LysM Domain Receptor Kinases Regulating Rhizobial Nod Factor–Induced Infection

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The rhizobial infection of legumes has the most stringent demand toward Nod factor structure of all host responses, and therefore a specific Nod factor entry receptor has been proposed. The SYM2 gene identified in certain ecotypes of pea (*Pisum sativum*) is a good candidate for such an entry receptor. We exploited the close phylogenetic relationship of pea and the model legume *Medicago truncatula* to identify genes specifically involved in rhizobial infection. The SYM2 orthologous region of *M. truncatula* contains 15 putative receptor-like genes, of which 7 are LysM domain–containing receptor-like kinases (LYKs). Using reverse genetics in *M. truncatula*, we show that two LYK genes are specifically involved in infection thread formation. This, as well as the properties of the LysM domains, strongly suggests that they are Nod factor entry receptors.

The establishment of a nitrogen-fixing nodule symbiosis by rhizobial bacteria on the roots of legumes requires that the bacteria enter the root in a host-controlled manner. In most legumes, this infection starts with curling of root hairs. A bacterium becomes trapped in a cavity formed by the curl, where it forms a microcolony (1) (Fig. 1A). Infection thread formation is initiated within this cavity by invagination of the root hair plasma membrane. In this way, a tubelike structure is formed by which the bacteria enter the plant and reach the base of the root hair (Fig. 1B). Ultimately, the infection thread reaches a nodule primordium formed in the root cortex that develops into a nitrogen-fixing nodule upon release of the bacteria.

Rhizobia secrete specific lipochitooligosaccharides, the so-called Nod factors, when they colonize the roots of their legume host. A Nod factor consists of a β-1,4-linked N-acetyl-D-glucosamine backbone of four or five residues, of which an acyl chain is attached at the C-2 position of the nonreducing terminal glucosamine residue (2). Depending on the rhizobial species, the structure of the acyl chain can vary, and substitutions at the reducing and nonreducing terminal glucosamine residues can be present (3). Nod factors are involved in induction of the early steps of nodulation and are also a major determinant of host specificity of nodulation.

Because responses are induced in the plant by Nod factor concentrations in a nano- to picomolar range, it seems probable that Nod factors are recognized by specific receptors (3–7).

Infection thread formation shows the highest demand toward Nod factor structure of all Rhizobium–induced responses (7–10). This has been studied in detail in the interaction of *Sinorhizobium meliloti* (Sm) and the model legume *Medicago truncatula* (7, 8). The nodF::Mdld mutation of Sm produces Nod factors that, in comparison to those secreted by wild-type bacteria, lack an acetate substitution at the nonreducing terminal glucosamine residue and in which the specific acyl chain of 16 C atoms containing two double bonds (C16:2) is replaced by vaccenic acid (C18:1). This *nodF::Mdld* mutant is able to induce most steps of the nodulation process (e.g., cortical cell activation and the formation of root hair curls in which bacteria form a microcolony), but the trapped bacteria are unable to induce infection thread formation. Therefore, it has been proposed that the induction of infection thread formation requires a highly specific Nod factor receptor, which was named the entry receptor (7, 8).

We used confocal laser scanning microscopy (CLSM) to analyze in more detail the stage at which infection thread formation is blocked when *M. truncatula* is inoculated with a *nodF::Mdld* mutant (Sm 2011ΔnodF::Mdld::Tns5-GFP). Most (~80%) of the infections were blocked at the initiation of infection thread formation, because only microcolonies were formed. However, in about 20% of the cases, infection thread–like structures were initiated. However, instead of a continuous tube, a structure composed of tube- and sac-like structures was formed, the growth of

**References and Notes**

4. Materials and methods are available as supporting material on Science Online.
5. Spectroscopy is defined as “the study of light as a function of wavelength that has been emitted, reflected, or scattered from a solid, liquid, or gas” (11).
7. In **F**<sub>0</sub> <sub>x</sub> = \( \frac{\text{mol} \% \text{Mg}}{\left(\text{mol} \% \text{Mg} + \text{mol} \% \text{Fe}\right)} \times 100 \)
12. An ock (Orbit Counter Keeper) is a TES orbit number from orbit insertion on 12 September 1997.
30. We thank T. V. V. King for the use of and insight into her olivine collection and Arizona State University and NASA for their support of our MGS TES research.

**Supporting Online Material**

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Materials and Methods

Table S1

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which was aborted very early in the hair (Fig. 1C). Most likely, this means that during infection thread formation, the polar mode of growth was lost and regained. These data suggest that the recognition of a Nod factor with a specific structure is required for the initiation of infection thread formation (entry), as well as for the maintenance of infection thread growth.

