PLANT SCIENCE

Nanobody-driven signaling reveals the core receptor complex in root nodule symbiosis

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Understanding the composition and activation of multicomponent receptor complexes is a challenge in biology. To address this, we developed a synthetic approach based on nanobodies to drive assembly and activation of cell surface receptors and apply the concept by manipulating receptors that govern plant symbiosis with nitrogen-fixing bacteria. We show that the *Lotus japonicus* Nod factor receptors NFR1 and NFR5 constitute the core receptor complex initiating the cortical root nodule organogenesis program as well as the epidermal program controlling infection. We find that organogenesis signaling is mediated by the intracellular kinase domains whereas infection requires functional ectodomains. Finally, we identify evolutionarily distant barley receptors that activate root nodule organogenesis, which could enable engineering of biological nitrogen-fixation into cereals.

Living cells must constantly monitor their environment to integrate changes and to communicate with neighboring cells and microorganisms. Cell surface receptors perceive external signals and convert stimuli into intracellular responses. There is, however, a limited understanding and a lack of methodology for exploring how individual receptors assemble and function in signaling-competent complexes that coordinate the downstream responses. We tackle this problem in vivo using single-pass transmembrane cell surface receptors that govern plant-microbe interactions. Plants interact with a wealth of microbes and need to distinguish between those that pose a risk and those that offer a potential benefit. To this end, plants use pattern-recognition receptors, including lysine motif (LysM) receptors, that perceive microbial-derived carbohydrate signals and mount an intracellular response (*1*–*4*). Plant LysM receptors recognize conserved cell wall components, such as chitin from pathogenic fungi (*5*, *6*), Myc factors in arbuscular mycorrhizal symbiosis (*7*, *8*), and Nod factors in nitrogen-fixing symbiosis with rhizobia (*2*, *9*–*12*). Genetic studies in the model legume *Lotus japonicus* (*Lotus*) have identified two LysM Nod factor receptors, NFR1 and NFR5 (*10*), along with the symbiosis receptor-like kinase SYMRK (*13*), which initiate and control the two developmental programs leading to nodule organogenesis and infection (intracellular accommodation), respectively. Loss-of-function mutations in these three receptors render plants incapable of establishing root nodule symbiosis. To study how the receptors collaborate during signaling and to define the active receptor complexes, we developed a synthetic approach based on single-domain antibody fragments from camelids (called nanobodies) to actively promote receptor assembly. We used the nanobody intervention to identify the core receptor complex that induces signaling in root nodule symbiosis in legumes.

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Nanobody-driven assembly defines the receptor complex initiating organogenesis

To investigate receptor complex activation, we developed technology that enables the controlled assembly of receptors in living cells and used symbiotic receptors as proof-of-principle targets. To drive assembly, we used llama-derived nanobodies as they are small, specific, high-affinity binders that are active in the reducing environment of the cytosol (*14*). We generated a nanobody with high affinity to the *Lotus* NFR5 kinase (Nb_{NFR5}) and used it as a C-terminal tag on the *Lotus* NFR1 receptor (Fig. 1A and fig. S1). We reasoned that Nb_{NFR5} fused to the C-terminal tail of NFR1 would specifically recognize and bind the kinase domain of the native NFR5 receptor and drive the formation of a heteromeric complex. This was tested by expressing nanobody-tagged NFR1 receptor constructs from the native *Nfr1* promoter and terminator in *Agrobacterium rhizogenes*–induced roots of *nfr1* mutant plants (Fig. 1B). We observe nodule formation independent of rhizobia or Nod factor, illustrating that receptor assembly is the key step in symbiotic signaling (Fig. 1C). In a control experiment, we omit the nanobody and observe that no spontaneous nodules are formed (Fig. 1C). To establish a general approach that does not rely on nanobody generation for each protein of interest, we next used the well-characterized LaG16 nanobody that binds superfolder green fluorescent protein (GFP) (*15*). We fused GFP to NFR5 and the LaG16 nanobody to NFR1 and expressed the constructs from their native promoters and terminators in *nfr1 nfr5* double mutant plants (Fig. 1E). When NFR1 is associated to NFR5 through nanobody binding to GFP, nodules are formed independently of rhizobia (Fig. 1, D and F). Similarly, organogenesis is activated in the reverse situation with GFP fused to NFR1 and the nanobody fused to NFR5 (fig. S2). To test whether this phenotype is dependent on the direct binding between the nanobody and GFP, we engineered a mutant version of LaG16 with three bulky tryptophan residues in the nanobody paratope. In contrast to functional LaG16, mutated LaG16 (LaG16m) lost the ability to bind GFP-tagged NFR5 in tobacco pull-down assays (Fig. 1G). Furthermore, when LaG16m is fused to NFR1, it fails to initiate signaling leading to organogenesis (Fig. 1F), demonstrating that NFR1-NFR5 receptor association is essential for signal transduction. To explore whether signaling is enhanced by assembling more receptors, we next coupled two LaG16 nanobodies in series to either NFR1 or NFR5, separated by a 40-residue-long (~130 Å) flexible linker which should sterically allow one receptor to associate with two co-receptors (Fig. 1F and fig. S2). We see no difference in nodule formation when one or two nanobodies are used, indicating that a single association event between NFR1 and NFR5 receptors is sufficient to initiate nodule organogenesis (Fig. 1F and fig. S2).

