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A Lotus japonicus cytoplasmic kinase connects Nod factor perception by the NFR5 LysM receptor to nodulation

Jasly E. M. M. Wong,1,2, Marcin Nadziej,1, Lene H. Madsen,4, Christoph A. Bücheler,5 Svend Dam,6 Niels N. Sandal,3 Daniel Couto,2,3, Paul Derbyshire,6 Mette Uldum-Berentsen,4 Sina Schroeder,4,5 Veit Schwämmle,6 Fabio C. S. Nogueira,6 Mette H. Asmussen,6 Soren Thirup,8 Simona Radutoiu,9 Mickaël Blaise,6,5 Kasper R. Andersen,6 Frank L. H. Menke,6 Cyril Zipfel,6,8, and Jens Stougaard,6,7

1Department of Molecular Biology and Genetics, Aarhus University, DK-8000 Aarhus, Denmark; 2The Sainsbury Laboratory, University of East Anglia, Norwich NR4 7HH, United Kingdom; 3Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense, Denmark; and Proteomics Unit, Chemistry Institute, Federal University of Rio de Janeiro, 21941-909, Rio de Janeiro, Brazil

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The establishment of nitrogen-fixing root nodules in legume–rhizobia symbiosis requires an intricate communication between the host plant and its symbiont. We are, however, limited in our understanding of the symbiosis signaling process. In particular, how membrane-localized receptors of legumes activate signal transduction following perception of rhizobial signaling molecules has mostly remained elusive. To address this, we performed a communoprecipitation-based proteomics screen to identify proteins associated with Nod factor receptor 5 (NFR5) in Lotus japonicus. Out of 51 NFR5-associated proteins, we focused on a receptor-like cytoplasmic kinase (RLCK), which we named NFR5-interacting cytoplasmic kinase 4 (NiCK4). NiCK4 associates with heterologously expressed NFR5 in Nicotiana benthamiana, and directly binds and phosphorylates the cytoplasmic domains of NFR5 and NFR1 in vitro. At the cellular level, NiCK4 is coexpressed with NFR5 in root hairs and nodule cells, and the NiCK4 protein relocates to the nucleus in an NFR5/NFR1-dependent manner upon Nod factor treatment. Phenotyping of retrotransposon insertion mutants revealed that NiCK4 promotes nodule organogenesis. Together, these results suggest that the identified RLCK, NiCK4, acts as a component of the Nod factor signaling pathway downstream of NFR5.

NFR5 | NiCK4 | RLCK | Lotus | nodulation

Legumes and rhizobia initiate symbiosis by exchanging signal molecules in a bidirectional communication, which ultimately leads to the formation of nitrogen-fixing root nodules in the host plant (1, 2). Flavones or isoflavones secreted into the rhizosphere by legume plants associate with the rhizobial NodD protein that activates a set of genes synthesizing lipo-chitooligosaccharides called Nod factor (NF) (3). In turn, these rhizobial NFs are perceived by LysM-type receptors that trigger nodule organogenesis and infection thread formation (4). In Lotus japonicus, dedicated plasma membrane (PM)-localized receptors—NFR1, NFR5, and NFR6 (5–7)—perceive NFs that constitute the major rhizobial signal (8–11). Both NFR1 and NFR5 are indispensable for NF signaling (5, 6) while NFR6 was suggested to amplify signaling in root epidermal cells (7). NFR1, NFR5, and NFR6 are lymkin type 1 (LysM) receptor kinases (RKs) composed of 3 LysM domains in the extracellular region, a single-pass transmembrane domain, and an intracellular kinase domain. Purified NFR1, NFR5, and NFR6 can directly and independently bind NF through their extracellular domains (7, 12). The kinase domains of NFR1 and NFR6 are active in vitro (7, 13). However, the pseudokinase domain of NFR5 has a truncated activation loop, an altered DFG motif, lacks an APE motif, and is inactive in vitro (12, 13).

The earliest responses of NF from Mesorhizobium loti that nodulates L. japonicus include depolarization of the PM and alkalization of root hair extracellular space (6, 14, 15). Application of NF in nanomolar concentrations also results in calcium influx and perinuclear calcium oscillations (16–18). Shortly after M. loti inoculation, root hair deformation and curling responses occur (6). In later stages of the developmental process, infection threads are formed and nodule primordia develop in the root cortex (6, 19, 20).

Single nfr1 or nfr5 mutants are unresponsive to M. loti and NF treatments (5, 6). This phenotypic similarity suggests that NFR1 and NFR5 may be part of the same signaling complex. Several lines of evidence support this notion. First, NFR5 associates with NFR5 in bimolecular fluorescence complementation (BBMFL) experiments (19). Second, pull-down experiments show that NFR5 interacts with NFR1 in Nod factor-treated Lotus japonicus cells (20). Third, in a yeast two-hybrid system, NFR5 interacts with NFR1, and NFR1 and NFR5 may be part of the same signaling complex. These results suggest that the identified RLCK, NiCK4, acts as a component of the Nod factor signaling pathway downstream of NFR5.

Significance

Legume receptors perceive Nod factor signal molecules at the plasma membrane of epidermal cells and initiate a signal transduction process that leads to the development of root nodules that house nitrogen-fixing rhizobia. Nodule organs are formed by reinitiation of cell divisions in already differentiated root cells. Previous genetic screens have identified plant genes involved in nodulation; however, the receptor-triggered relay mechanism activating the developmental program in the nucleus is still unknown. We present a proteomics approach that identified proteins that associate with the Lotus japonicus Nod factor receptor 5 (NFR5), among which the NFR5-interacting cytoplasmic kinase 4 (NiCK4) appears to be an important link between Nod factor perception by NFR5 and nodule organogenesis.

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1J.E.M.M.W. and M.N. contributed equally to this work.
2Present address: Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 0QH, United Kingdom.
3Present address: Structural Plant Biology Laboratory, Department of Botany and Plant Biology, University of Geneva, 1211 Geneva, Switzerland.
4Present address: Institute of Recherche en Infectiologie de Montpellier, CNRS, UMR 9904, Université de Montpellier, 34293 Montpellier Cedex 5, France.
5Present address: Institute of Plant and Microbial Biology and Zurich–Basel Plant Science Center, University of Zurich, 8008 Zurich, Switzerland.
6To whom correspondence may be addressed. Email: stougaard@mbg.au.dk.

