Atypical Receptor Kinase RINRK1 Required for Rhizobial Infection But Not Nodule Development in Lotus japonicus

Xiaolin Li,a,2 Zhiqiong Zheng,a,b,2 Xiangxiao Kong,a,b,2 Ji Xu,a Liping Qiu,a Jongho Sun,c Dugald Reid,d Haojie Jin,d Stig U. Andersen,d Giles E. D. Oldroyd,c Jens Stougaard,d J. Allan Downie,e and Fang Xiea,3,4

aNational Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai 200032, China bUniversity of the Chinese Academy of Sciences, Beijing 100049, China cSainsbury Laboratory, University of Cambridge, Cambridge CB2 1LR, United Kingdom dDepartment of Molecular Biology and Genetics, Aarhus University, Aarhus 8000 C, Denmark eJohn Innes Centre, Norwich Research Park, Norwich NR4 7UH, United Kingdom

During the legume-rhizobium symbiotic interaction, rhizobial invasion of legumes is primarily mediated by a plant-made tubular invagination called an infection thread (IT). Here, we identify a gene in Lotus japonicus encoding a Leu-rich repeat receptor-like kinase (LRR-RLK), RINRK1 (Rhizobial Infection Receptor-like Kinase1), that is induced by Nod factors (NFs) and is involved in IT formation but not nodule organogenesis. A paralog, RINRK2, plays a relatively minor role in infection. RINRK1 is required for full induction of early infection genes, including Nodule Inception (NIN), encoding an essential nodulation transcription factor. RINRK1 displayed an infection-specific expression pattern, and NIN bound to the RINRK1 promoter, inducing its expression. RINRK1 was found to be an atypical kinase localized to the plasma membrane and did not require kinase activity for rhizobial infection. We propose RINRK1 is an infection-specific RLK, which may specifically coordinate output from NF signaling or perceive an unknown signal required for rhizobial infection.

During the initiation of symbiotic nitrogen-fixation in legumes, signaling receptor complexes perceive nodulation factors (NFs) synthesized by rhizobia, which induce and coordinate rhizobial infection and nodule organogenesis (Oldroyd and Downie, 2008). NFs are lipochitin-oligosaccharides (LCOs), which, in different rhizobia, carry different acyl chains (C16-20) with variable degrees of saturation together with different substitutions on the oligosaccharide backbone (Lerouge et al., 1990; Spank et al., 1991; Cullimore et al., 2001; Bek et al., 2010). These substitutions are determinants of the host-specific interactions between a given rhizobial strain and its host legume (Lerouge et al., 1990; Spank et al., 1991).

In Lotus japonicus, NFs made by Mesorhizobium loti are perceived by a receptor complex containing NFR1 and NFR5 (Nod factor receptors 1 and 5; Madsen et al., 2003; Radutoiu et al., 2003). NFR1 and NFR5 encode LysM receptor kinases that are located in the plasma membrane, interact with each other, and directly bind to NFs using high-affinity binding sites in their ectodomains (Madsen et al., 2011; Broghammer et al., 2012). Knockout mutations in either NFR1 or NFR5 eliminate almost all NF-inducible responses, including induction of root hair deformation, perinuclear calcium oscillations (calcium spiking), calcium influx, and induction of genes required for the development and infection of nodules (Madsen et al., 2003; Radutoiu et al., 2003; Miwa et al., 2006; Høgslund et al., 2009), fitting with their function as signaling receptors. In Medicago truncatula, NFP (Nod factor perception) corresponds to NFR5, and mutations in this gene also cause complete loss of

1This work was funded by National Key R&D Program of China (2016YFA0500500), Chinese Academy of Sciences (CAS) (the Strategic Priority Research Program: XDB27040208 and IPP 153D31KYSB20160074), and National Natural Science Foundation of China (NSFC) (31470344) and received support from Biotechnology and Biological Sciences Research Council (BBSRC) (Award E017045/1) and from the John Innes Foundation (JIF) (to J.A.D.).
2These authors contributed equally to this work.
3Author for contact: xiefang@sibs.ac.cn.
4Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Fang Xie (xiefang@sibs.ac.cn).

X.-L.L., X.-X.K., and F.X. designed the experiments; X.-L.L., Z.-Q. Z., and X.-X.K. performed the experiments; X.-L.L., X.-X.K., J.A.D., and F.X. analyzed the data; H.J, S.U.A., and J.S. sequenced and X.-X.K. performed the experiments; X.-L.L., X.-X.K., J.A.D., and

Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.19.00509

804 Plant Physiology®, October 2019, Vol. 181, pp. 804–816, www.plantphysiol.org © 2019 American Society of Plant Biologists. All Rights Reserved.
NF-inducible responses (Arrighi et al., 2006). However, although LYK3 corresponds to NFR1, lyk3 mutations block formation of infection threads but do not impact cortical cell divisions associated with nodule morphogenesis, suggesting that LYK3 might act as an NF receptor (Catoira et al., 2001; Smit et al., 2007). Although M. loti mutants imply a similar differentiation in NF perception in L. japonicus (Rodpoothong et al., 2009), an equivalent NF receptor controlling entry has not been identified.

Coexpression of the L. japonicus NFR1 and NFR5 genes in roots of M. truncatula enables the formation of some uninfected nodules in response to M. loti (Radutoiu et al., 2007). Because M. loti normally does not induce nodule formation on M. truncatula, this indicates NFR1 and NFR5 play a role in host-specific nodulation and additional signaling is required for host recognition during the infection process. Symbiosis receptor kinase (SYMRRK) is a plasma membrane-located receptor-like kinase (RLK) involved in symbiotic signaling in L. japonicus (Stracke et al., 2002), and can interact with NFR5, implying a heteromeric receptor complex that mediates NF signaling (Antolin-Llovera et al., 2014). Overexpression of NFR1, NFR5, or SYMRRK can activate spontaneous nodule formation in the absence of rhizobia (Ried et al., 2014). In addition, some lipid raft proteins, such as flotilins (Haney and Long, 2010), and symbiosis-specific remorins (SYMREM) interact with symbiotic RLKs to help mediate rhizobial infection (Lefebvre et al., 2010; Tóth et al., 2012; Liang et al., 2018).

Rhizobial infection in most legumes is mediated by a tubular invagination called an infection thread. This often initiates in root-hair cells, in which rhizobia attach to the tip, inducing deformation of the tip region that leads to entrapment of the rhizobia in so-called ‘infection pockets’ formed by the root hair tip curling back on itself around the attached bacteria (van Brussel et al., 1992; Gage, 2004). Cell wall and membrane remodeling accompanied by cytoskeletal rearrangements are initiated at this early stage and lead to the inward growth of the infection thread toward the base of root hairs (van Sprosen et al., 1994; Brewin, 2004; Murray, 2011; Fournier et al., 2015). Its then crosses the walls of the root hair and adjacent cortical cell and continues growing down through layers of cortical cells, eventually extending into those dividing cortical cells that correspond to the developing nodule primordium. Several proteins required for infection thread formation have been identified including a nodulation-specific pectate lyase (NPL; Xie et al., 2012); several components of the WAVE/SCAR-APR2/3 complex (NAP, PIR, SCARN, and ARPC1; Yokota et al., 2009; Miyahara et al., 2010; Hossain et al., 2012; Qiu et al., 2015) required for actin nucleation; a putative ubiquitin-E3 ligase LIN/CERBERUS; a coil-coil domain protein RPC; and Vapyrin, which contains a VAMP-associated protein (VAP)/ major sperm protein (MSP) domain and several ankyrin-repeat domains (Arrighi et al., 2008; Kiss et al., 2009; Yano et al., 2009; Murray et al., 2011).