The core signaling complex is conserved in legumes

To understand whether the heteromeric receptor complex corresponds to the activated receptor state in other legume-rhizobia symbioses, we performed analogous experiments in *Medicago truncatula* (*Medicago*), in which we associated the two Nod factor receptors LYK3 and NFP (*9*, *16*) using nanobodies. We observe that a heteromeric LYK3-NFP receptor complex is sufficient to initiate organogenesis in *Medicago* in the absence of external signals (Fig. 2, A and B), demonstrating that the triggering event for signal activation leading to organogenesis is the association of a conserved core receptor complex comprising one NFR1/LYK3 receptor and one NFR5/NFP receptor. To understand whether symbiotic signaling is exclusively dependent on the physical assembly of the receptors or if it also requires a phosphorylation event, we introduced a point mutation in NFR1 impairing its kinase function (*17*). We find that plants expressing the kinase-dead NFR1 fail to form nodules (Fig. 2C), showing that the protein kinase activity mediates downstream signaling. The SYMRK receptor containing a malectin-like domain and leucine-rich repeats in the ectodomain

was shown to interact with NFR5 and to induce spontaneous nodules when overexpressed (*18*). To decipher the composition and functional requirements of the core complex, we tested nanobody-assembled NFR1-NFR5 signaling in *nfr1 nfr5 symrk* triple mutant plants. The transformed roots show no nodulation (Fig. 2C), verifying the dependence of signaling on the common symbiotic pathway and confirming the vital downstream function of SYMRK. To investigate whether it is possible to drive symbiotic signaling from SYMRK independently of the formed NFR1-NFR5 complex, we next used nanobodies to associate SYMRK to either NFR1 or NFR5 (Fig. 2C). We observe that neither the SYMRK-NFR1 nor the SYMRK-NFR5 complex can drive organogenesis (Fig. 2C), showing that the essential role of SYMRK in nodulation requires either a ligand- or nanobody-induced NFR1-NFR5 complex. To verify that the activated receptor complex operates through the known organogenesis pathways, we next explored its dependency on cytokinin signaling (*19*, *20*). We find that the activated NFR1-NFR5 complex is unable to initiate organogenesis in the *lhk1* loss-of-function cytokinin receptor mutant (Fig. 2C). Together, the data show that the NFR1-NFR5 complex is essential to initiate nodule organogenesis and that the downstream signaling cascade requires the symbiotic pathway.

Activated complex initiates both cortical and epidermal programs

We next investigated the effect of the nanobody-induced NFR1-NFR5 complex on the infection program by inoculating plants with *Mesorhizobium loti* (*M. loti*) bacteria, the symbiont of *Lotus*. We monitored infection by *M. loti* through the appearance of pink, leghemoglobin-producing nodules on the roots. Although an increased number of white nodules form on plants expressing the nanobody-associated NFR1-NFR5 complex, we see that formation of infected pink nodules is reduced compared with plants expressing the native receptors (Fig. 3A and fig. S3). This suggests that the activated NFR1-NFR5 complex initiates parallel developmental programs for both infection and organogenesis. But compared with organogenesis, infection is hampered by the presence of the nanobody-driven heteromeric NFR1-NFR5 state. We find that plants expressing the nanobody-induced complexes of either NFR1-SYMRK or NFR5-SYMRK are efficiently infected by rhizobia (fig. S4), suggesting that only the activated NFR1-NFR5 complex affects the infection program. Next, we investigated whether rhizobia that use an intercellular infection mechanism and circumvent the epidermal root hair infection program can colonize nodules induced by the nanobody-mediated receptor complexes. For this, we analyzed infection with IRBG74,