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(BiFC) experiments using *Nicotiana benthamiana* leaves (13). In the BiFC assays, cell death responses were observed when NFR5 was coexpressed with wild-type NFR1 but not with the T483A kinase-dead variant of NFR1 (13). Second, spontaneous nodulation that occurs upon overexpression of Nfr5 in *L. japonicus* transformed roots was not observed in an nfr1 mutant background (21). Finally, the extension of the host range of *Medicago truncatula* (Mt) to include *M. loti* requires the transfer of both Nfr1 and Nfr5 (22).

Genetic approaches have identified several symbiosis components acting downstream of NFR1 and NFR5. A putative coreceptor is the symbiosis receptor kinase (SymRK), which associates with NFR1 or NFR5 upon overexpression in *N. benthamiana* leaves and transformed *L. japonicus* root systems (21). SymRK (23, 24), nucleoporins NUP85 (25), NUP133 (26), and NENA (27), potassium channels Castor and Pollux (28–30), and calcium channels of the CNGC15 family (31) all act upstream of NF-induced perinuclear calcium oscillations. The calcium signature generated is then decoded by the calcium/calmodulin-dependent kinase (CaMK) (32–34) and the CYCLOPS transcription factor (35, 36) that resides in the nucleus. This triggers the expression and activation of additional transcription factors such as *Nin, Nsp1, Nsp2, Em1*, and *NF-Ys* (35–45) that regulate genes required for nodule formation and function.

The mechanisms of the early steps of this pathway connecting ligand perception by NF receptors at the PM to downstream signaling components are missing. An explanation for this paucity of interacting components and secondary signal molecules could be gene redundancy or functional compensation preventing their identification using molecular genetic approaches. Recent development in commounoprecipitation (co-IP) and proteomics technologies have led to the identification of interactors of plant RKs and receptor-like proteins (46, 47). Here, we present a proteomics approach in which proteins associated with enhanced yellow fluorescent protein (eYFP)- and HA-tagged NFR5 (NFR5-eYFP-HA) in *L. japonicus* were isolated in co-IP experiments and identified by mass spectrometry (MS). One of the NFR5-associated proteins, which we named NFR5-interacting cytoplasmic kinase 4 (NiCK4), was characterized biochemically and genetically. The discovery of NiCK4 as a signaling component that links NF perception at the PM to downstream nodulation signaling indicates that an intricate phosphorylation cascade mechanism involving the NFR5 pseudokinase activates the signal transduction process.

**Results**

**Identification of NFR5-Associated Proteins.** To identify components of the NFR5 signaling pathway, we generated *L. japonicus* transgenic plants expressing NFR5-eYFP-HA (hereafter referred to as NFR5-eYFP) to perform co-IP experiments. We first assessed that the NFR5-eYFP construct was capable of rescuing the *nfr5-2* nonnodulating phenotype (*SI Appendix, Fig. S1A*) before generating p35S:Nfr5-eYFP lines. In epidermal root cells and root hairs, NFR5-eYFP localized predominantly to the cell periphery and mobile endomembrane compartments including the endoplasmic reticulum (*SI Appendix, Fig. S1B*). NF treatments also produced the expected root hair deformations (*SI Appendix, Fig. S1C*), thus attesting to the responsiveness of our transgenic lines. We then carried out large-scale triplicate co-IP experiments using ∼3,000 NFR5-eYFP roots for each of the triplicate experiments, as summarized in *SI Appendix, Fig. S1D*. The transgenic roots were treated either with 200 nM purified *M. loti* NF or water (mock) for 15 min. Nontreated wild-type *Gifu* roots were used as a specificity control.

NFR5-eYFP was successfully captured on GFP-trap beads (Fig. 1A) and was identified with 78% coverage by MS (*SI Appendix, Fig. S2A*). Remarkably, we discovered 2 in vivo phosphorylation sites, S574 and T576 (*SI Appendix, Fig. S2C and D*), in addition to S282 (*SI Appendix, Fig. S2B*), which was previously shown to be phosphorylated by NFR1 and SymRK (13). This suggests that NFR5 may be phosphorylated by hitherto-unknown components (*SI Appendix, Fig. S2 B–D*). We defined NFR5-associated proteins as those that are represented by at least 2 unique peptides (with Mascot ion scores above 20) in all 3 biological replicates (*SI Appendix, Table S1) while absent from the list of unspecific proteins (*SI Appendix, Table S2*). Two hundred and fifteen putative NFR5-associated proteins, represented by over 2,000 peptide spectra, fulfilled these criteria (*SI Appendix, Table S1*).

Two hundred and nine proteins that were present in both mock- and NF-treated samples were ranked according to spectral count (*SI Appendix, Table S1*). The remaining 6 NFR5-associated proteins that were either enriched in NF- or mock-treated samples were placed at the bottom of the list (*SI Appendix, Table S1*) and were ranked 210–213, and 214 and 215, respectively. We further
filtered our candidate list by removing highly abundant and related proteins by comparison with previously published proteomics data (48) and additional data generated from liquid chromatography–mass spectrometry (LC-MS) proteomics studies of *L. japonicus* roots (*SI Appendix, Table S3 and Materials and Methods*) to reach a reduced set of 51 root-hair–expressed NFR5-associated proteins (Table 1) represented by 246 unique peptides (*SI Appendix, Table S4*). This includes high-interest signaling candidates such as receptor kinases, receptor-like cytoplasmic kinases (RLCKs), phosphatases, as well as cell wall remodeling proteins (Table 1).

**NiCK4 Is an RLCK That Could Relay Signals Downstream of NFR5.** RLCKs are pivotal in linking ligand perception of PM-localized pattern recognition receptors to downstream components (49–59). The fourth NFR5-associated RLCK, which we have renamed NiCK4 for NFR5-interacting cytoplasmic kinase 4, was therefore selected for further in vivo and in vitro investigations regarding a possible role in symbiosis signaling and to assess the validity and accuracy of our IP-MS approach. NiCK4 was selected over the other 3 RLCKs because we were interested in identifying a dynamic protein component that could potentially link NF perception at the PM to nuclear events that lead to nodulation. NiCK4 is a prime candidate as the closest *Arabidopsis thaliana* (*At*) homolog of *At* CRPK1 (*At*1G16670) (*Fig. 1B* and *SI Appendix*, *Fig. S4*), which is involved in transducing cold signal from the PM to the nucleus through phosphorylation of 14-3-3 proteins (58). Another interesting observation is that the number of *L. japonicus* and *M. truncatula* RLCKs in the CRPK1 family is 7 and 8, respectively, compared with 2 in *A. thaliana* (*Fig. 1B* and *SI Appendix*, *Fig. S4*). As *L. japonicus* and *M. truncatula* have the capacity to establish symbiotic relationships with rhizobia and arbuscular mycorrhiza fungi, one is tempted to speculate that some of these RLCK paralogs may have been nonfunctionalized to serve symbiotic functions. Finally, gene expression analyses of the 4 NFR5-associated RLCKs available in the *Lotus* Base (60, 61) revealed that the expression of *Nick4* is the lowest among the 4 RLCKs in *L. japonicus* root hairs (*SI Appendix, *Fig. S5*) (61). The detection of NiCK4 in 3 independent co-IP experiment, despite relatively low gene expression, therefore suggests that the association of NiCK4 and NFR5 is robust. Two spectra representing unique peptides of NiCK4 are shown in *Fig. 1* C and *D*.