The phenotypes caused by mutations in these various genes show they are required for infection, but their precise biological/biochemical functions are not known. In addition, mutations causing other infection defects have been identified in L. japonicus (lot1, crinkle, sym8, sym104, sym105, and alb1), but the genes affected have not yet been described (Imazumi-Anraku et al., 1997; Sandal et al., 2006; Murray, 2011; Oldroyd et al., 2011). Rhizobial secreted exopolysaccharides (EPSs) are important for infection thread initiation and formation, and EPS has been suggested to function as a signal in the chronic intracellular infection of plant cells of nitrogen-fixing root nodules (Gibson et al., 2008; Kelly et al., 2013). Recently, a LysM receptor kinase EPR3 (Exopolysaccharide receptor 3) was reported to be an EPS receptor in L. japonicus (Kawaharada et al., 2015, 2017b).

The transcription factors NIN (Nodule inception), ENR1 (ERF required for nodulation 1), NSP1 and NSP2 (Nodulation signaling pathway 1 and 2) also play crucial roles in rhizobial infection (Heckmann et al., 2006; Bek et al., 2010; Madsen et al., 2010; Cerri et al., 2017; Kawaharada et al., 2017a). NIN was the first essential nodulation gene identified in L. japonicus (Schauer et al., 1999), and it coordinates the regulation of rhizobial infection and nodule organogenesis (Vernié et al., 2015). The DNA-binding motif for NIN has been identified (Soyano et al., 2013, 2014), and NIN can directly bind to and activate expression of several infection-specific genes, such as NPL and SCARN, as well as genes required for nodule organogenesis, such as CRE1 and NF-YA1 (Xie et al., 2012; Soyano et al., 2013; Qiu et al., 2015; Vernié et al., 2015).

In this work, we identified a gene necessary for infection thread development encoding a Leu-rich-repeat (LRR) type RLK that we have named RINRK1 (Rhizobial infection receptor-like kinase 1). RINRK1 showed a specific expression pattern related to rhizobial infection. Because RINRK1 is required for full NIN induction and NIN is required for RINRK1 induction, there may be positive feedback involving RINRK1 and NIN, possibly resulting in amplification of NF signaling associated with rhizobial infection.

RESULTS

Identification of a LRR-RLK Required for Rhizobial Infection

The infection thread-defective mutant itd4 (SL3055-2) was isolated from an ethyl methanesulfonate (EMS) mutagenized pool of L. japonicus Gifu B-129 as having a 10-fold reduction in the number of infection threads based on histochemical staining of lacZ-marked M. loti R7A (Lombardo et al., 2006). The mutation causing the infection defect was previously mapped to the short arm of linkage group I, using a mapping population generated by crossing itd4 with L. japonicus Miyako-jima (MG20; Lombardo et al., 2006). Another L. japonicus
infection-defective mutant, \( alb1 \), was described previously (Imazumi-Anraku et al., 1997, 2000), and rough-mapping indicated \( ALB1 \) was also located on Chromosome I in the same region that precluded precise mapping in crosses with \( L. japonicus \) MG20. The \( alb1 \) mutation was reported not to be allelic with \( itd4 \), even though they mapped to a similar region, and had similar phenotypes (Lombardo et al., 2006). To check if this was correct, we conducted new crosses between the \( itd4 \) and \( alb1 \) mutants. From these crosses, 15 individual F1 plants (from 2 independent crosses) were obtained, all of which produced only white nodules 3 weeks after inoculation. Based on identification of the gene, these F1 plants were validated by sequencing PCR products, confirming that \( itd4 \) and \( alb1 \) are indeed allelic. This region has a translocation between the distal part of the short arm of chromosome I of Gifu B-129 and the long arm of chromosome II of MG20. This causes strong suppression of recombination in this region precluding precise mapping (Hayashi et al., 2001). Therefore, we made a new mapping population by crossing \( itd4 \) (pollen donor) with \( L. burttii \), which is an alternative parent to MG20 for mapping genes at this region (Kawaguchi et al., 2005; Sandal et al., 2012). From about 4000 F2 plants, 944 mutant progeny were obtained, suggesting inheritance of a recessive mutation; genotyping of these plants allowed us to map the mutation between the markers BM1741 and TM1842. Within this interval we identified no recombination with TM1840 (Supplemental Fig. S1A). Sequencing of candidate genes within this region identified one gene (Lj1g3v0415090.1) encoding a putative LRR-RLK, which contained a mutation causing a premature stop in \( itd4 \).

To confirm this identified mutation caused the infection defects, we cloned the full-length gene from Gifu B-129 including a 1.9-kb putative promoter region and 1.2 kb downstream of the translation stop. We transformed wild-type or \( itd4 \) mutant roots with empty vector (EV) or the cloned gene using \( M. rhizogenes \) for root transformation (Fig. S2A). The roots of the \( itd4 \) mutant transformed with the EV retained the infection defect and did not produce pink mature nodules (Table 1; Supplemental Fig. S2A). The roots of the \( itd4 \) mutant transformed with the EV retained the infection defect and did not produce pink mature nodules at this time point (Table 1; Supplemental Fig. S2A). Meanwhile, when the \( itd4 \) mutant was transformed with RINRK1, nearly all of transformed root systems had mature pink nodules 18 d after inoculation with \( M. loti \) R7A (Table 1; Supplemental Fig. S2A). At this time point, normal infection threads were formed on these complemented transgenic roots, and the pink nodules were fully infected (Supplemental Fig. S2, B–D). This complementation assay demonstrated that we had identified the gene causing the infection defect in \( itd4 \).

The predicted coding sequence consists of 1878 nucleotides in two exons (Supplemental Fig. S1B) encoding a 626-amino acid protein. The deduced sequence revealed a predicted signal peptide (SP) and three LRR motifs at the N-terminal region, a single transmembrane domain, and a cytoplasmic Ser/Thr protein kinase domain at the C terminus (Fig. 1A). The \( itd4 \) mutant carries a point mutation (G153 to A) converting W51 to a premature stop codon between the regions encoding the predicted signal peptide and the first LRR domain (Fig. 1A; Supplemental Fig. S1B). Although the mutation was previously called \( itd4 \) (Lombardo et al., 2006), for clarity, we renamed the gene RINRK1 and the mutant allele \( itd4 \) was renamed rinkr1-1.

**Table 1.** Complementation of rinkr1-1 nodulation phenotype by hairy root transformation

| Transformation Construct and Line | Nodulation Ratio | Nodules (50) |
|-----------------------------------|-----------------|--------------|
| pKGWR Wild type                   | 21/21           | 4.75 (0.3)   |
| rinkr1-1                          | 0/18            | 0            |
| pRINRK1:RINRK1 Wild type          | 11/11           | 4.7 (0.6)    |
| rinkr1-1                          | 22/24           | 4.4 (1.7)    |
| pRINRK1:RINRK1 (G364A) Wild type  | 15/15           | 4.6 (0.4)    |
| rinkr1-1                          | 6/10            | 5.0 (1.0)    |
| pRINRK1:RINRK1 (K383E) Wild type  | 20/20           | 4.9 (0.4)    |
| rinkr1-1                          | 6/6             | 3.0 (0.9)    |

*Ratios indicate numbers of successfully transformed plants that formed nodules versus all plants of the indicated line that were successfully transformed with the indicated construct. *b*Mean nodule number per successfully transformed plant.