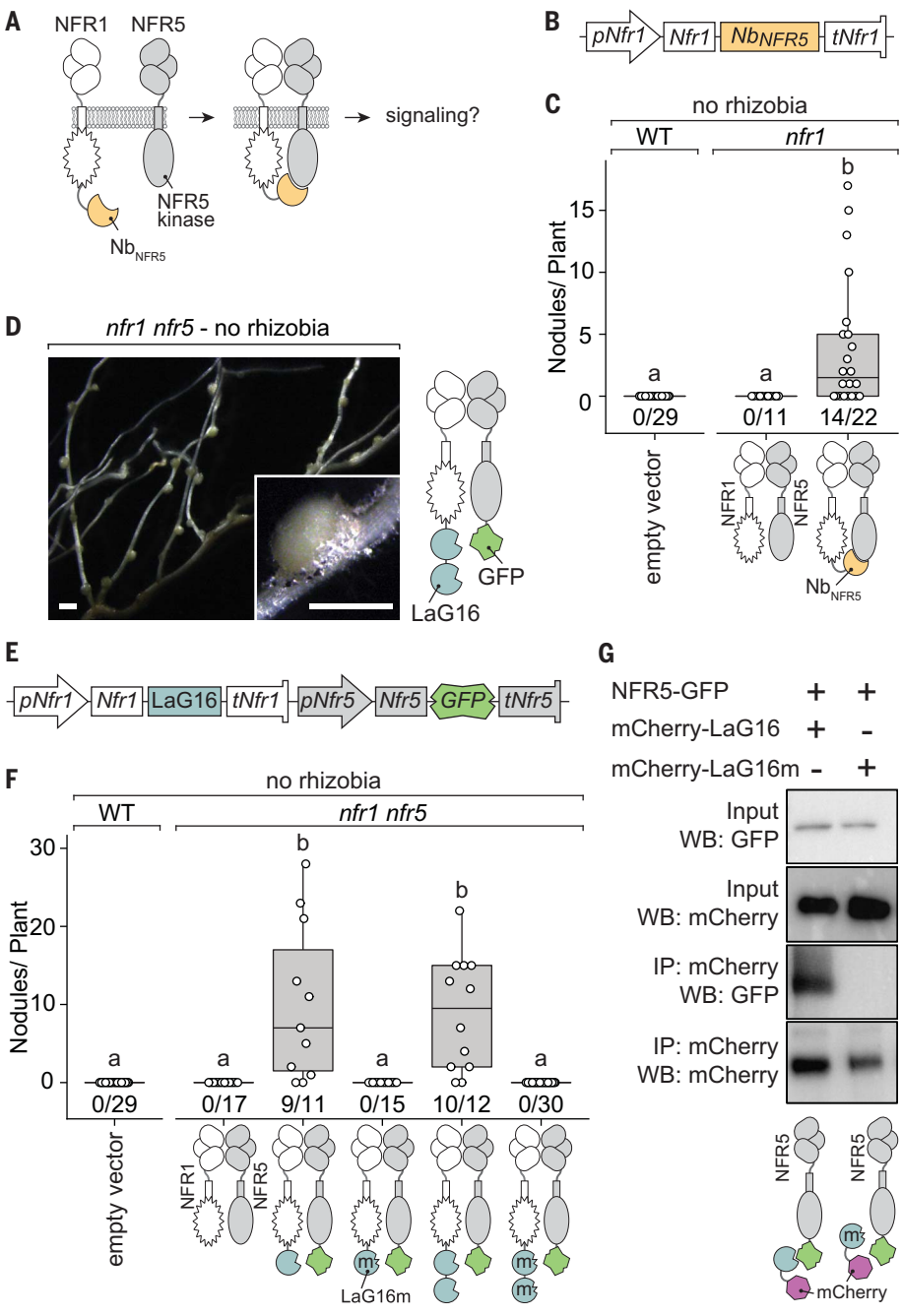

Fig. 1. Nanobody-driven assembly defines the receptor complex activating organogenesis. (**A**) Experimental design for nanobody-induced complex formation to activate symbiotic signaling. (**B**) Schematic of nanobody-tagged NFR1 receptor construct for expression in *Lotus* roots. (**C**) Number of nodules formed on WT and *nfr1* mutant plants expressing the indicated constructs in the absence of rhizobia. Nodules were counted 9 weeks after hairy root transformation. Circles represent individual plants. Numbers below boxplots specify number of nodulating plants out of the total number of plants. (**D**) Representative brightfield images of *Lotus* roots that express the depicted constructs, showing the nodulation phenotype in the absence of rhizobia. Scale bar: 1 mm. (**E**) Schematic of NFR1 and NFR5 receptor constructs for expression in *Lotus* roots. (**F**) Number of nodules formed on WT and *nfr1 nfr5* double mutant plants expressing the indicated constructs in the absence of rhizobia. The letter "m" represents the mutated nanobody with no binding to GFP. (**G**) Coimmunoprecipitation of LaG16- or LaG16m-tagged mCherry and GFP-tagged NFR5 from *Nicotiana benthamiana* leaf extracts. Anti-GFP and anti-mCherry antibodies were used for immunoblotting. LaG16m is the mutated nanobody with no binding to GFP. In (C) and (F), lowercase letters indicate significant differences between samples [analysis of variance (Kruskal-Wallis) and post-hoc analysis (Dunn's test), $P < 0.05$].