**NiCK4 Localizes to the PM and Directly Interacts with NFR5.** A prerequisite for complex formation is that NiCK4 and NFR5 should both be found at the same subcellular location. To assess this, NiCK4-mCherry and NFR5-eGFP fusion proteins were overexpressed in *N. benthamiana* leaves for localization studies. As a control, eGFP was N-terminally fused to *L. japonicus* low-temperature–induced protein 6B (*LjLTI6b*) PM marker (62). Our results indicate that individually expressed NiCK4-mCherry, NFR5-eGFP, and eGFP-LjLTI6b or coexpressed NiCK4 and NFR5-eGFP or eGFP-LjLTI6b all localize at the PM of leaf epidermal cells, as confirmed by plasmolysis (*SI Appendix, Figs. S6 and S7*). The PM localization of NiCK4-c-eGFP was further confirmed in *Lotus* roots (*SI Appendix, *Fig. S8*) (61). Despite being a predicted cytoplasmic protein with no transmembrane domains, the PM localization of NiCK4 may be explained by interactions with PM-bound RKs or via lipid-anchoring posttranslational modifications. Fluorescence resonance energy transfer (FRET) measurements by acceptor photobleaching revealed that NiCK4-mCherry interacts with NFR5-eGFP (*Fig. 2A*) but not eGFP-LjLTI6b in *N. benthamiana* (*Fig. 2B*).

Upon verifying that NiCK4 and NFR5 both localize to the PM, reciprocal co-IP experiments were performed using NiCK4-FLAG as a bait to validate the interaction between NiCK4 and NFR5. Anti-FLAG pulldown experiments were performed using NiCK4-FLAG, eGFP-LjLTI6b, and NFR5-eGFP fusion proteins that were expressed in *N. benthamiana* leaves. NiCK4-FLAG was successfully enriched in communoprecipitated samples (IP) as detected on Western blots with anti-FLAG antibodies (*Fig. 2C*). The eGFP-LjLTI6b PM marker and NFR5-eGFP were well detected in the crude lysate (input) when expressed individually or when coexpressed with NiCK4-FLAG (*Fig. 2C*). However, some proteins were named based on homologs identified in *A. thaliana* (*At*) or *M. truncatula* (*Mt*). NFR5-associated proteins that were NF- or mock-enriched are indicated in green or red, respectively.

### Table 1. Filtered list of 51 putative NFR5-associated proteins

| No. | Protein |
|-----|---------|
| Receptor kinases | |
| 1 | AtCRCK29-like protein |
| 2 | AT2G3200-like CrRLK1L1 protein |
| 3 | AtBIR1-like protein 1 |
| 4 | AtRLK7-like protein |
| 5 | AtPERK1-like protein |
| 6 | LjCERK6 LysM protein |
| 7 | AThERK1-like CrRLK1L1 protein |
| 8 | AtBIR1-like protein 2 |
| 9 | LjLYS13 LysM protein |
| Receptor-like cytoplasmic kinases | |
| 10 | AtPTI1-like protein |
| 11 | MSKPT1-like protein |
| 12 | AtBSK8-like protein |
| 13 | NiCK4/AtCRPK1-like protein |
| Phosphatases | |
| 14 | Phosphatase/Lyptase/AtPHD4-like protein |
| 15 | AtPP2C76-like protein |
| 16 | PP6 regulatory subunit/AtASAL1-like protein |
| 17 | AtPP2A B’ regulatory subunit-like protein |
| 18 | LjLNP2 |
| Cell wall remodeling proteins | |
| 19 | Putative pectin-acetylase |
| 20 | Putative O-fucosyltransferase |
| 21 | Putative cellulose synthase/AtGLS5-like protein |
| 22 | Putative cellulose synthase/AtGLS10-like protein |
| 23 | LjLENTY2/Hyp O-arabinoxylosyltransferase |
| 24 | Putative xylanogen xylosyltransferase |
| 25 | AtCESA2-like protein |
| 26 | Putative galacturonosyltransferase |
| 27 | AtCESA3-like protein |
| Ion pumps | |
| 28 | AtAH2-like PM proton pump |
| 29 | AtMA-like protein |
| 30 | MtmC8-like ER calcium pump |
| 31 | AtACA10-like PM calcium pump |
| Others | |
| 32 | AtAR8-like protein |
| 33 | Putative E ubiquitin-protein ligase |
| 34 | 12X transmembrane protein |
| 35 | ER membrane protein complex subunit-like protein 1 |
| 36 | DUF1682-containing protein |
| 37 | AtDayleper-like protein |
| 38 | AtUTX3-like protein |
| 39 | AtRLC3-like protein |
| 40 | AtERD4-like protein |
| 41 | Putative carboxypeptidase |
| 42 | FG-GAP repeat-containing protein |
| 43 | MthMGR1-like protein |
| 44 | PI4K-alpha-like protein |
| 45 | Calcium-dependent lipid-binding protein |
| 46 | AtTMPT-like protein |
| 47 | ER membrane protein complex subunit-like protein 2 |
| 48 | Putative calmodulin-binding transcription activator 2 |
| 49 | AtSMC1-like protein |
| 50 | Paladin-like protein |
| 51 | Uncharacterized protein |

*Some proteins were named based on homologs identified in *A. thaliana* (*At*) or *M. truncatula* (*Mt*). NFR5-associated proteins that were NF- or mock-enriched are indicated in green or red, respectively.*

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only NFR5-eGFP and not eGFP-LjLT16b communoprecipitated with NiCK4-FLAG (Fig. 2C). In addition, no unspecific binding of NFR5-eGFP to anti-FLAG beads coated with synthetic FLAG peptides was observed (Fig. 2C, lane 5). Altogether, our results indicate that the interaction of NFR5-eGFP and NiCK4-FLAG was not due to nonspecific contributions from the GFP or FLAG tags.