Identification of RINRK2, a homolog of RINRK1 with a Minor Role in Rhizobial Infection

Some infected nodules were found on SL3055-2 (rinkr1-1) after prolonged periods of infection. To determine if this delayed infection could be due to genetic redundancy, we used BLAST searches and identified proteins belonging to a legume-specific clade of homologs, including representatives from \( M. truncatula \), common bean (Phaseolus vulgaris), and soybean (Glycine max; Supplemental Fig. S3). A \( L. japonicus \) paralog of RINRK1 (Lj4g3v1535150.1) was identified from this phylogenetic analysis, and we named it RINRK2. All of these legume RINRK homologs had a similar gene structure containing two exons and one intron at equivalent locations (https://phytozome.jgi.doe.gov/pz/portal.html). The predicted RINRK1 proteins shared about 69%–72% identity and about 80%–3% similarity from different legume species.

A mutant line (30018122) was identified, which carries a LORE1 retrotransposon insertion in the first exon of RINRK2 (Supplemental Fig. S1B); therefore, this mutant was designated as rinkr2-1. The rinkr2-1 mutant produced similar infection threads as wild type (Fig. 1D). We crossed rinkr1-1 and rinkr2-1 mutants and identified a double mutant (rinkr1 rinkr2). Infection...
events in the rinrk1-1, rinrk2-1 and rinrk1 rinrk2 mutants were analyzed by confocal microscopy using *M. loti* constitutively expressing GFP. This confirmed most infection events in rinrk1-1 were blocked, resulting in the accumulation of many enlarged infection foci (Fig. 1C) compared with the normal elongated infection threads in wild type (Fig. 1B). In the double mutant, almost no infection threads were observed and almost all infections appeared to be blocked at the stage of the infection foci (Fig. 1E). In the rinrk1-1 and rinrk1 rinrk2 mutants, occasional infection threads appeared to degrade and release bacteria into root hairs of the mutant (Fig. 1F). Infection events were quantified 7 and 14 d postinoculation with *M. loti* R7A expressing cloned lacZ. This confirmed rinrk1-1 had fewer ITs and more infection foci (IF) than wild type and that rinrk2-1 was similar to wild type. The rinrk1 rinrk2 double mutant had even more IFs and fewer ITs than rinrk1-1 (Fig. 1G). Two weeks after inoculation with *M. loti*, wild-type and rinrk2-1 plants produced mature pink nodules, whereas the rinrk1-1 and rinrk1 rinrk2 mutants formed a few small white nodules. After 3 weeks a few pale-pink nodules appeared on the rinrk1-1 mutant, but this was not seen with the rinrk1 rinrk2 double mutant. Five weeks after inoculation, 10 out of 21 rinrk1-1 plants produced about two pale-pink nodules. In contrast, only two of 16 plants of the rinrk1 rinrk2 produced one pale-pink nodule (Fig. 1H; Supplemental Fig. S4, A–H). Examination of histochemically stained nodule sections revealed the pale-pink nodules were partially infected (Supplemental Fig. S4, I–N). The infections in the rinrk1 rinrk2 double mutant appeared to occur via crack entry (Supplemental Fig. S4N). These observations suggest RINRK2 has some functional redundancy with RINRK1 for rhizobial infection.

**RINRK1 Is Required for Normal Expression of Early Nodulin Genes**

Several genes (e.g. *NIN, ENDO40-1, NPL*) are induced during rhizobial infection and nodule organogenesis in infected root hairs and nearby cells (Schauser et al., 1999; Granlund et al., 2005; Xie et al., 2012). Therefore, we assayed (by reverse transcription-quantitative PCR [RT-qPCR]) the effect of the *rinrk* mutation on the expression pattern of some of these genes. NF induction of *NIN* was no different in wild type and rinrk2-1 showing a continuous increase in expression over 24 h. However, in the rinrk1-1 and rinrk1 rinrk2 mutants, *NIN* expression reached a maximum 6 h after NF addition but then did not increase in the next 12–24 h as seen in wild type (Fig. 2A), revealing a second phase of induction that is absent from the rinrk1 mutant and the rinrk1 rinrk2 double mutant. *NPL* was strongly induced in wild type and rinrk2-1 showing a continuous increase in expression over 24 h. However, in the rinrk1-1 and rinrk1 rinrk2 mutants, *NIN* expression reached a maximum 6 h after NF addition but then did not increase in the next 12–24 h as seen in wild type (Fig. 2A), revealing a second phase of induction that is absent from the rinrk1 mutant and the rinrk1 rinrk2 double mutant. *NPL* was strongly induced in wild type and in the rinrk2 mutant, but this strong induction was absent from the rinrk1 and rinrk1 rinrk2 mutants (Fig. 2B). There was no significant difference between rinrk1 and rinrk1 rinrk2 for *NIN* and *NPL* induction (Fig. 2, A and B). Following inoculation with *M. loti* R7A, normal induction of *NIN, RbohB, ENOD40-1, and N6* required RINRK1 (Fig. 2, C–F).
NFs added to L. japonicus roots induce nuclear-associated calcium spiking and a separate calcium influx at the root-hair tip (Miwa et al., 2006). Both responses are blocked in nfr1 and nfr5 mutants, whereas the calcium influx is retained in synrk mutants (Miwa et al., 2006). To assess if RINRK1 and RINRK2 were required for the NF-signaling, we assessed calcium responses in the rinrk1-1 and rinrk1 rinrk2 mutants. Both retained NF-induced calcium spiking and calcium influx (Supplemental Fig. S5), implying that RINRKs are not essential for these calcium responses.

RINRK1 and RINRK2 Are Induced by NF and Display an Infection-Specific Expression Pattern

We used RT-qPCR to analyze expression of RINRK1 and RINRK2 in roots of wild-type L. japonicus at different time points after the addition of M. loti NF or following inoculation with M. loti R7A or the M. loti R7A nodC mutant that produces no NF. RINRK1 and RINRK2 expression increased 6 h after the addition of NFs from M. loti, and RINRK1 was more strongly induced than RINRK2 (Fig. 3A). M. loti induced RINRK1 and RINRK2 24 h after inoculation, and again, RINRK1 was more strongly induced than RINRK2. The M. loti R7A nodC mutant did not induce RINRK1 or RINRK2 expression (Fig. 3B).

Roots transformed with a RINRK1-promoter GUS fusion (proRINRK1:GUS) revealed RINRK1 was expressed in epidermal cells 24 h after NF addition; this expression occurred in the zone of the root that is normally most susceptible to rhizobial infection (Fig. 3C). Expression of proRINRK1:GUS was particularly strongly induced in infected root hairs, as observed 3 d after inoculation with a strain of M. loti R7A, that could be visualized due to its constitutive expression of GFP (Fig. 3, D–F). Expression of proRINRK1:GUS was also strongly induced in both young and mature nodules (Fig. 3G); sectioning and examination of these nodules by light microscopy revealed proRINRK1:GUS was expressed in all cell layers of young uninfected nodules (Fig. 3, H and I). However, in mature nodules, the GUS staining was observed in the nodule parenchyma cells but not in the fixation zone containing nitrogen-fixing bacteroids (Fig. 3, J and K).

NIN Directly Binds to the RINRK1 Promoter and Induces RINRK1 Expression

Since the transcription factor NIN is associated with the regulation of several genes during rhizobial infection (Soyano et al., 2014; Liu et al., 2019), we tested whether RINRK1 was regulated by NIN. First, our RT-qPCR analysis revealed NF induction of RINRK1 was strongly reduced in the nin-2 mutant background (Fig. 4A). We further used proRINRK1:GUS to assess NIN-dependent RINRK1 induction. At 3 d after inoculation, strong proRINRK1:GUS induction was seen in root hairs and epidermal cells of transformed roots of wild type (Fig. 4, B and C), but very low levels of GUS expression were observed in transformed roots of the nin-2 mutant (Fig. 4, D and E). To test directly for effects of NIN on RINRK expression, we coexpressed 35S:GFP-NIN (NIN) with proRINRK1:GUS in Nicotiana benthamiana leaf cells. As negative controls, we coexpressed with proRINRK1:GUS either the EV lacking NIN (EV) or a construct expressing the NIN-like gene NLP1, which induces expression of other promoters (Lin et al., 2018). Measurements of GUS activity indicated NIN but not NLP1 can activate proRINRK1:GUS expression in this system (Fig. 4F). As another negative control, we coexpressed NIN and proNIN:GUS and confirmed there was no induction of GUS activity in N. benthamiana leaves (Fig. 4F).

