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

Wong, Jaslyn E.M.M.; Nadzieja, Marcin; Madsen, Lene H.; Bücherl, Christoph A.; Dam, Svend; Sandal, Niels N.; Couto, Daniel; Derbyshire, Paul; Uldum-Berentsen, Mette; Schroeder, Sina; Schwämmle, Veit; Nogueira, Fábio C.S.; Asmussen, Mette H.; Thirup, Søren; Radutoiu, Simona; Blaise, Mickaël; Andersen, Kasper R.; Menke, Frank L.H.; Zipfel, Cyril; Stougaard, Jens

Published in:
Proceedings of the National Academy of Sciences of the United States of America

DOI:
10.1073/pnas.1815425116

Publication date:
2019

Document version
Final published version

Document license
CC BY-NC-ND

Citation for published version (APA):

Terms of use
This work is brought to you by the University of Southern Denmark through the SDU Research Portal. Unless otherwise specified it has been shared according to the terms for self-archiving. If no other license is stated, these terms apply:

- You may download this work for personal use only.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying this open access version.
A Lotus japonicus cytoplasmic kinase connects Nod factor perception by the NFR5 LysM receptor to nodulation

Jaslyn E. M. M. Wong,1,2 Marcin Nadzieja,1, Lene H. Madsen,2 Christoph A. Bücheler,2 Svend Dam,2 Niels N. Sandal,3 Daniel Couto,3, Paul Derbyshire,2 Mette Ulum-Berentsen,1 Sina Schroeder,2,4 Veit Schwämmle,5 Fábio C. S. Nogueira,6 Mette H. Asmussen,6 Søren Thirup,7 Simona Radutoiu,6 Mickaël Blaise,5 Kasper R. Andersen,8 Frank L. H. Menke,8 Cyril Zipfel,9,6 and Jens Stougaard1,7

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

Edited by David C. Baulcombe, University of Cambridge, Cambridge, United Kingdom, and approved June 4, 2019 (received for review September 7, 2018)

The establishment of nitrogen-fixing root nodules in legume–rhizobium 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 NFRe (5–7)—perceive NFs that constitute the major rhizobial signal (8–11). Both NFR1 and NFR5 are indispensable for NF signaling (5, 6) while NFRe was suggested to amplify signaling in root epidermal cells (7). NFR1, NFR5, and NFRe are ligin motif (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 NFRe can directly and independently bind NF through their extracellular domains (7, 12). The kinase domains of NFR1 and NFRe 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, NFR1 associates with NFR5 in bimolecular fluorescence complementation (BFC) assays (19). Second, the expression pattern of NFR5 and NFR1 is similar in root hairs and nodule cells (20). Third, a bimolecular fluorescence complementation (BFC) assay identified Nick4, which is coexpressed with NFR5 in legume root hairs and nodule cells, as a potential NFR5-interacting RLCK (20). In this paper, we sought to characterize Nick4 functionally and in vivo in order to test the hypothesis that Nick4 is an important link in the signal transduction process that leads to the development of root nodules that house nitrogen-fixing rhizobia.

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.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

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.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1815425116/-/DCSupplemental.
(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 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 Nfp1 and Nfp5 (22).

Genetic approaches have identified several symbiosis components acting downstream of NFR1 and NFR5. A putative co-receptor 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 (CcAmK) (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 coimmunoprecipitation (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 (NcCK4), was characterized biochemically and genetically. The discovery of NcCK4 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). NFR 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 triniturate co-IP experiments using ~3,000 NFR5-eYFP root hairs for each of the triniturate experiments, as summarized in SI Appendix, Fig. S1D. The transgenic roots were treated either with 200 mM 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, S282 and T576 (SI Appendix, Fig. S2 C 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

Fig. 1. Co-IP of NFR5-eYFP and MS discovery of NcCK4. (A) SimplyBlue SafeStained gel of proteins coimmunoprecipitated with GFP-trap beads. The molecular weights of the marker proteins (in kilodaltons) are indicated next to their corresponding protein bands. The corresponding Western blot of the GFP-HRP antibodies used for visualization. (B) Co-IP of NFR5-eYFP and MS discovery of NcCK4. (C) SimplyBlue SafeStained gel of proteins coimmunoprecipitated with GFP-trap beads. The molecular weights of the marker proteins (in kilodaltons) are indicated next to their corresponding protein bands. The corresponding Western blot of the GFP-HRP antibodies used for visualization. (D) Co-IP of NFR5-eYFP and MS discovery of NcCK4.
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 the fourth NFR5-associated RLCKs in the CRPK1 family is 7 and 8, respectively, compared with 2 in *A. thaliana* (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 neofunctionalized 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. 1C 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). 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