A single nodL mutation (Sm 2011 nodL::Tn5-GFP), resulting in Nod factors that lack the acetate substitution at the nonreducing terminal glucosamine, caused a comparable but weaker infection phenotype that resulted in infection threads with sac-like structures (Fig. 1, D and E). A nodL mutant is still able to infect nodule primordia. In contrast, inoculation with a nodFE mutant (Sm 2011 nodFE-GFP) that produces Nod factors containing a C18:1 acyl chain resulted in the formation of infection threads that by number and morphology were indistinguishable from those formed by wild-type bacteria. This shows that nodL and nodFE mutations have a synergistic effect.

Mutations causing a specific block of the formation of infection threads in a Nod factor structure–dependent manner have not been identified in M. truncatula or Lotus japonicus, two legume species that are amenable to positional cloning. However, a good candidate gene is described in pea (Pisum sativum), namely, SYM2. SYM2 has been identified in several pea ecotypes and the allele of the ecotype Afghanistan is best characterized (10, 11). Pea lines with an introgressed SYM2 allele from Afghanistan pea (SYM2*), have a high demand on Nod factor structure with respect to infection thread formation. They efficiently form infected nitrogen-fixing nodules that by number and morphology were indistinguishable from those formed by wild-type bacteria. This shows that nodL and nodFE mutations have a synergistic effect.

To study gene function in the symbiotic interaction, we performed a survey-sequencing of sequence homologies to N. tabacum–like structures are formed that infect nodule primordia, although sac-like structures were present. (F and G) A LYK3 knockdown root inoculated with Sm 2011 nodFE-GFP (14 dpi). Red-orange coloring is the result of DsRED1 fluorescence, which is used as a selection marker for transgenic roots. (F) An infection event arrested at the microcolony stage. (G) An infection thread consisting of tube- and sac-like structures. Scale bars, 10 μm. All Sm strains used carry the plasmid pCH60 containing green fluorescent protein under the control of a constitutive promoter (24). Microcolonies, infection threads (tubes), and sac-like structures are marked with asterisks, arrowheads, and arrows, respectively.

Pea is not amenable to positional cloning, because of its large genome and the lack of efficient transformation methods. Because M. truncatula and pea are phylogenetically closely related and therefore highly syntenic (14, 15), we decided to characterize the SYM2 orthologous region in M. truncatula and test whether it contains genes specifically required for the rhizobial infection process. A 300-kb orthologous region in M. truncatula delineated by markers that flank SYM2 has been previously identified (15). As a first step to characterize the gene content within this region, we survey-sequenced four bacterial artificial chromosome (BAC) clones that represent a minimum tiling path spanning this region (16). A total of 21 putative genes were identified and annotated on the basis of sequence homologies and expressed sequence tag (EST) data (Fig. 2). BLAST searches revealed 15 putative genes that encode receptor-like proteins; 1 gene homologous to the tobacco mosaic virus (TMV)–resistance gene N, belonging to a curled root hair 4 days post-inoculation (dpi) with a wild-type Sm 2011-GFP microcolony. (A) A macrocolony and infection thread (tube) formed by wild-type Sm 2011-GFP (7 dpi). (C) An infection structure formed by Sm 2011 nodFdnodL::Tn5-GFP, consisting of tube- and sac-like structures (7 dpi). (D and E) Infection threads formed by Sm 2011 nodFdnodL::Tn5-GFP (7 dpi). These infection threads could successfully infect nodule primordia, although sac-like structures were present. (F and G) A LYK3 knockdown root inoculated with Sm 2011 nodFE-GFP (14 dpi). Red-orange coloring is the result of DsRED1 fluorescence, which is used as a selection marker for transgenic roots. (F) An infection event arrested at the microcolony stage. (G) An infection thread consisting of tube- and sac-like structures. Scale bars, 10 μm. All Sm strains used carry the plasmid pCH60 containing green fluorescent protein under the control of a constitutive promoter (24). Microcolonies, infection threads (tubes), and sac-like structures are marked with asterisks, arrowheads, and arrows, respectively.
ing to the toll interleukin-1 receptor–nucleotide binding site–leucine-rich repeat (TIR-NB-LRR) class of resistance genes and named MiHTR (for *M. truncatula* HOMOLOG OF TMV RESISTANCE), 7 genes homologous to *Cladosporium fulvum* (Cf) resistance genes (comprising 18 to 20 extracellular LRR domains), named MhHCR1 through MhHCR7 (for *M. truncatula* HOMOLOG OF CF RESISTANCE), and 7 receptor-like kinases, each predicted to contain one or two domains with homology to lysin motives (LysM) and named LYK1 to LYK7 (for LysM DOMAIN–CONTAINING RECEPTOR-LIKE KINASE). The remaining putative genes were named A to F (Fig. 2). Cross-hybridization and restriction fragment length polymorphism analyses showed that homologous sequences were present in the pea *SYM2* region, confirming the microsynteny between these two legumes (13).

To identify candidate genes involved in the rhizobial infection process, we first selected the genes that are expressed in roots. Reverse transcription–polymerase chain reaction (RT-PCR) was performed on RNA isolated from nodules, leaves, and roots to determine the expression pattern of the identified genes. MiHTR, MhHCR4, MhHCR5, MhHCR6, LYK3, LYK6, LYK7, and unknown-function genes B, C, E, and F could be detected in roots (Fig. 2).