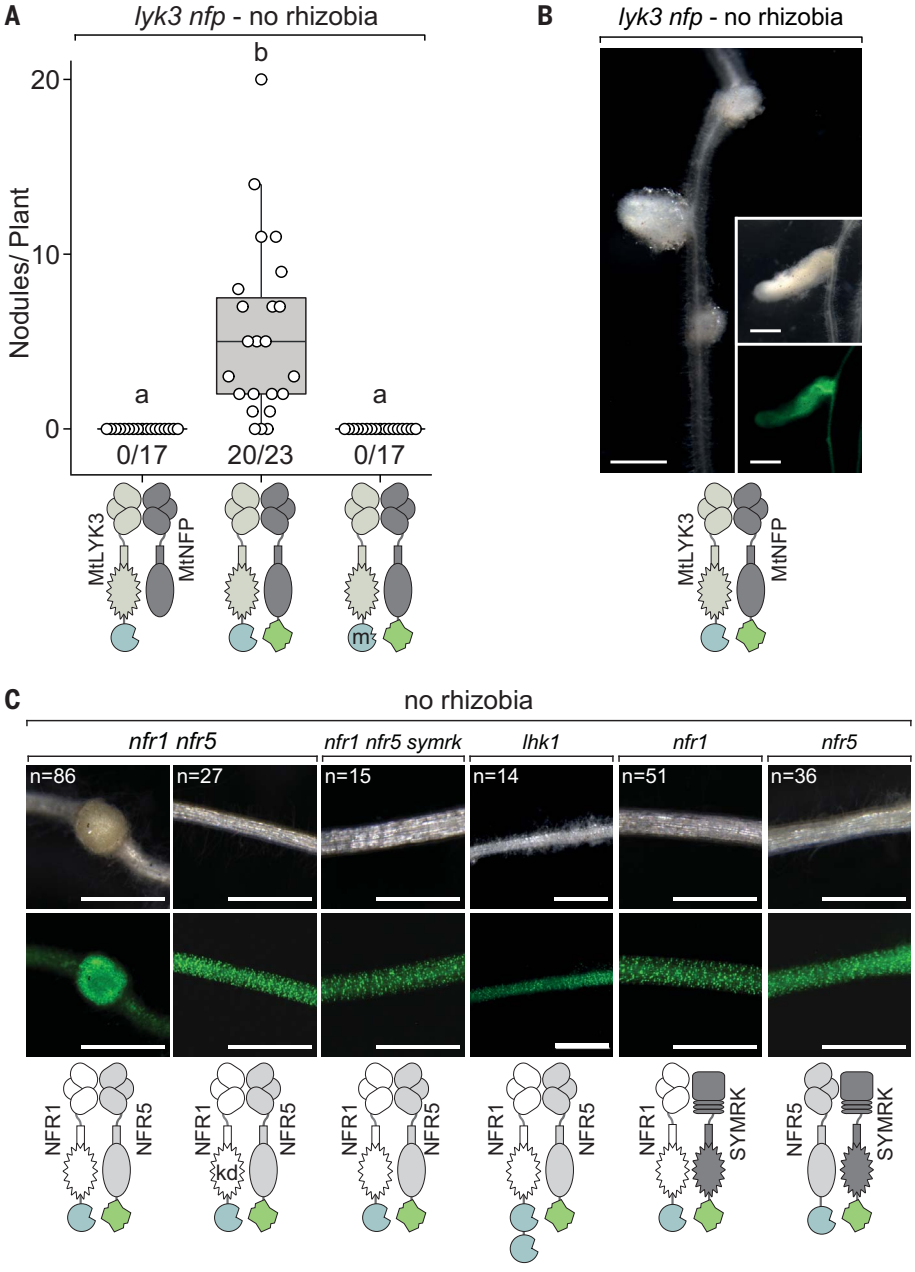

Fig. 2. The core symbiotic signaling complex activating organogenesis is conserved. (**A**) Number of nodules formed on *Medicago lyk3 nfp* double mutant plants expressing the indicated constructs in the absence of rhizobia. Nodules were counted 9 weeks after hairy root transformation. Circles represent individual plants. Numbers below the boxplots specify number of nodulated plants out of the total number of plants. The letter "m" represents the mutated nanobody with no binding to GFP. Lowercase letters indicate significant differences between samples [analysis of variance (Kruskal-Wallis) and post-hoc analysis (Dunn's test), $P < 0.05$]. (**B**) Representative brightfield and YFP fluorescence (transformation control) images of *Medicago* roots, showing the nodulation phenotype in the absence of rhizobia. Scale bar: 1 mm. (**C**) Representative brightfield and YFP fluorescence (transformation control) images of *Lotus* roots expressing NFR1-LaG16 with NFR5-GFP, kinase-dead (kd) NFR1-LaG16 with NFR5-GFP, NFR1-LaG16 with SYMRK-GFP or NFR5-LaG16 with SYMRK-GFP. *n* = total number of plants. Scale bars: 1 mm.