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AtCRK29-like protein</td>
</tr>
<tr>
<td>2</td>
<td>AT2G32200-like CrRLK1 protein</td>
</tr>
<tr>
<td>3</td>
<td>AtBIR1-like protein 1</td>
</tr>
<tr>
<td>4</td>
<td>AtRLK7-like protein</td>
</tr>
<tr>
<td>5</td>
<td>AtPERK1-like protein</td>
</tr>
<tr>
<td>6</td>
<td>LjCERK6 LysM protein</td>
</tr>
<tr>
<td>7</td>
<td>AthERK1-like CrRLK1 protein</td>
</tr>
<tr>
<td>8</td>
<td>AtBIR1-like protein 2</td>
</tr>
<tr>
<td>9</td>
<td>LjLYS13 LysM protein</td>
</tr>
<tr>
<td>10</td>
<td>AtPT11-like protein</td>
</tr>
<tr>
<td>11</td>
<td>AtMSK1-like protein</td>
</tr>
<tr>
<td>12</td>
<td>AtBSK1-like protein</td>
</tr>
<tr>
<td>13</td>
<td>NiCK4/AtCRPK1-like protein</td>
</tr>
<tr>
<td>14</td>
<td>Phosphoinositide phosphatase/AtRHD4-like protein</td>
</tr>
<tr>
<td>15</td>
<td>AtPP2C76-like protein</td>
</tr>
<tr>
<td>16</td>
<td>PP6 regulatory subunit/AtSAL1-like protein</td>
</tr>
<tr>
<td>17</td>
<td>PP2A B’ regulatory subunit-like protein</td>
</tr>
<tr>
<td>18</td>
<td>LjLP2</td>
</tr>
</tbody>
</table>

**Cell wall remodeling proteins**

19 | Putative pectinacetylesterase                 |
20 | Putative O-fucosyltransferase                 |
21 | Putative callose synthase/AtGLS5-like protein |
22 | Putative callose synthase/AtGLS10-like protein |
23 | AtLPLENTY2/Hyp O-arabinosyltransferase        |
24 | Putative xyloglucan xylosyltransferase        |
25 | AtCesA2-like protein                          |
26 | Putative galacturonosyltransferase            |
27 | AtCesA3-like protein                          |

**Ion pumps**

28 | AtAHAlike PM proton pump                     |
29 | AtMIA-like protein                           |
30 | MtMCA8-like ER calcium pump                  |
31 | AtACA10-like PM calcium pump                 |

**Others**

32 | AtAIR9-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 | AtDaylleeper-like protein                   |
38 | AtUXT3-like protein                          |
39 | AtRFC3-like protein                          |
40 | AtERD4-like protein                          |
41 | Putative carboxypeptidase                    |
42 | FG-GAP repeat-containing protein             |
43 | MTHMGRI-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 | AtSMD1-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.
only NFR5-eGFP and not eGFP-Lj/LTI6b 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, Figs. 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 glycin-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 scaffoldings (64), in which phosphorylation of the pseudokinase domain could potentially result in the formation of docking platforms for downstream signaling components that modulate signaling outputs (65). Unlike NFR5, NicK4 possesses the hallmark features of an active kinase such as the DFG, the AXK 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 to 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 auto-phosphorylation 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
basic protein more strongly than TRX-NFR1-CD and SymRK-CD (Fig. 3B), but none of the 3 active kinases phosphorylated BSA (Fig. 3B and C). All E. coli-expressed proteins used in these kinase assays were soluble and nonaggregated 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 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 MAF730399 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 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 Nfr5 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 predominantly observed in noninoculated 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 pNiCK4 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. 4 K–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-eGFP 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-eGFP transformed wild-type Lotus roots, we detected NiCK4-eGFP relocalization to the nucleus in roots hairs of 6 out of 8 roots observed after 90 min of NF treatment (Fig. 5A). This NF-induced movement is dependent on both NFR5 and NFR1. No nuclear localization of NiCK4-eGFP was detected in any of the root hairs of 11 nfr5-2 or 9 nfr1-1 transformed hairy roots (Fig. 5A).