We also investigated whether NiCK4-FLAG associates with NFR1-eGFP and SymRK-eGFP, the proposed coreceptors of NFR5 (6, 13, 21, 22, 63). NFR1-eGFP and SymRK-eGFP were also expressed individually or coexpressed with NiCK4-FLAG in N. benthamiana leaves. While no association of NFR1-eGFP or SymRK-eGFP with NiCK4-FLAG was observed in the anti-FLAG pulldown experiments (Fig. 2C), we cannot exclude that NFR1-eGFP and SymRK-eGFP were not detected due to lower expression levels compared with NFR5-eGFP (Fig. 2C).

Furthermore, we employed the microscale thermophoresis (MST) technique to further investigate whether NiCK4 directly associates with the cytoplasmic domain of NFR5, NFR1, and SymRK. For this purpose, NiCK4, as well as the cytoplasmic domains of NFR5 (NFR5-CD), NFR1 (NFR1-CD), and SymRK (SymRK-CD) were expressed in Escherichia coli and purified by affinity and size-exclusion chromatography (SEC) (SI Appendix, Fig. S9 A–C, and E). MST experiments revealed that NiCK4 directly binds NFR5-CD298-595 and NFR1-CD with dissociation constants of 1.36 ± 0.62 μM (Fig. 2D) and 0.39 ± 0.22 μM (Fig. 2E), respectively, but does not directly bind SymRK-CD (Fig. 2F).

**NiCK4 Phosphorylates NFR5-CD In Vitro.** The cytoplasmic domain of NFR5 contains a degenerated glycine-rich loop and truncated activation loop (Fig. 3A), and has consistently been demonstrated to be inactive (7, 12, 13). It has thus remained unclear how the pseudokinase NFR5 contributes to signal initiation following NF perception. Pseudokinases are proposed to act as scaffolding platforms for downstream signaling components that modulate signaling outputs (65). Unlike NFR5, NiCK4 possesses the hallmark features of an active kinase, such as the AKX motif, the HRD motif in the catalytic loop, as well as intact DFG and APE motifs in the activation loop (Fig. 3A). This, together with our finding that NiCK4 directly interacts with NFR5-CD298-595, led us to investigate whether NiCK4 was an active kinase capable of phosphorylating NFR5-CD. Consistent with previous reports (12, 13), in vitro kinase assays revealed that NFR5-CD does not display any detectable kinase activity (Fig. 3B and D, and SI Appendix, Figs. S10 and S11B). NiCK4, however, is an active kinase capable of autophosphorylation (Fig. 3B and D, and SI Appendix, Figs. S10 and S11). Remarkably, Fig. 3A is also capable of transphosphorylating NFR5-CD298-595 and NFR1-CD-T483A, the kinase-dead variant of NFR1-CD (SI Appendix, Fig. S10).

To investigate how the phosphorylation of NFR5 by NiCK4 compares to NFR1 or SymRK, we performed additional in vitro kinase experiments using an extended NFR5-CD276-595 construct that contains all juxtamembrane residues, including the single S282 residue phosphorylated by NFR1-CD (13). Here, NFR1-CD was N-terminally fused to a thioredoxin tag (TRX-NFR1-CD) to facilitate the separation of NFR1-CD and NFR5-CD276-595 on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) gels (Fig. 3B and SI Appendix, Fig. S10). We first determined the conditions in which the kinase activities of NiCK4, TRX-NFR1-CD, and SymRK-CD were optimal by assessing the autophosphorylation abilities of these kinases in buffers containing different divalent metals (Ca2+, Mg2+, Mn2+, and Zn2+) at various concentrations (SI Appendix, Fig. S11A). As NiCK4 and TRX-NFR1-CD were most active in the presence of 10 mM MgCl2 and SymRK-CD was most active in the presence of 5 mM MnCl2 (SI Appendix, Fig. S11A), we performed NFR5-CD276-595 phosphorylation studies using a buffer containing 10 mM MgCl2 and 5 mM MnCl2. As observed previously (12, 13), NFR5-CD276-595 lacks kinase activity, and TRX-NFR1-CD, SymRK-CD, and NiCK4 possess both autophosphorylation and transphosphorylation activities (Fig. 3B and SI Appendix, Fig. S11). The results from these kinase assays suggest that NiCK4 seems to phosphorylate NFR5-CD276-595 and myelin...
E. coli). All -expressed proteins used in these kinase assays were soluble and nonaggregated as indicated by monodisperse peaks in their SEC profiles (SI Appendix, Fig. S8). Following NF treatment of Lotus roots before and after treatment with NF. As mentioned previously, Nick4 localizes to the PM when overexpressed (SI Appendix, Fig. S8). Plants cold signals from the PM to the nucleus (58), we investigated the localization of Nick4-cGFP in the susceptible zone of Lotus roots before and after treatment with NF. As mentioned previously, Nick4 localizes to the PM when overexpressed (SI Appendix, Fig. S8). Following NF treatment of pLjUb:Nick4-cGFP transformed wild-type Lotus roots, we detected Nick4-cGFP relocalization to the nucleus in roots hairs of 6 out of 8 roots observed after 90 min of NF treatment (Fig. S4). This NF-induced movement is dependent on both Nick4 and NFR5. No nuclear localization of Nick4-cGFP was detected in any of the root hairs of 11 nfr5-2 or 9 nfr1-1 transformed hairy roots (Fig. S4).

**Nick4 Promotes Nodule Organogenesis but Not Infection Thread Formation.** To probe whether Nick4 was implicated in processes that control nodule organogenesis or infection thread formation, loss-of-function mutants were subsequently obtained from the Lotus retrotransposon 1 (LORE1) mutant resource (73, 74). Three homozygous LORE1 insertions in exonic regions of the Nick4 gene, named nick4-1 to nick4-3, were isolated for phenotypic analyses (Fig. S4). Plants not containing any LORE1 insertion from the nick4-2 LORE1 segregating population were also isolated and served as the wild-type (WT) outsegregant control in these experiments. The WT plant contains an intact Nick4 gene encoding a 387-aa protein (60). However, insertion of the LORE1 element in exons 1, 2, and subsequently activates downstream components involved in symbiosis signaling.