Since the NF-induced expression of RINRK1 requires NIN, it seemed likely RINRK1 may be regulated by NIN. Genome wide NIN binding sites were identified previously using chromatin immunoprecipitation (Soyano et al., 2014), and that work identified a NIN-binding site upstream of RINRK1 (referred to as chr1.CM0166.760.r2.a in that work). In the promoter region of RINRK1 we identified four sequences (Fig. 4G; Supplemental Fig. S6) similar to a consensus
NIN-binding site (NBS; Soyano et al., 2014). These four putative NBS sequences are 2320 to 2346 (S1), 2709 to 2734 (S2), 2800 to 2826 (S3), and 21296 to 21322 (S4) nucleotides upstream of the predicted translation start site (Fig. 4G). Electrophoresis mobility shift assays revealed specific retardation of RINRK1 promoter fragments containing S1 or S2 when incubated with the carboxyl-terminal half of NIN, which contains the predicted DNA-binding domain (Fig. 4H). Addition of unlabeled competitor DNA confirmed NIN specifically bound to these DNA fragments of the promoter region (Fig. 4H). No retardation was seen with promoter fragments containing S3 or S4 (Fig. 4H). Based on all these results, we conclude NIN can bind to the RINRK1 promoter to induce RINRK1 expression.

RINRK1 Is an Atypical Pseudokinase and Does Not Requires Its Kinase Activity for Rhizobial Infection

To determine the subcellular localization of the RINRK1 protein, we expressed a RINRK1-GFP fusion driven by its native promoter, but we were unable to detect any florescence signals, either in N. benthamiana leaves or L. japonicus roots. When RINRK1-GFP expression was driven by the constitutive cauliflower mosaic virus 35S promoter in transgenic L. japonicus roots, a GFP signal was observed in the plasma membranes of root, root hair cells and L. japonicus root protoplasts (Supplemental Fig. S7).

Comparison of the kinase domain of RINRK1 and RINRK2 with other RLKs revealed both have sequences and structural conservation typical of protein kinases.
but lack several conserved amino acid residues essential for kinase activity (Hanks et al., 1988); the Gly-rich loop (I), VALK domain (II), the HRD domain (VIb), and DFG (VII) motifs are conserved (Castells and Casacuberta, 2007; Supplemental Fig. S8). However, RINRK1 and RINRK2 and the nearest homologs of RINRK1 in *M. truncatula* (Medtr3g078250.1) and in *Arabidopsis* (*Arabidopsis thaliana*; At2g26730) possess an Asn (N) in the HRD domain where active protein kinases usually contain Asp (D; e.g. in NFR1, SYMRK, and AtBAK1). Inactive RLKs, such as STRUBBELIG, BAK1-Interacting Receptor-like Kinase 2 (BIK2), and Receptor Dead Kinase 1 (RDK1), also have Asn at this position (Halter et al., 2014; Kumar et al., 2017). Moreover, RINRK1 and RINRK2 have EYG residues instead of the DFG motif of subdomain VII, which is involved in cation binding and orientation of the ATP gamma phosphate for phosphate transfer (Hanks et al., 1988; Fig. 5A). These differences with active kinases suggest RINRK1 and RINRK2 lack kinase activity.

We tested the in vitro kinase activity of RINRK1 compared with NFR1, NFR5, and SYMRK (NFR1-CD, NFR5-CD, or SYMRK-CD). The NFR1 and SYMRK kinase domains showed strong auto-phosphorylation, whereas no auto-phosphorylation was observed for the RINRK1 and NFR5 kinase domains (Fig. 5B; Supplemental Fig. S9). Cloned RINRK1 carrying mutations of residues predicted to be essential for kinase activity (RINRK1 G364A or RINRK1K383E) could still rescue the infection defects of the rinrk1-1 mutant (Fig. 5C; Table 1), supporting the idea that kinase activity is not essential for RINRK1 function. We tested whether RINRK1 is a substrate of NFR1 or SYMRK by coincubating RINRK1 with NFR1 or SYMRK in the presence of *g*32P-labeled ATP. It appears not to be a substrate, because we found no phosphorylated band corresponding to RINRK1; under the same conditions, NFR5 was phosphorylated by NFR1 (Fig. 5B; Supplemental Fig. S9).

**RINRK1 Is Not Required for Spontaneous Nodule Formation Induced by Overexpression of SYMRK**

Overexpression of NFR1, NFR5, or SYMRK in *L. japonicus* leads to spontaneous nodule formation, and this has been used to show SYMRK functions downstream of NFR1 and NFR5 (Ried et al., 2014). Overexpression of RINRK1 (proUB:RINRK1) did not induce spontaneous nodules (143 transgenic root systems). We
also tested if proUB:SYMRK could induce spontaneous nodule formation in the \textit{rinrk1-1} mutant. Transformation of hairy roots with proUB-SYMRK induced spontaneous nodules in both \textit{L. japonicus} wild type and the \textit{rinrk1-1} mutant (Fig. 6, A–H). Spontaneous nodules formed on the transformed roots of the \textit{rinrk1-1} mutant. There were fewer nodules than seen with wild-type roots transformed with proUB-SYMRK (Fig. 6I); a similar reduction in SYMRK-induced spontaneous nodules was observed in \textit{nfr1} and \textit{nfr5} mutants (Ried et al., 2014). We conclude \textit{RINRK1} is not required for the induction of nodule organogenesis.