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 mutants containing LORE1 insertions in exonic regions of the Nick4 gene, named nick4-1 to nick4-3, were isolated for phenotypic analyses (Fig. 5B). Plants not containing any LORE1 insertion from the nick4-2 LORE1 segregating population were also isolated and served as the wild-type (WT) outgroup 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

Wong et al.
We hereby propose a model describing NiCK4 as an important signaling component of the NFR5 signaling pathway (Fig. 5D). 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:tYFP-NLS (A and C) or pNfr5:tYFP-NLS (B and D). In noninoculated roots, pNiCK4:tYFP-NLS (A) and pNfr5:tYFP-NLS (B) are expressed in epidermal cells including root hair cells. In nodule primordia, pNiCK4:tYFP-NLS expression is maintained in the nodule parenchyma (PA), cortex, and epidermis of mature nodules 14 d postinoculation (dpi) with *M. loti* strain MAFF303099 expressing dsRed. (D) The expression of pNfr5:tYFP-NLS is strongly down-regulated in mature nodules 14 dpi with *M. loti* strain MAFF303099. (E–M) Confocal microscopy of *L. japonicus* roots coexpressing pNiCK4:tYFP-NLS and pNfr5:mCherry-NLS. pNiCK4:tYFP-NLS and pNfr5:mCherry-NLS are coexpressed in cortical cells (E–G) and nodule primordia (H–J) 14 dpi with *M. loti* strain MAFF303099 expressing dsRed. pNiCK4:tYFP-NLS and pNfr5:mCherry-NLS are also coexpressed in root hair cells (K–M) 11 dpi with *M. loti* strain MAFF303099. The asterisks (*) indicate root hairs that coexpress pNiCK4:mCherry-NLS and pNiCK4:tYFP-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 *pLjUbi:nick4-eGFP* transformed root systems, NiCK4-eGFP relocates to the nucleus (indicated with arrowheads) 90 min after NF treatment in roots of *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 boxes, 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 NFR5 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 threads 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 AtCRP1K and AtBIRK1 have been recently shown to relay signaling from the PM to the nucleus in response to cold or immune signals, respectively (56, 59). Interestingly, NiCK4 also perceives and transmits PM-nuclear signals 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 1 (or more) coreceptor(s) and intracellular RLCK(s) (76, 77). The receptor/coreceptor/RLCK signaling mechanism is not limited to LysM-RLCKs, but is also found in receptor kinases that contain leucine-rich repeat (LRRs) or leucine-rich domains (MLDs), as summarized in SI Appendix, Fig. S12C. In rice [Oryza sativa (Os)], LysM receptor-like proteins lacking a kinase domain, OsCEBIP and OsLYP4/LYP6 bind chitin (78) and peptidoglycan (79). The coreceptor OsCERK1 and its associated OsRLCK185 are essential for downstream chitin- and peptidoglycan-induced immunity signaling (52). In A. thaliana, a similar LysM-pseudokinase/LysM-RLK/RLCK complex involving AtLYK5/AtCERK1/AtPBL27, respectively, is responsible for chitin-induced MAP kinase activation (80). LRR-RLCKs such as flg22-binding AtFLS2 (81) and elf18-binding AtEFR (82) also require a coreceptor, AtBAK1 (83), and the RLCK, AtBIRK1, for initiating PAMP-induced reactive oxygen species burst and antibacterial immunity (50, 54). AtBAK1 is also recruited as a coreceptor for the brassinosteroid (BR) receptor, AtBRI1 (84–86), which is another LRR-RLK that interacts and phosphorylates RLCKs from the BSK family to activate BR signaling (87). Finally, the RALF peptide-binding MLD-containing AraANX1/ANX2 receptors most likely work with coreceptors such as other CRRLKL proteins, AtBUPS1/BUPS2 (88), or LRR extensin (LRX) proteins (89), as well as the RLCK, ArMARIS (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), LjPLNTY2 (94), and the L. japonicus homologs of MtHMG1 (95, 96), MsSPK1 (97), and MmCA8 (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 RK (Table 1). The up-regulation of LjLYS13 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 elongation protein is the L. japonicus homolog of AtSAL1 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 B’7 regulatory subunit of PPA2 (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 (95). Moreover, PPA2 has been shown to negatively regulate RR-mediated immune signaling in plants (108) and could possibly play a similar role in the NF signaling pathway. Other NFR5-associated RKs included a cysteine-rich RK (CRK), another CRRLKL and AtBIR1-like protein (101, 102), an AtRLK7-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), ArMARIS (56), and AtBSK8 (64) (SI Appendix, Fig. S4 and Table 1).