**Nick4 and Nfr5 Display Similar Expression Patterns.** To dissect the biological relevance of the Nick4–NFR5 interaction, we first studied the expression profile of Nick4 and Nfr5 in L. japonicus noninoculated roots or roots inoculated with M. loti strain MAFF303099 labeled with dsRed. We used Nick4 and Nfr5 promoters to drive the expression of nuclear-localized triple YFP reporter (YFP-NLS) (66). Expression of these pNick4:YFP-NLS and pNfr5:YFP-NLS constructs were then monitored in L. japonicus transformed roots (67–69).

The expression pattern we observed for pNfr5:YFP-NLS is consistent with previous reports (70). In noninoculated roots and inoculated roots, pNfr5:YFP-NLS expression was observed in epidermal cells including root hairs (Fig. 4B). Furthermore, the expression of Nfr5 was present in cortical cells of inoculated roots. The expression of Nick4 was strongest in nodule primordia but disappeared in fully developed nodules (Fig. 4D). Interestingly, pNick4:YFP-NLS mimics the expression pattern of pNfr5:YFP-NLS. In both noninoculated and inoculated roots, the pNick4:YFP-NLS reporter was expressed in epidermal cells including root hairs (Fig. 4A). Nick4 was also expressed in cortical cells of inoculated roots. Unlike Nfr5, however, the expression of Nick4 was retained in mature nodules, predominantly observed in noninfected cells in the nodule parenchyma, nodule epidermis, and outer cortex (71, 72) (Fig. 4C).

To investigate whether Nfr5 and Nick4 were expressed in the same root hair and nodule cells, pNick4:YFP-NLS and pNfr5:mCherry-NLS reporter proteins were coexpressed in L. japonicus transformed roots (67–69). The tYFP-NLS reporter was maintained for Nick4 since the expression of pNick4:YFP-NLS was generally weaker than pNfr5:YFP-NLS. However, pNfr5:mCherry-NLS was only detectable after M. loti inoculation, with the strongest expression observed in cortical cells. We observed that pNfr5:mCherry-NLS and pNick4:YFP-NLS coexpress in the same root hair cells (Fig. 4K–M), cortical cells (Fig. 4 E–G), and nodule primordia (Fig. 4 H–J). This suggests that Nick4 and NFR5 may function together from the earliest stages of NF perception to later stages of nodule maturation.

**Nick4 Shuttles from the PM to the Nucleus upon NF Treatment.** Intrigued by the observation that the closest A. thaliana homolog of Nick4, AtCRPK1 (Fig. 1B and SI Appendix, Fig. S4), relays cold signals from the PM to the nucleus (58), we investigated the localization of Nick4-cGFP in the susceptible zone of Lotus roots before and after treatment with NF. As mentioned previously, Nick4 localizes to the PM when overexpressed (SI Appendix, Fig. S8). Following NF treatment of pLjUb:Nick4-cGFP transformed wild-type Lotus roots, we detected Nick4-cGFP relocalization to the nucleus in roots hairs of 6 out of 8 roots observed after 90 min of NF treatment (Fig. S4). This NF-induced movement is dependent on both Nick4 and NFR5. No nuclear localization of Nick4-cGFP was detected in any of the root hairs of 11 nfr5-2 or 9 nfr1-1 transformed hairy roots (Fig. S4).

**Basic protein more strongly than TRX-NFR1-CD and SymRK-CD (Fig. 3B), but none of the 3 active kinases phosphorylated BSA (Fig. 3 B and C). All E. coli-expressed proteins used in these kinase assays were soluble and monodisperse as indicated by monodisperse peaks in their SEC profiles (SI Appendix, Fig. S9).**

Unfortunately, we were unable to assess whether Nick4 could be phosphorylated by NFR1 or SymRK, as kinase-dead variants of Nick4 could not be expressed despite numerous attempts. However, Nick4 is strongly phosphorylated in the presence of both NFR5-CD298–595 and kinase-active NFR1-CD but not kinase-active SymRK-CD (Fig. 3D). Similarly, NFR5-CD298–595 and kinase-active NFR1-CD, but not kinase-active SymRK-CD, are strongly phosphorylated in the presence of Nick4. It is plausible that NFR5, NFR1, and Nick4 may form a tripartite signaling complex that serves to amplify the initial NF signal and 

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organogenesis but not infection thread formation.Component downstream of NFR5, which is involved in nodule organogenesis but not infection thread formation.

In the presence of NF, NiCK4 phosphorylates NFR5, possibly leading to formation or modification of docking sites for NFR5 interactors including NiCK4 and NFR1. NiCK4 subsequently phosphorylates NFR1, which then phosphorylates and triggers...

Fig. 4. NiCK4 and nfr5 display similar expression patterns. (A–D) Confocal microscopy of L. japonicus roots individually expressing pNick4:eGFP-NLS (A and C) or pNfr5:tYFP-NLS (B and D). In noninoculated roots, pNick4:eGFP-NLS (A) and pNfr5:tYFP-NLS (B) are expressed in epidermal cells including root hair cells. In nodules primordia, pNick4:eGFP-NLS expression is maintained in the nodule parenchyma (PA), cortex, and epidermis of mature nodules 14 d postinoculation (dpi) with M. loti strain MAF303099 expressing dsRed. (D) The expression of pNfr5:tYFP-NLS is strongly down-regulated in mature nodules 14 dpi with M. loti strain MAF303099. (E–M) Confocal microscopy of L. japonicus roots coexpressing pNick4:eGFP-NLS and pNfr5:mCherry-NLS, pNick4:eGFP-NLS and pNfr5:mCherry-NLS are coexpressed in cortical cells (E–G) and nodule primordia (H–J) 14 dpi with M. loti strain MAF303099 expressing dsRed. pNick4:eGFP-NLS and pNfr5:mCherry-NLS are also coexpressed in root hair cells (K–M) 11 dpi with M. loti strain MAF303099. The asterisks (*) indicate root hairs that coexpress pNfr5:mCherry-NLS and pNick4:eGFP-NLS. Arrowheads depict infection threads. Autofluorescence, YFP, and mCherry/dsRed channels are represented in white, green, and magenta, respectively. White nuclei indicate merged green and magenta nuclei. (Scale bars: 50 μm.)