**DISCUSSION**

NF recognition is essential for all aspects of symbiotic nitrogen fixation in most legumes, coordinating the processes in the root cortex necessary for nodule organogenesis and in the root epidermis where rhizobial infection is initiated (Oldroyd and Downie, 2008). Evidence from both plant and bacterial genetic studies suggests at least two stringencies of NF perception with less stringent ‘early’ recognition initiating nodule organogenesis and more stringent ‘late’ recognition being required for bacterial infection (Ardourel et al., 1994; Morieri et al., 2013). Such differential perception of NF suggests multiple receptor complexes that can differentially perceive NF and activate the very different processes required for organogenesis and infection. Here we identify two new RLKs (RINRK1 and RINRK2) that are induced by NF and can promote rhizobial infection but not nodule organogenesis. Mutation of \textit{rinrk1} and \textit{rinrk1 rinrk2} revealed what appeared to be two phases of NF induction of the \textit{NIN} gene: an initial early phase of induction (up to 6 h) that was retained in the \textit{rinrk1} and \textit{rinrk1 rinrk2} mutants, and a second phase of induction (12–24 h) that was lost in these mutants. This is entirely consistent with RINRK1 and RINRK2 functioning in infection thread formation. However, RINRK1 and RINRK2 were not required for NF induced calcium oscillations, suggesting these RINRKs are not required for NF activation of the signaling pathway leading to nodule development. This does not exclude the possibility RINRK1 could interact with known NF receptors (e.g. NFR1 or NFR5) to generate a specific output that relates NF perception to rhizobial infection. The RINRKs are required for full induction of \textit{NIN} and other early nodulation genes such as \textit{ENOD40-1}, \textit{N6} and \textit{RbohB}. The interpretation of patterns of induction of these genes in \textit{M. loti} infection-defective mutants may be made a little more complicated by the observation that expression of these genes is associated with infection events; therefore, reducing the number of infection events in the \textit{rinrk} mutants could decrease overall expression levels of infection-related genes. However, another infection-thread-deficient mutant \textit{scarn}, which has similar infection phenotypes as \textit{rinrk1 rinrk2}, showed normal \textit{M. loti} induction of \textit{NIN}, \textit{NPL}, and \textit{ENOD40-1} (Qiu et al., 2015). This observation suggests the dynamics of these gene expression...
patterns in rinrk1 and rinrk1 rinrk2 is not directly linked to a reduction in infection but rather is related to their biological function. RINRKs lack several conserved motifs or amino acid residues that are important for kinase activity, and our in vitro assays suggest RINRK1 lacks auto-phosphorylation. We showed mutant forms of RINRK1, in which the conserved Lys in kinase domain II is altered to Glu RINRK1K383E or the conserved Gly RINRK1, which retains kinase activity, it is clear based on its sequence that, although RINRK1K383E or the conserved Gly RINRK1, which retains kinase activity, it is clear based on its sequence that, although RINRK1 lacks residues critical for kinase activity, it is clear based on its sequence that, like RINRK1, RINRK2 lacks residues critical for kinase activity. Although we did not test if RINRK2 has kinase activity, it is clear based on its sequence that, like RINRK1, RINRK2 lacks residues critical for kinase activity. Its nearest homolog in Arabidopsis, At2g26730, has also been suggested to be a kinase-dead protein, and it has been estimated the Arabidopsis genome contains 10%-20% atypical or kinase-dead RLKs (Castells and Casacuberta, 2007). These proteins were proposed to have diverged to become signal transduction proteins despite their lack of kinase activity (Castells and Casacuberta, 2007). Typical examples are BIR2, which acts in BAK1-mediated plant immunity (Halter et al., 2014); STRUBBELIG (SUB), which acts in leaf and floral organ development (Chevalier et al., 2005); POLLEN-SPECIFIC RECEPTOR-LIKE KINASE 5 (PRK5), which acts in PAMP associated plant immunity (Wrzaczek et al., 2015); and RDK1, which acts in abiotic stress signaling (Kumar et al., 2017). The mechanistic basis of signaling via atypical RLKs is not yet understood, but all these proteins are essential for signal transduction and are proposed to function via protein-protein interactions. For example, BIR2 interacts with BAK1 and RDK1 interacts with ABI2 in Arabidopsis (Halter et al., 2014; Kumar et al., 2017). Identification and characterization of proteins that interact with RINRK1 and RINRK2 will be needed to help us understand how these signaling kinase-like proteins promote formation and propagation of infection threads.

NIN is the key regulator that coordinates rhizobial infection and nodule organogenesis (Vernié et al., 2015). NIN is required for the induction of many of the genes known to be associated with rhizobial infection in root hairs of M. truncatula (Liu et al., 2019). Several genes with NIN binding sites were identified previously (Soyano et al., 2014), and we confirm NIN binds upstream of RINRK1. Since NIN is required for normal RINRK1 expression in L. japonicus and can promote RINRK1 expression in N. benthamiana, we can conclude NIN directly regulates RINRK1 expression. However, RINRK is required for full induction of NIN by NF. Since RINRK induction requires NIN and full NIN induction requires RINRK, we propose there may be a positive feedback loop involving RINRK and NIN; such positive feedback could enhance NF responses, and this could facilitate rhizobial infection.

As a NF-induced LRR-type pseudokinase, RINRK1 most probably acts to produce a signaling output in response to NF to promote the initiation and maintenance of infection threads along which rhizobia can grow. Thus RINRK1 may form part of a branch of the nodulation signaling pathway that promotes infection rather than nodule organogenesis. This could facilitate the spatial and temporal coordination of infection-related signaling. We do not yet know how this branch could be activated. One possibility is that RINRK1 could interact with component(s) of the NF recognition
complex and function along with an active kinase to phosphorylate target proteins. Another possibility is that RINRK1 may be involved in perceiving an unknown rhizobial signal that activates formation of infection threads once the nodulation signaling pathway has been activated by NF. The potential positive feedback loop, resulting from NIN induction of RINRK1 expression and RINRK1 promoting NIN expression, could facilitate the commitment required by the plant to promote a structure that enables root infection by rhizobia.

MATERIAL AND METHODS

Plant Materials and Strains

Lotus japonicus Gifu B-129 (Handberg and Stougaard, 1992) and Lotus burttii (Kawaguchi et al., 2005; Fukai et al., 2012) were used for this study. The rinrk1-1 (tid4/LJ3055-2) EMS-induced mutant was a derivative of Gifu B-129 (Lombardo et al., 2006). rinrk-2 was obtained from a pool of L. japonicus mutants with LORE1 transposon insertions (Urbaník et al., 2012). rinrk1-1 and rinrk2-1 were crossed, then F1 plants were generated, and the rinrk1 rinrk2 double mutant was identified among the F2 plants by genotyping and sequencing. L. japonicus seeds were prepared and inoculated with Mesorhizobium loti R7A as described previously (Qu et al., 2015). Plasmids were introduced by transformation into Escherichia coli DH10B or DH5α for cloning, into E.coli Rosetta (DE3) for protein expression, into Agrobacterium rhizogenes AR1193 (Stougaard et al., 1987) for expression in L. japonicus roots and into A. tumefaciens EHA105 for expression in Nicotiana benthamiana.

Map-Based Cloning

Rinrk1-1 (SL3055-2) was crossed with L. burttii, and nodule phenotype was scored in F2 progeny from self-pollinated F1 plants. Genomic DNA was extracted (Perry and Parniske, 2005) using simple sequence repeats markers described previously (Kawaguchi et al., 2005).

Nodulation and Infection Thread Analysis

L. japonicus seedlings were grown in 1:1 mix of vermiculite and perlite and after 1 week were inoculated with M. loti R7A carrying pMP2444 (GFP). Nodules were counted 3 to 6 weeks after inoculation. Infection events were visualized by laser scanning confocal microscopy (Olympus FV1000) using M. loti R7A carrying pKT7WG2. This construct was transformed into A. rhizogenes AR1193 by electroporation and then introduced into wild-type roots by hairy-root transformation. For transient expression in L. japonicus root protoplasts, the RINRK1 genomic DNA was amplified from Gifu B-129 by RINRK1-GFP-F and RINRK1-GFP-R, and the PCR products were digested with Spel and then inserted into pA7-GFP using CloneExpress II One Step Cloning Kit (Vazyme) to generate pA7*RINRK1-GFP. The sequence was confirmed by DNA sequencing, the constructs were transiently expressed in L. japonicus root protoplasts using DNA-PEG-calcium transfection, and images were taken by laser scanning confocal microscopy (Leica TCS SP8; Jia et al., 2018).

RINRK1 Promoter:GUS Analysis

A 1.9-kb region upstream of the RINRK1 translation start codon was amplified from Gifu leaf DNA using primers pRINRK1-attB-F and pRINRK1-attB-R, and the PCR products were cloned into pDONR207 and then recombined into the destination vector pK7FWG2 to form proRINRK1:GUS. The construct was cloned into pDONR207 and then confirmed by DNA sequencing, proRINRK1:GUS was introduced into A. rhizogenes AR1193 by electroporation and then transformed into roots of wild type by hairy-root transformation on 1/2 B5 medium. The transformed chimeric plants were transplanted into vermiculite/perlite pots and after 5–7 d inoculated with M. loti R7A containing lacZ or GFP. The roots were harvested at different time points for GUS staining, and some samples were then stained with Magenta-gal (Sangon Biotech) for visualization of lacZ-tagged M. loti (Lotus japonicus handbook, 2005). The double stained nodules were sectioned, and images were taken with a Nikon Eclipse Ni light microscope.