The PM-localized proton pump, identified as the top NFR5-associated protein of the L. japonicus homolog of the PM and alkalization 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 Gifu3535/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 supplementation with 10 μg/mL phosphinothrin and by checking for YFP signal under a DM5500 B fluorescence microscope (Leica). At 8 d post-germination (dp), WT and selected plants were transferred to 1.4% Agar Noble (Difco) plates, and NFR5-eYFP overexpressing plants were selected by supplementation with 10 μg/mL phosphinothrin and by checking for YFP signal under a DM5500 B fluorescence microscope (Leica). Images were collected at 512–518 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 nM 18:0 NF from 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 NaN3, 1 mM NaF, and 0.5% (wt/vol) PVPP. IGEPA CAS-630 (1.0% (vol/vol)) (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. Preequilibrated G-Trap beads (ChromoTek) were 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% (instead of 1.0%) IGEPA CAS-630 and no PVPP. Coimmunoprecipitated 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 an SDS-PAGE gel, and NFR5-eYFP IP samples were 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/μl 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 Orbitrap Fusion Trihybrid mass spectrometer (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 SymRk, was available in house, while Nick4 and Lii166 cDNA sequences were commercially synthesized. Triparental mating followed by hairy root induction in N. benthamiana Gifu plants was used as controls. Root tissues were extracted during the bleaching process. Bleaching parameters were described in ref. 68. Leaves 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 nM 18:1 NF from M. loti strain 7RA.

Protein Expression in E. coli. The sequences of Nick4 and Symrk-cd were amplified from N. 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, Trx-Nfr1-cd, Nfr5-cd, Symrk-cd, and Symrk-cd (SI Appendix, Table S5). The amino-acid subunit composition of NFR1-T483A was introduced with the QuikChange-Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) using the primer (SI Appendix, Table S5). Verified plasmids were then transformed into Heat Competent Rosetta 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 25 mM Tris-HCl, pH 8, 1 M NaCl, 50 mM imidazole, 10% (vol/vol) glycerol, 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 ÄKTA 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. Proteins were labeled with 100 nM 18:0 NF from M. loti strain R7A were conjugated into pIV10 plasmid using Golden Gate cloning system (114). The plasmids were introduced with the QuikChange-Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and incubated for 1 h at room temperature. Prior to MST measurements, the labeled protein and ligands were spun down at 16,843 g at 4 °C for 30 min to remove aggregates. MST experiments were performed in buffer containing 50 mM phosphate, pH 7.5, 200 mM NaCl, 10% glycerol, 200 μM 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 NFR1-CDS, NFR5-CDS, or Symrk-CDS was added to the first tube and diluted 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 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 [γ-32P]ATP (3000 Ci/mmol) in 50 mM Tris-HCl, pH 8, 80 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 TRIO scanner (Amersham Biosciences).
Phenotyping of LORF1 Mutants. From segregating LORF1 population, plants homozygous for the WT gene or LORF1 insertion in the gene of interest were identified via PCR amplification using gene-specific forward and reverse primers, or in LORF1 revertor and LORF1 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 (Damarol). Seedlings were treated with M. loti strain NZP2235 (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 dtd (OD600nm = 0.01) for 10 d before infection threads were counted.

ACKNOWLEDGMENTS. We thank Anita Bek for performing the NFR5-eYFP complementation experiments in nfr5-2 plants, Finn Pedersen for greenhouse assistance, Simon Kelly for providing cDNA from Lotus root, Noor de Jong for help with plant transformation, Rikke Jespersen and Dugald Reid for generating and analyzing gene expression data, Hongtao Ji for generating LORF1 mutant plants, and Stig U. Andersen and Peter Røepstorff for scientific discussions. This work was supported by the Danish National Research Foundation Grant DNRF79 (to J.S.), the Gatsby Charitable Foundation and the European Research Council Grant "PHOSPHinATE" (to C.Z.), a Short-Term Fellowship from the European Molecular Biology Organization (ASTF, 615-2014) (to J.E.M.M.W.), and a scholarship (SFRH/BDE/90882/2011) from the Portuguese Government (Fundaçao para a Ciência e a Tecnologia) (to D.C.).

C. Zipfel

D. Couto, C. Zipfel, Regulation of pattern recognition receptor signalling in plants.

M. Hayafune


H. Shi

Y. Ao

Z. Liu


B. S. Blaum

C. Grütter, S. Sreeramulu, G. Sessa, D. Rauh, Structural characterization of the RLCK family member BSK8: A pseudokinase with an unprecedented architecture.


D. F. Urba


F. C. Güel, Genetic dissection of the legume nodule 1: The structure of the peripheral zone in four nodule types. Botany 87, 1117–1138 (2009).