which uses an intercellular, crack-entry mechanism (*21*, *22*). The transgenic *nfr1 nfr5* mutant roots form only white, uninfected nodules (fig. S5), suggesting that both infection modes are impaired when NFR1 and NFR5 are part of a nanobody-bound complex. To understand how downstream processes are affected by the presence of the nanobody-activated receptor complex, we created stable *Lotus* lines expressing GFP-tagged NFR5 and LaG16-tagged NFR1. The stable lines were generated in the *nfr1 nfr5* double mutant background with a β-glucuronidase (*GUS*) reporter gene driven by the *NIN* promoter. Looking at expression of the transcription factor *NIN*, which orchestrates both the epidermal infection program and the cortical organogenesis program, we can evaluate the effect of the activated receptor state on both programs (*23*). We observe nodule organogenesis independent of rhizobia 7 to 10 days after germination in three independent stable lines. GUS staining reveals *NIN* activation in nodules as well as in dividing cortical cells, which mark sites of emerging nodule primordia (Fig. 3B). This cortical *NIN* activation is very similar to the native condition observed for wild-type (WT) plants inoculated with *M. loti* (Fig. 3B). In the wild type, we see that the epidermal infection program is tightly regulated, as *NIN* is expressed only in a few epidermal root hair cells. By contrast, plants expressing the nanobody-driven NFR1-NFR5 complex show broad *NIN* expression in numerous epidermal cells along the root (Fig. 3B). This wider, less-regulated epidermal *NIN* activation might interfere with the coordinated timing of organogenesis and infection. We quantified infection efficiencies by comparing WT plants with the stable *Lotus* lines expressing the NFR1-NFR5 complex 10 days after inoculation with *M. loti*. We observe a strong reduction in the number of infection threads formed when nanobodies are used to constitutively activate NFR1-NFR5 (Fig. 3D). Because both organogenesis and infection are initiated in transgenic roots expressing the nanobody-driven receptor complex, we took the opportunity to ask whether certain receptor domains contribute differently to the two developmental processes. For this, we engineered nanobody-mediated receptor complexes lacking the ectodomains of NFR1, NFR5, or both and expressed them in transgenic *Lotus* roots. We find that all ectodomain-lacking versions of the complexes are still able to activate organogenesis, albeit with a reduced efficiency compared with full-length receptors (Fig. 3C). By contrast, removal of any ectodomains results in complete loss of infection by *M. loti* as no infected pink nodules appear (Fig. 3A). These data show that the infection program is dependent on functional Nod factor–recognizing ectodomains, whereas organogenesis signaling can be mediated solely from the intracellular NFR1-NFR5 domains. Furthermore, our findings demonstrate that it is possible to bypass the ligand-induced signaling function conferred by the ectodomains to induce the cortical organogenesis program.

Cereal receptors can activate root nodule organogenesis

The ability to develop nitrogen-fixing root nodules is restricted to a single clade of flowering