Fig. 5. NiCK4 shuttles to the nucleus after Nod factor (NF) treatment and promotes nodulation. (A) In plJUb/NiCK4-eGFP transformed root systems, NiCK4-eGFP relocates to the nucleus (indicated with arrowheads) 90 min after NF treatment in roots of WT Lotus plants but not in transformed nfr1-1 or nfr5-2 mutant roots. Inset shows nuclear localization of NiCK4-eGFP at higher magnification. T = 0 and T = 90 represent images obtained from the same root hairs before and 90 min after NF treatment, respectively. The number of WT, nfr5-2, and nfr1-1 mutant plants imaged are 8, 11, and 9, respectively. (Scale bars: 50 μm.) (B) The predicted gene structure of NiCK4 with six exons indicated. The untranslated regions and coding sequences are represented by filled dark gray and light gray boxes, respectively, and the intronic regions are represented by black lines. (C) Nodulation counts of nick4 mutants and WT plants grown on agar plates 21 d after inoculation with M. loti strain NZP2235. Reduced nodulation was observed in nick4 mutants compared with WT plants. *P < 0.05 and **P < 0.01 (t test). White and pink nodules are represented by filled dark gray and light gray bars, respectively. (D) Working model for NiCK4 involvement in symbiosis signaling. In the presence of NF, NiCK4 phosphorylates NFR5, possibly improving its own docking site(s) or sites for hitherto-unknown NRFR5 interactors. NiCK4 then phosphorylates NFR1, which in turn phosphorylates NiCK4 and leads to NiCK4 dissociation and migration to the nucleus. This mechanism would relay the NF signal from the PM-localized receptors to the nuclear components involved in promoting nodule organogenesis. Given the nodulation phenotype of nick4 mutants, NiCK4 is not solely responsible for this relay.
the release of NiCK4 from the tripartite complex. NiCK4, in turn, migrates to the nucleus as one of the signal transduction components that relays the NF signal from PM-localized receptors to nuclear components involved in promoting nodule organogenesis.

Discussion

This study reports a proteomics approach for isolating proteins in the symbiotic receptor complex and the identification of NiCK4 in the nodulation signaling pathway. Nick4 and Nfr5 are expressed in the same root hair and cortical cells of L. japonicus roots, and the 2 proteins most likely form a complex on the PM as suggested by the FRET analysis in Fig. 2A. Upon NF treatment, phosphorylation events in the NFR1–NFR5–NiCK4 tripartite complex result in NiCK4 migrating from the PM to the nucleus. Symbiosis signaling processes that govern nodule organogenesis are then initiated, while the signaling processes required for the formation of root hair infection treads remain unperturbed. Such bifurcation of the signal transduction downstream of NFR1 and NFR5 was previously observed using a genetic dissection of the symbiosis signaling (4). Future work should define how NiCK4 ultimately regulates nodulation. This would involve detailed studies investigating how phosphorylation patterns of NFR5 and NFR1 influence activation of the downstream signal transduction, the role of NiCK4-mediated phosphorylation in this process, and notably the identification of NiCK4 substrates. The reduced nodulation observed in nick4 mutants indicates that NiCK4 plays a positive role either through modification of NFR5 docking platforms for downstream signaling components or by modification of the NFR1 activity. NiCK4 interaction and phosphorylation of other substrates that may influence signal transduction can also not be excluded.

RLCKs such as AtCRPK1 and ArabiB1K1 have been recently shown to relay signaling from the PM to the nucleus in response to cold or immune signals, respectively (58, 59). Interestingly, NiCK4 also acts as a receptor/coreceptor on the PM to nodule organogenesis that lead to nodule organogenesis. NiCK4 and several other NFR5-associated proteins contain closely related family members (Fig. 1B and SI Appendix, Figs. S3 and S4). This could explain why components downstream of NFR5 have remained elusive in genetic screens and suggest that higher-order mutants may be required to fully uncover their roles in symbiosis signaling.

Perception of extracellular signals in plants commonly involves PM-localized ligand-binding RLCKs, which typically require a receptor (s) and intracellular RLCK(s) (76, 77). The receptor/coreceptor/RLCK signaling mechanism is not limited to LysM-RKs, but is also found in receptor kinases that contain leucine-rich-repeat (LRRs) or malectin-like domains (MLDs), as summarized in SI Appendix, Fig. S12C. In rice [Oryza sativa (O)], LysM receptor-like kinases lacking a kinase domain, OsCEBIP and OsLYP4/YP6 bind chitin (78) and peptideglycan (79). The coreceptor OxCERK1 and its associated OsRLCK185 are essential for downstream chitin- and peptideglycan-induced immunity signaling (52). In A. thaliana, a similar LysM-pseudokinase/LysM-RK/RLCK complex involving A. thaliana ATR5/AtCERK1/ArPBL27, respectively, is responsible for chitin-induced MAP kinase activation (80). LRR-RKs such as flg22-binding ATRFLS2 (81) and elf18-binding ATRFLR (82) also require a coreceptor, ArabiB1K1 (83), and the RLCK, ATRCBL1, for initiating PAMP-induced reactive oxygen species burst and antibiotic immunity (50, 54). ATRB1K1 is also recruited as a coreceptor for the brassinosteroid (BR) receptor, ArabiB1R1 (84–86), which is another LRR-RR that interacts and phosphorylates RLCKs from the BSK family to activate BR signaling (87). Finally, the RALF peptide-binding MLD-containing ArANX1/ANX2 receptors most likely work with coreceptors such as other CRRLKL proteins, ATRBUPS1/BUPS2 (88), or LRR extensin (LRX) proteins (89), as well as the RLCK, ArMARI5 (56), to control pollen tube growth. The identification of NiCK4 as an interactor of NFR5 is therefore in line with these recent discoveries in various signaling processes that illustrate how RLCKs are of paramount importance in mediating signaling downstream of transmembrane receptors (90).

The association of NiCK4 and NFR5 in reciprocal co-IP experiments and the elucidation of the biological role of NiCK4 in promoting nodule organogenesis confirm that our proteomics approach to isolate interactors of NFR5 is valid and technically sound. NFR5-associated proteins with proposed symbiotic functions include LjLNP2 (91–93), LjPLLENTY2 (94), and the L. japonicus homologs of MhHMG1 (95, 96), MsSPK1 (97), and MmCMA8 (98). In addition to NiCK4, other interesting interactors that merit functional studies were found.