Electrophoresis Mobility Shift Assays

The RINRK1 promoter regions: S1 (–283 to –461 bp), S2 (–618 to –771 bp), S3 (–763 to –924 bp) and S4 (–1223 to –1378 bp) were amplified by primers shown in Supplemental Table S1 using proRINRK1:GUS as a template. The PCR products were fluorescently labeled at the 5′ ends with Cy5 (Yangguang Corp.) and purified by gel extraction (OGMA Bio-TEK) and then fluorescence-labeled DNA was detected using a Biophotometer Plus (Eppendorf). NIN carrying a C-terminal His tag was purified as described previously (Xie et al., 2012). Fluorescence-labeled DNA (1 nm) was incubated with the purified NIN protein in 20 μl of binding buffer (20 mm Tris pH 7.5; 5% (v/v) glycerol; 10 mm MgCl2; 0.25 mm dithiothreitol; 0.5 μg bovine serum albumin; and 1 μg salmon sperm DNA). After incubation at 30°C for 20 min, the products were electrophoresed at 4°C on a 6% native polyacrylamide gel in Tris-borate/EDTA buffer for 2 h at 100 V. Fluorescence in the gel was detected with a Stariion FLA-9000 (Fujifilm).

Transient Activation of proRINRK1:GUS Expression in N. benthamiana Leaves by NIN

LjNIN pKT7WG2 (Qiu et al., 2015) or MtNLPI (Lin et al., 2018) and proRINRK1:GUS, or LjNLN and proNIN:GUS (Qiu et al., 2015) were introduced into
A. tumefaciens strain EHA105 and then coinfiltrated into N. benthamiana leaves as described previously (Qiu et al., 2015). Samples from at least 5 leaves were collected 48 h after inoculation, frozen in liquid nitrogen, and used for protein extraction. GUS activity was measured with 4-methylumbelliferyl-β-d-glucuronide as substrate (Sigma-Aldrich) using Thermo scientific Varioskan flash. Mean values and standard deviations were determined from three biological replicates.

In Vitro Kinase Assays
RINRK1-CD pDONR207 was recombined into pMW GWA by LR reaction to generate RINRK1-CD-MBP. NFR1, NFR5, and SYMRK cytoplasmic domains were tagged with 6xHis in pMW GWA (Busso et al., 2005). These constructs were introduced into E. coli BL21-Codon Plus (DE3)-RIL (Stratagene). Protein expression was induced during exponential growing using 0.2 mm isopropylthio-β-galactoside for 9 h at 20°C. The protein was purified using MBP or His beads (Smart) according to the manufacturer’s instructions. For auto-phosphorylation assays, 1 µg purified RINRK1-MBP protein was incubated in 20 µL kinase buffer (50 mm Tris·HCl, pH 7.4; 10 mm MgCl2; 1 mm diithiothreitol; 200 µM ATP; 10 µCi [γ-32P]ATP) at 30°C for 1 h. Then the proteins were separated by SDS-PAGE, and phosphorylated proteins were analyzed by autoradiography and/or phosphor-imaging by the Starion FLA-9000 (Fujifilm).

All constructs in entry vectors were sequenced, and primers used in this paper were available in Supplemental Table 1.

Accession Numbers
Sequence data from this article can be found in the GenBank data libraries under accession numbers MN109982 for RINRK1 and MN109983 for RINRK2 or from the L. japonicus genome version 3.0 (http://www.kazusa.or.jp/lotus/) for RINRK1 Ljg3v0141509.0 and RINRK2 Ljg3v0155150.1.

Supplemental Data
The following supplemental materials are available.
Supplemental Figure S1. Positional cloning of RINRK1 and gene structure of RINRK1 and RINRK2.
Supplemental Figure S2. RINRK1 can rescue rinrk1-1 infection deficiency.
Supplemental Figure S3. Phylogenetic analysis of RINRK proteins.
Supplemental Figure S4. Nodule phenotype of wild type and rinrk mutants.
Supplemental Figure S5. Analysis of calcium oscillation in wild type and rinrk mutants.
Supplemental Figure S6. Alignment of putative NIN-binding sites (NBS) in the RINRK1 promoter region.
Supplemental Figure S7. Assay of RINRK1 subcellular localization using transgenic expression of RINRK1-GFP in L. japonicus roots and root protoplasts.
Supplemental Figure S8. Alignment of the sequence of RINRK and related kinases with typical and atypical kinase domains.
Supplemental Figure S9. A Coomassie staining of the corresponding SDS-PAGE gel.
Supplemental Table S1. Primers used in this study.

ACKNOWLEDGMENTS
We thank Jeremy Murray (Institute of Plant Physiology and Ecology, China) and Jie Zhang (Institute of Microbiology, China) for constructive suggestions on the work and helpful comments on the manuscript. We thank Tracey Welham, Anne B. Heckman, Fabien Lombardo, and Trevor Wang (Joint Intelligence Committee, United Kingdom) as well as Martin Parniske (University of Munich, Germany) for the mutant and for help with the preliminary mutant screen.

Received May 1, 2019; accepted August 1, 2019; published August 13, 2019.