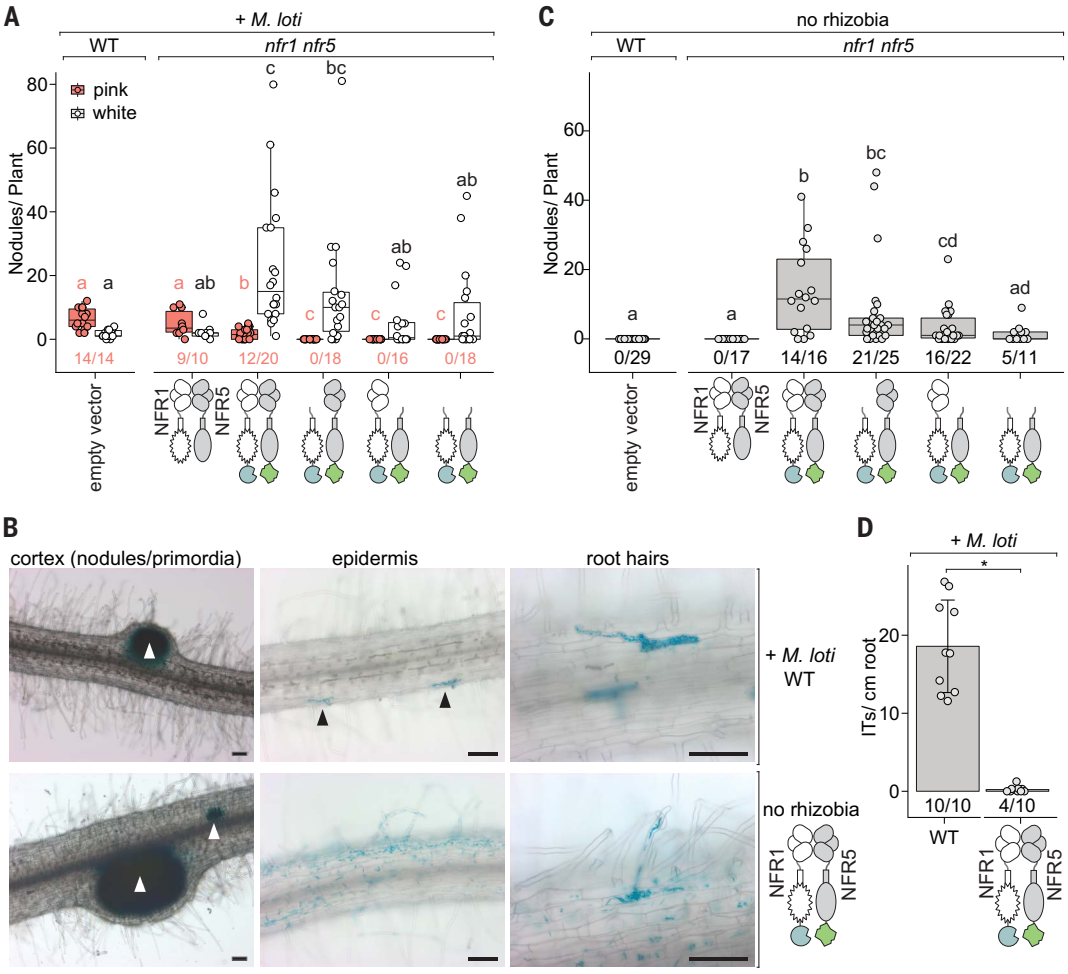

Fig. 3. Signaling activates both cortical and epidermal programs. (**A**) Number of nodules formed on WT and *nfr1 nfr5* double mutant plants expressing the indicated constructs. Nodules were counted 6 weeks after inoculation with *M. loti*. Red numbers below boxplots specify the number of plants with pink (infected) nodules out of the total number of plants. (**B**) Representative images of *pNIN*:GUS expression in WT plants inoculated with *M. loti* and non-inoculated roots of stable *Lotus* lines expressing NFR1-LaG16 and NFR5-GFP. Triangles pinpoint cells expressing GUS. For the stable line root hair image, three images were overlaid. Scale bars: 0.1 mm. (**C**) Number of nodules formed on WT and *nfr1 nfr5* double mutant plants expressing the indicated constructs. Nodules were counted 9 weeks after hairy root transformation. Numbers below the boxplots specify number of nodulated plants out of the total number of plants. In (A) and (C), lowercase letters indicate significant differences between samples [analysis of variance (Kruskal-Wallis) and post-hoc analysis (Dunn's test), $P < 0.05$]. Note that the empty vector and complementation controls are the same as in Fig. 1F. (**D**) Number of infection threads (ITs) formed per cm of root at 10 days post infection with *M. loti* in WT and stable *Lotus* lines expressing NFR1-LaG16 and NFR5-GFP. Error bars represent standard deviation. For statistical analysis a Kruskal-Wallis test was performed, $*P < 0.001$. Numbers below the bars specify the number of plants with ITs out of the total number of plants.