Three NFR5-associated RLCKs that were enriched in NF-treated samples include homologs of A. thaliana HERK1 CRRLKL protein that is involved in cell elongation processes (99, 100) and the LRR-RK BIR1 that negatively regulates several plant defense signaling pathways (101, 102), and the LjLYS13 LysM pseudokinase. LjLYS13 is the putative coreceptor of the LjCERK6 chitin receptor (103), which was also identified as an NFR5-associated receptor (Table 1). The up-regulation of Llys13 upon treatments/inoculations with M. loti, chitin, or Phytophthora palmivora (an oomycete plant pathogen) (104, 105) suggests that it could be implicated in symbiotic and defense signaling. Another NF-enriched NFR5-associated cell wall protein is the L. japonicus homolog of ArSAL1 that regulates auxin signaling (106) (Table 1). Interestingly, auxin signaling has been proposed to occur in parallel with the common symbiosis signaling pathway as NF-induced auxin accumulation was absent in nfr1-1 loss-of-function mutants (107).

The 2 NFR5-associated proteins that were found exclusively in mock-treated samples included the LjLNP2 and a β’ regulatory subunit of PP2A (Table 1 and SI Appendix, Table S1). LjLNP2 is closely related to LjLNP that has been proposed to function in parallel or downstream of the NF receptors (93). Moreover, PP2A has been shown to negatively regulate RK-mediated immune signaling in plants (108) and could possibly play a similar role in the NF signaling pathway. Other NFR5-associated RLCKs included a cysteine-rich RK (CRK), another CRRLKL and ArabiB1-like protein (101, 102), an AtRKL7-like protein (109), and a proline-rich extensin-like receptor kinase (PERK) (Table 1 and SI Appendix, Fig. S3). Three other RLCKs that associated with NFR5 include homologs of MsSPK1 (97), ArMARI5 (56), and ArBSK8 (64) (SI Appendix, Fig. S4 and Table 1). The PM-localized proton pump, identified as the top NFR5-associated protein (98), is responsible for depolarization of the PM and alakalization of root hair extracellular space occurs within minutes of NF application (6).

These remaining NFR5-associated proteins should be probed for their roles in symbiosis, defense, or cell wall remodeling. This would allow us to understand how the plant elegantly remodels its cell wall to accommodate rhizobia without mounting full-scale defense responses against them. A greater understanding of the functions of NFR5-associated proteins could also help to shed light on the mechanisms that NFR5 uses to elicit NF-triggered responses such as perinuclear calcium oscillations, extensive gene activation, root hair deformations, and invagination of the PM and cell wall to form infection threads.

Experimental Procedures

Plant Material and Growth Conditions for IP-MS Experiments. For IP-MS studies, heterozygous T1 Gifuup353; NFR5–eYFP-HA Lotus japonicus transgenic lines were generated by stable transformation of the WT L. japonicus Gifu plants (110, 111). WT and T1 seeds were germinated using the sulfuric acid method as previously described (112). Seedlings were grown on 1.4% Agar supplemented with 10 μM phosphothionin and by checking for YFP signal under a DM5500 B fluorescence microscope (Leica). At 8 d postgermination (dpg), WT and selected plants were transferred to 1.4% Agar Noble (Sigma-Aldrich) plates containing quarter-strength B and D media. At 10 dpg, the entire set of NFR5–eYFP-HA transgenic seedlings was harvested for PM localization and response to 200 nM NF using a DM6000 B microscope (Leica). Images were collected at 512–558 nm for YFP using a DAF filter. The plants were incubated at 22 °C under 16-h light and 8-h dark cycle.
Co-IP. At 17 dpg, roots of NFR5-eYFP overexpressing Lotus seedlings were individually treated with water (mock) or 200 mM 180.0 NF from M. loti strain R7A for 15 min. The roots were harvested and flash frozen in liquid nitrogen. Nontreated WT Gifu plants were used as controls. Root tissues were ground with a mortar and pestle. The samples were further homogenized in a Potter tube filled with extraction buffer containing 150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% (vol/vol) glycerol, 10 mM DTT, 1 mM PMFS, p9599 protease inhibitor mixture (Sigma-Aldrich), phosphatase inhibitor mixture 2 (Sigma-Aldrich), phosphatase inhibitor mixture 3 (Sigma-Aldrich), 1.5 mM Na3VO4, 1 mM NaF, and 0.5% (wt/vol) PVPP. IGEPAL CA-630 (Sigma-Aldrich), 0.1% (vol/vol) Triton X-100, and 0.5% (vol/vol) PVPP. IGEPAL CA-630 inhibitor mixture 2 (Sigma-Aldrich), phosphatase inhibitor mixture 3 (Sigma-Aldrich), 1.5 mM Na3VO4, 1 mM NaF, and 0.5% (wt/vol) PVPP. IGEPAL CA-630 (Sigma-Aldrich) was added to the extract and the sample was gently agitated at 4 °C for 40 min before centrifugation at 16,000 × g at 4 °C. The extract was then added to the supernatant, and the sample was gently agitated at 4 °C for 2.5 h. The beads were then spun at 100 × g for 1 min. The solution was aspirated out, and the beads were washed 3 times with extraction buffer containing 0.5% (wt/vol) IGEPAL CA-630 and 0.5% PVPP. Coinmunoprecipitated proteins were eluted by vortexing the samples thoroughly before heating them at 70 °C for 15 min. One microliter of eluted protein samples was separated on a SDS/PAGE gel, and NFR5-eYFP IP samples was done as previously described (113). Gel slices were destained in 50% acetonitrile. Reduction and alkylation was done by incubation for 45 min in 10 mM DTT, followed by 30 min in the dark in 55 mM chloroacetamide. After several washes with 25 mM ammonium bicarbonate, 50% acetonitrile gel slices were dehydrated in 100% acetonitrile. Gel pieces were rehydrated with 50 mM ammonium bicarbonate and 5% acetonitrile containing 20 ng/l YFP trypsin (Pierce), and digestion proceeded overnight at 37 °C. Tryptic peptides were sonicated from the gel in 5% formic acid and 50% acetonitrile, and the total extracts were evaporated until dry. LC-MS/MS analysis was performed with an EASY-nLC 1000 RPLC system (Thermo Scientific) and a nanoflow-HPLC system (Dionex Ultimate3000; Thermo Scientific). The peptide identification was performed by searching the Lotus japonicus proteome database (version 2.5) using Mascot (version 2.4.1; Matrix Science) with the modification of allowing trypsin peptide termini. Scaffold (version 4; Proteome Software) was used to validate MS/MS-based peptide and protein identifications and to annotate spectra using search criteria of a minimum of 2 peptides with Mascot ion scores above 20% and 95% peptide identity. Selected spectra were manually inspected before acceptance.

