels as described previously (Gregory et al., 1996). DNA-binding activity of SIP1 was examined using γ-³²P-end-labeled double-stranded oligonucleotide trimer of the consensus Engrailed binding site NP₃ [(TCAATTAATGA)X3] or trimer of TTA₉ [(TTATTATTA)X3; Gregory et al., 1996]. To test if SIP1 specifically binds to the promoter of the *NIN* gene, Oligo1, Oligo2, and Oligo3 (see Fig. 4A for sequences) were synthesized and used for EMSA. His-tagged SIP1 was purified with nickel-agarose beads and eluted using 500 mM imidazole followed by dialysis. Approximately 10 ng of SIP1 protein was added in a mixture containing 0.3 µM of γ-³²P-labeled double-stranded DNA in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, 50 µg bovine serum albumin, and 5% glycerol. After incubation at room temperature for 20 min, the samples were subjected to electrophoresis on a 5% polyacrylamide/bis (29:1) gel in 0.5× Tris-borate/EDTA buffer at a constant voltage of 100 V. The gel was dried, and the radioactive bands were detected using PhosphorImager (FUJIFILM).

Transient Expression

The *SIP1* cDNA amplified using primers 5'-TATACTAGTATGGAAA-TTGTGAT-3', and 5'-AAGGTCACCTTACACGGCTCCCTG-3' was cloned into the *Spe1/BstEII* site of pCAMBIA1302 vector (CAMBIA). We amplified DsRed coding sequence using primer 5'-CAACTAGTATGAGGCTTCCAA-GAATGT-3', and 5'-TTACTAGTAAGGAACAGATGGTGGCGTC-3' from the pDsRed (CLONTECH), and it was inserted into the *Spe1* site of the pCAMBIA1302-SIP1 construct, resulting in p35S-DsRed-SIP1. The plasmid was used for transient expression in the onion (*Allium cepa*) epidermal cells by particles bombardment using a Biolistic PDS-1000/He particle delivery system (Bio-Rad). After incubation for 24 to 48 h at 25°C in dark, the epidermal cell layers were examined using a confocal laser-scanning microscope (Leica).

Yeast One-Hybrid Assay

The *SIP1* cDNA was fused to the GAL4 AD in pGADT7-Rec2 (CLONTECH), generating pGADT7-SIP1. The promoter (-1 bp to -500 bp) of the *NIN* gene (Schauer et al., 1999) was amplified using primers 5'-GGAATTCGGCGTG-AGACTTTACATTAC-3' and 5'-CGACGCGTGAAAATGATTGCCCTTGCC-3'. A 430-bp fragment of the *NIN* promoter (-70 to -500 bp) lacking the Oligo3 AT-rich site (Δ *NIN*) was amplified by PCR using primers 5'-GGAATTCGGCGTGAGACTTTACATTAC-3' and 5'-TTTACGCGTCTTGCTAGG-ACC-3'. The promoter of the *LjCBP1* gene (Webb et al., 2000) was amplified from genomic DNA using primers 5'-GGAATTCCTCAAGTCAGTCAATTT-TTG-3' and 5'-CGACGCGTCCACTGACTTAATTCATG-3'. The promoter fragments of the *NIN*, Δ *NIN*, and *LjCBP1* genes were inserted into the *EcoRI-MluI* site of pHIS2 (CLONTECH), producing *NIN_{pro}::HIS3*, Δ *NIN_{pro}::HIS3*, and *CBP1_{pro}::HIS3*, respectively, which would express the HIS3 reporter under the control of the corresponding promoter.

The plasmid was transformed into yeast Y187 cells harboring pGADT7-SIP1, which expresses SIP1 fused with the GAL4 transcription AD. The DNA-binding activity of SIP1 was determined by the expression of the HIS3 reporter.

Measurement of Transcript Level by Quantitative PCR

Total RNA was isolated from stems, leaves, control roots, and roots inoculated with *M. loti*, using a TRIZOL reagent (Invitrogen). RNA samples were treated with DNase I to remove potential contaminating genomic DNA, followed by extraction with phenol:chloroform. The DNA-free RNA samples were confirmed by lack of a PCR product using primers specific to the untranscribed *NIN* gene promoter (5'-GTTTCAAGAATGGGAGGGG-3', 5'-CTCCTCTGTTTCATTGGTG-3'). First-strand cDNA was prepared using Oligo(dT) primer, and quantitative PCR was performed on a Mini Opticon real-time PCR system (Bio-Rad) using One-Step SYBR PrimeScript RT-PCR kit II (Takara). The same cDNA pool was used for amplification of all tested transcripts. The relative quantification software (Bio-Rad) was used to determine the efficiency-corrected relative transcript concentration, normalized to a calibrator sample (Radutoiu et al., 2003). ATP synthase was used as a reference gene (AW719841). Melting curve analysis and sequencing of the amplified products was used to determine the identity of the amplified PCR products. Intron spanning primers were used for transcript amplification of the ATP synthase (5'-CAATGTCGCAAGGCCATGGTG-3' and 5'-AACACCACT-CTCGATCATTTCTCTG-3'), *SIP1* (5'-CTTGCAAGTAAGTTCACAAC-3' and 5'-GTACGAGTCCAATCAGATCCAG-3'), and *NIN* (5'-AATGCTCTTGAT-CAGGCTG-3' and 5'-AGGAGCCCAAGTGAGTGCTA-3') genes. The same experiment was performed three times, and the averages of measurements were presented.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU559710.

Supplemental Data

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

Supplemental Figure S1. Full-length cDNA sequence of *L. japonicus* SIP1.

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