LITERATURE CITED
Antolin-Llovera M, Ried MK, Parniske M (2014) Cleaveage of the SYM-BIOSIS RECEPTOR-LIKE KINASE ectodomain promotes complex formation with Nod factor receptor 5. Curr Biol 24: 422–427
Ardourel M, Demont N, Debelé F, Maillet F, de Billy F, Promé JC, Dénarié J, Truchet G (1994) Rhizobium meliloti lipopolysaccharide nodulation factors: Different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell 6: 1357–1374
Arrighi JF, Barre A, Amor BB, Boursol A, Soriano LC, Mirabella R, de Carvalho-Neibel F, Journet EP, Gheardi M, Huguet T, et al (2006) The Medicago truncatula lysine motif-receptor-like kinase gene family includes NIP and new nodule-expressed genes. Plant Physiol 142: 265–279
Arrighi JF, Godfrey O, de Billy F, Saurat O, Jauneau A, Gough C (2008) The RPG gene of Medicago truncatula controls Rhizobium-directed polar growth during infection. Proc Natl Acad Sci USA 105: 9820–9825
Bek A, Sauer J, Thyesen MR, Dume O, Petersen BO, Thrarp S, James E, Jensen KJ, Stougaard J, Radutoiu S (2010) Improved characterization of nod factors and genetically based variation in LysM Receptor domains identify amino acids expendable for nod factor recognition in Lotus spp. Mol Plant Microbe Interact 23: 58–66
Brewin NJ (2004) Plant cell wall remodelling in the rhizobium-legume symbiosis. Crit Rev Plant Sci 23: 293–316
Broghammer A, Krusell L, Blaise M, Sauer J, Sullivan JT, Maolanon N, Vinther M, Lorentzen A, Madsen EB, Jensen KJ, et al (2012) Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proc Natl Acad Sci USA 109: 13859–13864
Busso D, Delagoutte-Busso B, Moras D (2005) Construction of a set Gateway-based destination vectors for high-throughput cloning and expression screening in Escherichia coli. Anal Biochem 343: 313–322
Castells E, Casacuberta JM (2007) Signalling through kinase-defective domains: The prevalence of atypical receptor-like kinases in plants. J Exp Bot 58: 3503–3511
Catoira R, Timmers AC, Maillet F, Galera C, Pennmetsa RV, Cook D, Dénarié J, Gough C (2001) The HCL gene of Medicago truncatula controls Rhizobium-induced root hair curling. Development 128: 1507–1518
Cerri MR, Wang Q, Stolz P, Folkmann J, Frances L, Katzer K, Li X, Heckmann AR, Wang TL, Downie JA, et al (2017) The ERN1 transcription factor gene is a target of the CaCaMK/CYCLOPS complex and controls rhizobial infection in Lotus japonicus. New Phytol 215: 323–337
Chevalier D, Batoux M, Fulton L, Pfister K, Yadav RK, Schellenberg M, Schneitz K (2005) STRUBBELIG defines a receptor kinase-mediated signalling pathway regulating organ development in Arabidopsis. Proc Natl Acad Sci USA 102: 9074–9079
Cullimore JV, Ranjeva R, Bono JJ (2001) Perception of lipochitooligosaccharide nod factors in legumes. Trends Plant Sci 6: 24–30
Fournier J, Teillet A, Chabaud M, Ivanov S, Genre A, Limpens E, de Carvalho-Niebel F, Barker DG (2015) Remodeling of the infection chamber before infection thread formation reveals a two-step mechanism for rhizobial entry into the host legume root hair. Plant Physiol 167: 1233–1242
Fukai E, Soyano T, Umehara Y, Nakayama S, Hirakawa H, Tabata S, Sato S, Hayashi M (2012) Establishment of a Lotus japonicus gene tagging population using the exon-targeting endogenous retrotransposon LORÉT. Plant J 69: 720–730
Gage DJ (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing, rhizobia during nodulation of temperate legumes. Microbiol Mol Biol Rev 68: 280–300
Gibson KE, Kobayashi H, Walker GC (2008) Molecular determinants of a symbiotic chronic infection. Annu Rev Genet 42: 413–441
Gronlund M, Roussis A, Flementakis E, Quaedvlieg NE, Schlamann HR, Umehara Y, Katinakis P, Stougaard J, Spanik HP (2005) Analysis of promoter activity of the early nodulin Enod40 in Lotus japonicus. Mol Plant Microbe Interact 18: 414–427
Halter T, Imkampe J, Mazzotta S, Wierzbza M, Postel S, Bäuerle C, Kiefer C, Stahl M, Chinchilla D, Wang X, et al (2014) The leucine-rich repeat receptor kinase BIR2 is a negative regulator of BAK1 in plant immunity. Curr Biol 24: 134–143

814 Plant Physiol. Vol. 181, 2019
Handberg K, Stougaard J (1992) Lotus japonicus, an autogamous, diploid legume species for classical and molecular genetics. Plant J 2: 487–496
Haney CH, Long SR (2010) Plant flotillins are required for infection by nitrogen-fixing bacteria. Proc Natl Acad Sci USA 107: 478–483
Hanks SK, Quinn AM, Hunter T (1988) The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. Science 241: 42–52
Hayashi M, Miyahara A, Sato S, Kato T, Yoshikawa M, Taketa M, Harada M, Pedrosa A, Onda R, Imaizumi-Anraku H, et al (2001) Construction of a genetic linkage map of the model legume Lotus japonicus using an intranspecific F2 population. DNA Res 8: 301–310
Heckmann AB, Lombardo F, Miwa H, Perry JA, Bunnewell S, Parmiske M, Wang TL, Downie JA (2006) Lotus japonicus nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. Plant Physiol 142: 1739–1750
Høgslund N, Radutoiu S, Krusell L, Voroshilova V, Hannah MA, Goffard H, Høgslund N, Radutoiu S, Krusell L, Voroshilova V, Hannah MA, Goffard H (2010) Plant pathogens cause auxin redistribution and cell death in infected cells of non-legume. Plant Physiol 154: 901–912
Hossain MS, Liao J, James EK, Sato S, Tabata S, Jurkiewicz A, Madsen MB (1992) Lotus burttii, as symbiotic to Lotus japonicus, forms empty nodules with incompletely developed nodule maturation. Plant Cell Physiol 33: 301–307
Kawaguchi M, Pedrosa-Harand A, Yano K, Hayashi M, Murooka Y, Saito K, Imaizumi-Anraku H, Kawaguchi M, Koiwa H, Akao S, Syono K (2001) Dissection of nodulation functions as a positive regulator in plant responses to ABA. Mol Plant Microbe Interact 14: 1444–1450
Li CW, Breakspear A, Guan D, Cerri MR, Jackson K, Jiang S, Robson F, Radhakrishnan GV, Roy S, Bone C, et al (2019) NIN acts as a network hub controlling a growth module required for rhizobial infection. Plant Physiol 179: 1704–1722
Liang P, Stratil TF, Pop C, Marin M, Folgmann J, Mysore KS, Wen J, Ott T (2018) Symbiotic root infections in Medicago truncatula require remorin-mediated receptor stabilization in membrane nanodomains. Proc Natl Acad Sci USA 115: 5289–5294
Lin JS, Li X, Luo Z, Mysore KS, Wen J, Xie F (2018) NIN interacts with NLPs to mediate nitrate inhibition of nodulation in Medicago truncatula. Nat Plants 4: 942–952
Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szymczynski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425: 637–640
Madsen EB, Antolin-Llovera M, Grossmann C, Ye J, Vieweg S, Broghammer A, Krusell L, Radutoiu S, Jensen ON, Stougaard J, Parmiske M (2011) Autophosphorylation is essential for the in vitro function of the Lotus japonicus Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. Plant J 65: 404–417
Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, Bek AS, Ronow CW, James EK, Stougaard J (2010) The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nat Commun 1: 1–12
Miwa H, Sun J, Oldroyd GE, Downie JA (2006) Analysis of Nod-factor-induced calcium signaling in root hairs of symbiotically defective mutants of Lotus japonicas. Mol Plant Microbe Interact 19: 914–923
Miyahara A, Richens J, Starker C, Morieri G, Smith L, Long S, Downie JA, Oldroyd GE (2010) Conservation in function of a SCAR/WAVE component during infection thread and root hair growth in Medicago truncatula. Mol Plant Microbe Interact 23: 1553–1562
Morieri G, Martinez EA, Jarynowski A, Driguez H, Morris R, Oldroyd GE, Downie JA (2013) Host-specific Nod-factors associated with Medicago truncatula nodule infection differentially induce calcium influx and calcium spiking in root hairs. New Phytol 200: 656–662
Murray JD (2011) Invasion by invitation: Rhizobial infection in legumes. Mol Plant Microbe Interact 24: 631–639
Murray JD, Muni RRD, Torres-Jerez I, Tang Y, Allen S, Andriankaja M, Li G, Laxmi A, Cheng X, Wen J, et al (2011) Vapryn, a gene essential for intracellular progression of arbuscular mycorrhizal symbiosis, is also essential for infection by rhizobia in the nodule symbioses of Medicago truncatula. Plant J 65: 244–252
Oldroyd GE, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59: 519–546
Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45: 119–144
Perry J, Parmiske M (2005) 96-Well DNA isolation method. In J Stougaard, M Udvardy, M Parmiske, H Spahn, G Saibbach, J Webb, M Chiurazzi, M Márquez, eds, Lotus japonicas Handbook. Springer Netherlands, Heidelberg, Germany, pp 129–131
Qi L, Lin JS, Xu J, Sato S, Parmiske M, Wang TL, Downie JA, Xie F (2015) SCARN a novel class of SCAR protein that is required for root-hair infection during legume nodulation. PLoS Genet 11: e1005623
Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature 425: 583–592
Radutoiu S, Madsen LH, Madsen EB, Jurkiewicz A, Fukui E, Quistgaard EM, Albrektsen AS, James EK, Thirup S, Stougaard J (2007) LysM domains mediate lipochitin-oligosaccharide recognition and Nfr genes extend the symbiotic host range. EMBO J 26: 3923–3933
Ried MK, Antolin-Llovera M, Parmiske M (2014) Spontaneous symbiotic reprogramming of plant roots triggered by receptor-like kinases. eLife 3: e01389
Rodpothong P, Sullivan JT, Songsrirute K, Sumpton D, Cheung KW, Thomas-Oates J, Radutoiu S, Stougaard J, Ronow CW (2009) Nodulation gene mutants of Mesorhizobium loti R7A-nodZ and R7A-nodL mutants provide a protocol for model legume root protoplast isolation and transformation. Front Plant Sci 9: 670
Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for sulphated and acylated glucosamine oligosaccharide signal. Nature 419: 670
Kawaguchi M, Pedrosa-Harand A, Yano K, Hayashi M, Murooka Y, Saito K, Nagata T, Namai K, Nishida H, Shibata D, et al (2005) Lotus burttii takes a position of the third corner in the lotus molecular genetics triangle. DNA Res 12: 69–77
Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gyssel K, Muszynski A, Carlson RW, Thygesen MB, Sandal N, Assmussen MH, Vinther M, Andersen SU, et al (2013) Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature 523: 308–312
Kawaharada Y, James EK, Kelly S, Sandal N, Stougaard J (2017a) The ethylene responsive factor required for nodulation 1 (ERN1) transcription factor is required for infection-thread formation in Lotus japonicus. Mol Plant Microbe Interact 30: 194–204
Kawaharada Y, Nielsen MW, Kelly S, James EK, Andersen KR, Rasmussen SR, Fütchbauer W, Madsen LH, Heckmann AB, Radutoiu S, Stougaard J (2017b) Differential regulation of the Erp3 receptor coordinates membrane-restricted rhizobial colonization of root nodule vasculature. Plant Physiol 173: 1453–1464
Kelly SJ, Muszynski A, Kawaharada Y, Hubber AM, Sullivan JT, Sandal N, Carlson RW, Stougaard J, Ronson CW (2013) Conditional requirement for exopolysaccharide in the Mesorhizobium-Lotus symbiosis. Mol Plant Microbe Interact 26: 319–329
Kiss E, Oláh B, Kaló P, Morales M, Heckmann AB, Boboła A, Lózsa A, Kontár K, Middleton P, Downie JA, Oldroyd GE, Endre G (2009) LIN, a novel type of U-box/WD40 protein, controls early infection by rhizobia in legumes. Plant Physiol 151: 1239–1249
Kumar D, Kumar R, Baek D, Hyun TK, Chung WS, Yun DJ, Kim YJ (2017) An Arabidopsis thaliana RECEPTOR DEAD KINASE1 (RDK1) gene functions as a positive regulator in plant responses to ABA. Mol Plant 10: 223–243
Lefebvre B, Timmers T, Mbengue M, Moreau S, Hervé C, Tóth K, Bittencourt-Silvestre J, Klaus D, Deslandes L, Godiard L, et al (2010) A remorin protein interacts with symbiotic receptors and regulates bacterial infection. Proc Natl Acad Sci USA 107: 2343–2348
Leroux P, Roche P, Faucher C, Maillet F, Truchet G, Prome J-C, Dénarié J (1990) Symbiotic host specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344: 781–784
have host-specific phenotypes on Lotus spp. Mol Plant Microbe Interact 22: 1546-1554