Protein Expression and Imaging in N. benthamiana Leaves. The cDNA sequence of the Nfr1 and Nfr5, and genomic sequence of SycDK, was available in house, while Nick4 and LjLt6b cDNA sequences were commercially synthesized and inserted into a pSPTG6 vector with Golden Gate technology (114) to produce GFP, mCherry, or FLAG fusion constructs. Confocal microscopy images were obtained as described previously (115). Images of N. benthamiana epidermal cells were taken 2 d postinoculation with Agrobacterium tumefaciens strain GV3101 transformed with plasmid DNA. GFP and mCherry were excited at 488 and 543 nm, respectively. Fluorescence emissions were collected between 491 and 561 nm, respectively. Reciprocal co-IP studies were performed as previously described (116). MS-IP-MS analysis of Gifu control and NFR5-eYFP IP samples was done as previously described (68, 69). Lotus roots expressing Nfr1/eYFP were imaged using 488-nm excitation and 500- to 540-nm emission. Eight WT Gifu, 11 nfr5-2, and 9 nfr1-1 mutant plants were imaged before and 90 min after being treated with 100 mM 18.1% NF from M. loti strain R7A.

Protein Expression in E. coli. The sequences of Nick4 and Symk-cd were amplified from L. japonicus root cDNA. Nfr1-cd and Nfr5-cd sequences were amplified from in-house plasmids encoding cDNA sequences of full-length Nfr1 and Nfr5. The sequences were cloned into various vectors to generate Nick4, Nfr1-cd, Nfr5-cd, Nick5-cd, Nfr5-cd, and Symk-cd (SI Appendix, Table S5). The amino-acid-substitution analysis of NFR1-T483A was introduced with the QuikChange-Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) (Stratagene) to produce Nick4 (SI Appendix, Table S5). The verified plasmids were then transformed into Heat Competent Rosetta 2 E. coli cells (Novagen). The protein expression and purification procedures were performed as described in ref. 117. Briefly, the cell pellets were resuspended with lysis buffer containing 50 mM Tris-HCl, pH 8, 500 mM NaCl, 20 mM imidazole, pH 8, 1 mM benzamidine, 5 mM β-mercaptoethanol, and 10% (vol/vol) glycerol. The supernatants were then loaded onto Ni-NTA columns (Qiagen), which were washed with buffer containing 50 mM Tris-HCl, pH 8, 1 M NaCl, 500 mM imidazole, pH 8, 1 mM benzamidine, 5 mM β-mercaptoethanol, and 10% (vol/vol) glycerol. His-tagged proteins were finally eluted with buffer containing 50 mM Tris-HCl, pH 8, 500 mM NaCl, 500 mM imidazole, pH 8, 1 mM benzamidine, 5 mM β-mercaptoethanol, and 10% (vol/vol) glycerol. After TEV protease digestion and a reverse IMAC purification step, the protein samples were loaded onto ENrich 70 10/300 (Bio-Rad), Superdex 75 increase 10/300 (GE Healthcare) or Superdex 200 increase 10/300 (GE Healthcare) SEC columns connected to an AKTA PURIFIER system (GE Healthcare). Proteins were eluted with buffer containing 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM β-mercaptoethanol, and 5% (vol/vol) glycerol.

Binding Studies via MST. Protein ligands were dialyzed overnight in buffer containing 50 mM phosphate, pH 7.5, 200 mM NaCl, and 10% glycerol. GFP and mCherry-labeled proteins were dialyzed on a TALON protein purification column with buffer containing 50 mM phosphate, pH 7.5, 200 mM NaCl, and 5% glycerol before labeling with the Monolith Protein Labeling Kit BLUE (Nanotemper Technologies). Prior to MST measurements, the labeled and ligand proteins were spun down at 16,843 × g at 4 °C for 30 min to remove aggregated proteins. MST experiments were performed in buffer containing 50 mM phosphate, pH 7.5, 200 mM NaCl, 10% glycerol, 20 mM ATP, pH 7, 4 mM MgCl2, and 0.1% Tween 20. For each MST experiment, a 2-fold dilution series was prepared in which 200 μM of Nick1-cd, Nick5-cd, or Symk-cd was added to the first tube and titrated 1:1 across the following 15 tubes. An equal volume of 100 to 200 nM labeled Nick4 protein was then added to all 16 tubes. The proteins were mixed, loaded onto standard glass capillaries (Nanotemper Technologies), and incubated for 1 h at room temperature before analysis on a Monolith NT.115 apparatus (NanoTemper Technologies). MST experiments were run at room temperature with LED powers of 50% or 80%, MST powers of 20% or 50%, 30 s laser on time, and 5 s laser off time. The data were analyzed with the NT-Analysis software (Nanotemper Technologies) and fitted with GraphPad Prism 6 software using the sigmoidal dose–response model to obtain the equilibrium dissociation constant (95% confidence interval).

Phosphorylation Studies. Proteins were incubated with 100 nCi [32P]ATP (PerkinElmer) in 50 mM Tris-HCl, pH 8, buffer consisting of 20 mM MgCl2, 5 mM MnCl2, and 20 μM cold ATP at room temperature for 1 h. The samples were then separated on SDS/PAGE gels, which were exposed overnight on phosphor plates (Molecular Dynamics). The phosphor plates were scanned with the typhoon TRIOS scanner (Amersham Biosciences).
Phenotyping of LORE1 Mutants. From segregating LORE1 mutant populations, plants homozygous for the WT gene or LORE1 insertion in the gene of interest were identified via PCR amplification using gene-specific forward and reverse primers, or the gene-specific forward primer and LORE1 reverse primer, respectively (SI Appendix, Table S6). Homozygous mutants were isolated and assessed for nodulation capacity on 1.4% agar Noble (Sigma-Aldrich) plates containing quarter-strength B and D media, or in pots containing lightweight expanded clay aggregate (LECA; Saint-Gobain Weber) and vermiculite size M (Darmorlin). Seedlings were treated with M. loti strain N2P2235 (OD600 = 0.02) that were grown in yeast mannitol broth at 28 °C for 48 h with a rotational speed of 180 rpm. Plates were incubated at 22 °C under 16-h light and 8-h dark cycle and pots were incubated in the greenhouse. Nodules were counted after 3 and 8 wk, respectively. For infection thread counts, seedlings grown on plates were inoculated with M. loti strain MAFF303099 expressing dfr (OD600 = 0.01) for 10 d before infection threads were counted.

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