plants (*24*). However, most plants, including cereals, can form arbuscular mycorrhizal symbiosis by activating the common symbiosis pathway that is shared with root nodule symbiosis in legumes (*25*, *26*). This dichotomy indicates that the LysM receptors from cereals are unable to activate downstream signaling leading to nodulation. To test this, we analyzed the barley genome and found seven LysM receptor-like kinases (RLKs) belonging to the NFR1 and NFR5 families (Fig. 4A). Phylogenomic analysis and structural modeling of these barley RLKs revealed that RLK4 and RLK10 show the greatest similarity to NFR1/LYK3 and NFR5/NFP, respectively (Fig. 4, A and B, and fig. S8) (*11*, *12*, *27*). We first tested the ability of these individual barley RLKs to functionally complement the *nfr1* and *nfr5* single or *nfr1 nfr5* double mutants when expressed in *Lotus* from the native *Nfr1* and *Nfr5* promoters and terminators. Despite the similarity, RLK4 and RLK10 are unable to functionally complement NFR1 and NFR5 (fig. S6 and table S1). We then asked whether nanobody-driven association of *Lotus* NFR5 with barley RLK4 expressed from the native *Nfr1* promoter and terminator induces nodule formation in *nfr1 nfr5* double mutant plants. We observe that the RLK4-NFR5 complex leads to nodule organogenesis independent of rhizobia (Fig. 4C). Likewise, nodules form when barley RLK10 is in a nanobody-mediated complex with *Lotus* NFR1 (Fig. 4C). Moreover, we find that the barley RLK4-RLK10 receptor complex associated through nanobodies leads to nodule organogenesis independent of the Nod factor receptors in both *Lotus* and *Medicago* (Fig. 4, C and D, and fig. S7). Barley RLK4 and RLK10 fail to support infection as no pink nodules appear when plants are inoculated with *M. loti* (fig. S6). Together, these results demonstrate that LysM receptors from barley—which diverged from *Lotus* 200 million years ago (*28*)—have retained the ability to activate the symbiotic pathway leading to root nodule organogenesis in legumes. We next investigated whether the *Lotus* chitin receptor CERK6 (*29*)—the closest homolog to *Lotus* NFR1 and in the same receptor family as barley RLK4—can support nodulation. *Lotus* CERK6 shares 62% sequence identity with *Lotus* NFR1 (table S1), but only 2 out of 27 plants form a single nodule when CERK6 and NFR5 are associated through nanobodies in the *nfr1 nfr5* mutants (Fig. 4C). Because RLK4 shares only 51% sequence identity with *Lotus* NFR1 (table S1), signal activation cannot be solely explained by sequence similarity. Although the molecular details that enable certain receptors to activate organogenesis remain elusive, these results show that signaling specificity is ensured by receptor complex composition.

In this study, we developed a synthetic approach to investigate the composition and activation of multicomponent and branched receptor systems. Our nanobody technique for driving complex formation, together with other approaches such as chemical control dimerization (*30*), will be applicable when