Sandal N, Petersen TR, Murray J, Umehara Y, Karas B, Yano K, Kumagai H, Yoshikawa M, Saito K, Hayashi M, et al (2006) Genetics of symbiosis in Lotus japonicus: Reconstructing inbred lines, comparative genetic maps, and map position of 35 symbiotic loci. Mol Plant Microbe Interact 19: 80–91

Sandal N, Jin H, Rodriguez-Navarro DN, Temprano F, Cvi tanich C, Brachmann A, Sato S, Kawaguchi M, Tabata S, Parniske M, et al (2012) A set of Lotus japonicus Gita x Lotus burttii recombinant inbred lines facilitates map-based cloning and QTL mapping. DNA Res 19: 317–323

Schauer L, Roussis A, Stiller J, Stougaard S (1999) A plant regulator controlling development of symbiotic root nodules. Nature 402: 191–195

Smit P, Limpens E, Geurts R, Fedorova E, Dolgikh E, Gough C, Bisseling T (2007) Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling. Plant Physiol 145: 284–296

Soyano T, Hirakawa H, Sato S, Hayashi M (2014) Nodule Inception creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. Proc Natl Acad Sci USA 111: 14607–14612

Spaenk HP, Sheeley DM, van Brussel AA, Glushka J, Yorke WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJ (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of Rhizobium. Nature 354: 125–130

Stougaard J, Abildsten D, Marcker KA (1987) The Agrobacterium rhizogenes pRi TL-DNA segment as a gene vector system for transformation of plants. Mol Gen Genet 207: 251–255

Stracke S, Kistner C, Yoshida S, Mulder L, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J, Sczygloowski K, Parniske M (2002) A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417: 959–962

Tóth K, Stratil TF, Madsen EB, Ye J, Popp C, Antolin-Llovera M, Grossmann C, Jensen ON, Schüssler A, Parniske M, Ott T (2012) Functional domain analysis of the Remorin protein LjSYMREM1 in Lotus japonicus. PLoS One 7: e30817

Urbanški DF, Malolepszy A, Stougaard J, Andersen SU (2012) Genome-wide LOREI retrotransposon mutagenesis and high-throughput insertion detection in Lotus japonicus. Plant J 69: 731–741

van Brussel AA, Bakhuizen R, van Sprossen PC, Spaenk HP, Tak T, Lugtenberg BJ, Kijne JW (1992) Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of Rhizobium. Science 257: 70–72

van Sprossen PC, Bakhuizen R, van Brussel AA, Kijne JW (1994) Cell wall degradation during infection thread formation by the root nodule bacterium Rhizobium leguminosarum is a two-step process. Eur J Cell Biol 64: 88–94

Vernié T, Kim J, Frances L, Ding Y, Sun J, Guan D, Niebel A, Gifford ML, de Carvalho-Niebel F, Oldroyd GE (2015) The Nin transcription factor coordinates diverse nodulation programs in different tissues of the Medicago truncatula root. Plant Cell 27: 3410–3424

Wrzaczek M, Vainonen JP, Stael S, Tsatsiani I, Help-Rinta-Rahko H, Gauthier A, Kaufholdt D, Bollhöner B, Lamminmäki A, Staes A, et al (2015) GRIM REAPER peptide binds to receptor kinase PRK5 to trigger cell death in Arabidopsis. EMBO J 34: 55–66

Xie F, Murray JD, Kim J, Heckmann AB, Edwards A, Oldroyd GE, Downie JA (2012) Legume pectate lyase required for root infection by rhizobia. Proc Natl Acad Sci USA 109: 633–638

Yano K, Shibata S, Chen WL, Sato S, Kaneko T, Jurkiewicz A, Sandal N, Banba M, Imaiizumi-Anraku H, Kojima T, et al (2009) CERBERUS, a novel U-box protein containing WD-40 repeats, is required for formation of the infection thread and nodule development in the legume-Rhizobium symbiosis. Plant J 60: 168–180

Yokota K, Fukai E, Madsen LH, Jurkiewicz A, Rueda P, Radutoiu S, Held M, Hassain MS, Sczygloowski K, Morieri G, et al (2009) Rearrangement of actin cytoskeleton mediates invasion of Lotus japonicus roots by Mesorhizobium loti. Plant Cell 21: 267–284