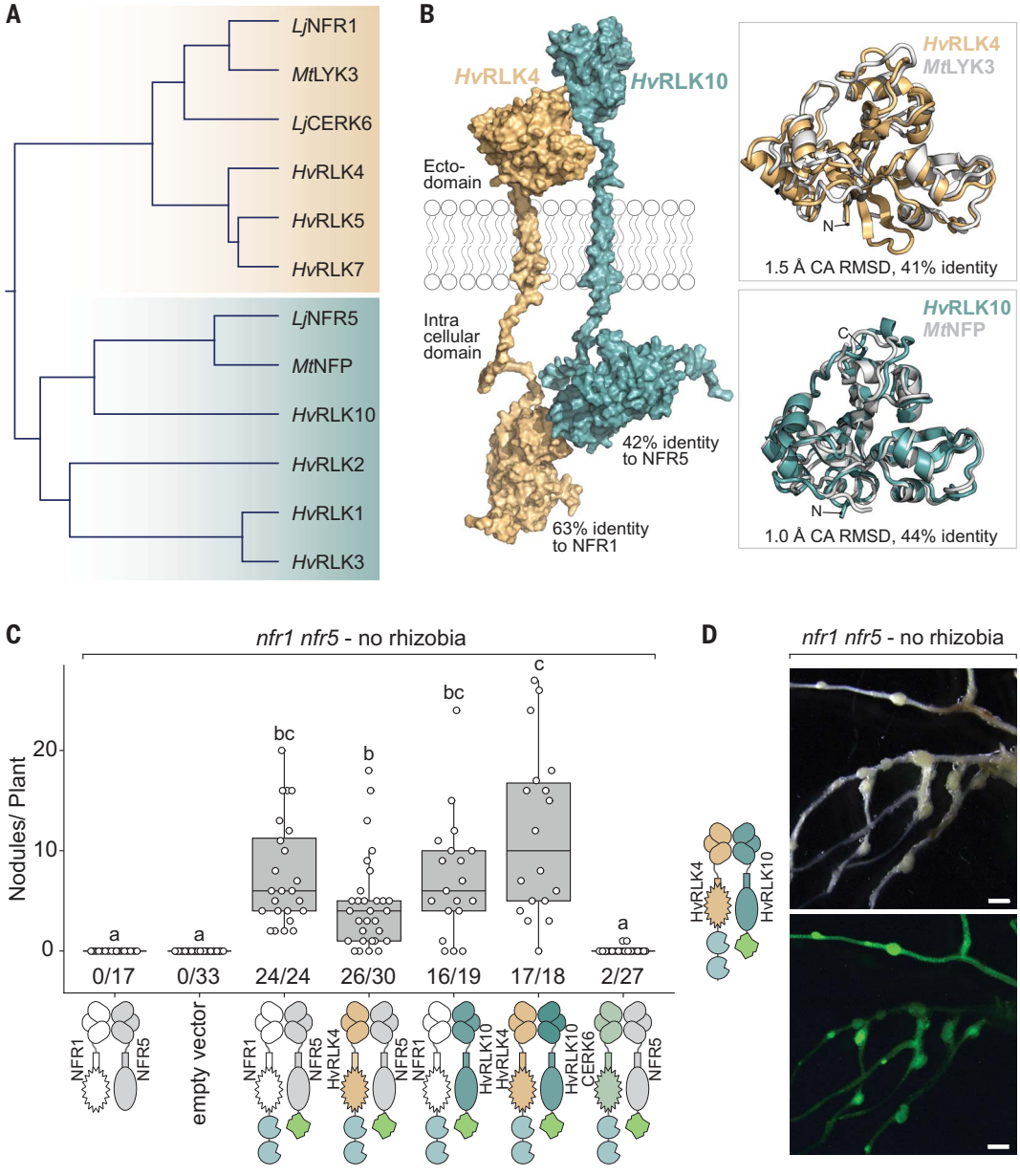

Fig. 4. Barley receptors can activate the symbiotic signaling pathway. (**A**) Phylogeny of selected barley (*Hv*), *Lotus* (*Lj*) and *Medicago* (*Mt*) full-length LysM receptors. Yellow and teal shaded backgrounds highlight NFR1- and NFR5-type receptors, respectively. (**B**) Structural modelling of barley RLK4 and RLK10. The amino acid sequence identities compared with the intracellular parts of *Lotus* NFR1 and NFR5 are indicated. Superposition of the modelled ectodomains of barley RLK4 and RLK10 compared with the crystal structures of *Medicago* LYK3 (PDB-ID: 6XWE) and NFP (PDB-ID: 7AU7), respectively. C-α root-mean-square deviations (RMSD) are indicated in angstroms (Å). (**C**) Number of nodules formed on *nfr1 nfr5* double mutant plants expressing the indicated constructs in the absence of rhizobia. Nodules were counted 9 weeks after hairy root transformation. Numbers below the boxplots specify number of nodulated plants out of the total number of plants. Lowercase letters indicate significant differences between samples [analysis of variance (Kruskal-Wallis) and post-hoc analysis (Dunn's test), $P < 0.05$]. Note that the complementation control is the same as in Fig. 1F. (**D**) Phenotype of nodules formed in *nfr1 nfr5* roots expressing barley RLK4-LaG16 with RLK10-GFP are depicted to the left. YFP fluorescence serves as a transformation control. Scale bars: 1 mm.

studying other cell surface receptor systems. We used the nanobody-based approach to address a longstanding question in the field of plant-microbe interactions: namely, what constitutes an activated LysM receptor signaling complex. We demonstrate that NFR1 and NFR5 form the core receptor complex initiating root nodule symbiosis in *Lotus*. Our data match earlier findings showing that root hair deformation and calcium influx as the initial responses to rhizobia are maintained in the *symrk* mutant but abolished in the *nfr1* and *nfr5* mutants (*13*, *31*, *32*), suggesting that the NFR1-NFR5 complex has a signaling function independent of SYMRK. The data support that the first critical event for signal activation involves assembly of a core NFR1-NFR5 complex, which then interacts with SYMRK. We show that although the heteromeric NFR1-NFR5 complex activates the epidermal program essential for infection by rhizobia, nanobody-driven activation leads to inefficient colonization by rhizobia. A tight spatial and temporal synchronization between the infection program and the nodule organogenesis program is essential for efficient root nodule symbiosis. *NIN* is critical for both infection and organogenesis and continuous signaling from NFR1-NFR5 through nanobody activation and misregulation of *NIN* likely compromises the synchronization of bacterial attachment, root hair curling, and infection thread progression with the formation of nodule primordia leading to inefficient infection. Our data show that the intracellular kinase domains are necessary and sufficient for organogenesis signaling when artificially assembled using nanobodies whereas functional receptor ectodomains are important for infection. The main function of the NFR1 and NFR5 ectodomains is to perceive Nod factors, which naturally allow synchronization between receptor complex activation and infection and organogenesis. The ectodomains may serve additional functions like interaction scaffolds to mark the site of infection. We demonstrate that barley RLK4 and RLK10 receptors have the capacity to activate symbiotic signaling in legumes, suggesting that the ability to activate organogenesis is deeply rooted in the LysM receptor family, and was co-opted by plants in the nitrogen-fixing clade for root nodule symbiosis. Evidence for this conserved signaling function

in barley suggests routes for engineering biological nitrogen-fixation into cereals for more sustainable food production in the future.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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MDAR Reproducibility Checklist
Data S1 and S2

View/request a protocol for this paper from *Bio-protocol*.

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