We then decided to knock down gene expression by RNA interference (RNAi) using *Agrobacterium rhizogenes*-mediated root transformation (17, 18). *A. rhizogenes*-mediated root transformation results in cotransformation of ~30% of the roots formed. Each root is the result of an independent transformation event. Some of these roots are chimeric. The selection of homogeneously cotransformed roots was facilitated with a binary vector that carries *DsRed1* as a fluorescent selection marker. Cotransformed roots expressing *DsRed1* can be efficiently nodulated by compatible *Sinorhizobium meliloti* strains (including *Sm 2011-GFP* and *Sm 2011ΔnodF-GFP*), and infection threads are similar to those of wild-type roots. To trigger RNAi, we used hairpin constructs that contained ~500-hp regions of the target gene under the control of the CaMV 35S promoter (18, 19). We first tested the effectiveness of A. *rhizogenes*-mediated RNAi in the roots of *M. truncatula* by targeting the *DM12* gene. *DM12* encodes an LRR domain–containing receptor kinase that is an essential element of Nod factor–induced signaling (20). Targeting *DM12* resulted in 75% of the transgenic roots (as judged by red fluorescence) showing a completely Nod*-* phenotype, and no infections or curled root hairs occurred on these roots. In contrast, 80 to 100% of the control roots were efficiently nodulated (with an average of 11 nodules per root). This shows that *A. rhizogenes*-mediated RNAi is a fast and efficient tool to knock down genes in the roots of *M. truncatula*.

Next, we knocked down, by RNAi, the genes of the *SYM2* orthologous region of *M. truncatula* that were expressed in roots. Plants carrying transgenic roots were inoculated and checked for nodulation efficiency 2 weeks post-inoculation (table S1). Because pea *SYM2* lines were completely blocked in nodulation when inoculated with Rh strains that lack *nodX* and *nodE*, we inoculated the *M. truncatula* knockdown plants with wild-type *Sm 2011-GFP* or *Sm 2011ΔnodF-GFP*, which have similar efficiencies when infecting wild-type roots. A marked reduction of nodulation occurred only in LYK3 knockdown roots inoculated with *Sm 2011ΔnodF-GFP*. In this case, 60% of the cotransformed roots (n = 55) completely lacked nodules. In contrast, inoculation with wild-type *Sm 2011-GFP* resulted in only a single Nod*-* root, whereas the remaining roots were Nod*+* (n = 13, averaging 7 nodules per root). Control roots inoculated with *Sm 2011-GFP* or *Sm 2011ΔnodF-GFP* were efficiently nodulated (averaging 11 and 9 nodules per root, respectively). To determine the reduction of LYK3 mRNA levels in the knockdown roots, we isolated RNA from three large roots. Quantitative RT-PCR showed a ~70% reduction of LYK3 mRNA (fig. S1).

We examined the effect of LYK3 knockdown on infection thread morphology by fluorescence microscopy and CLSM. LYK3 knockdown roots that did not contain nodules after inoculation with *Sm 2011ΔnodF-GFP* (n = 28) were still able to trap bacteria within curled root hairs. However, the number of infection threads was markedly reduced. Most infections (78%) were arrested at the microcolony stage (n = 482, averaging 17 microcolonies per root) (Fig. 1F). Furthermore, infection thread–like structures were formed but aborted in the root hair (n = 136, averaging 5 structures per root) and had aberrant morphologies that consisted of tube- and sac-like structures (Fig. 1G). In contrast, on control roots (n = 19), 81% of the infections resulted in tubular infection threads (averaging 22 threads per root). When LYK3 knockdown roots were inoculated with wild-type *Sm 2011-GFP*, the majority of the infection events resulted in tubular infection threads (n = 148, averaging 11 threads per root). In 20% of the cases, tubes containing sac-like structures were observed (n = 43, averaging 3 structures per root). Control roots inoculated with either *Sm 2011-GFP* or *Sm 2011ΔnodF-GFP* only occasionally showed infection threads with aberrant structures (<5%).

To confirm the knockdown phenotype, we made a second *LYK3* hairpin construct that covered a different region of the gene. Targeting *LYK3* with this second construct resulted in 90% of the transformed roots (n = 36) inoculated with *Sm 2011ΔnodF-GFP*, showing a Nod*-* phenotype. Twenty-three of these roots were further analyzed for infection events, showing that 91% of all infections (n = 172, averaging 8 infections per root) were arrested at the microcolony stage, whereas the infection thread–like structures (n = 18, averaging 1 structure per root) showed aberrant morphologies that consisted of tube- and sac-like structures. These data show that knockdown of *LYK3* with two independent hairpin constructs causes a similar phenotype: a block of infection thread initiation and growth in a Nod factor–dependent manner. This phenotype is similar to that observed with the *Sm 2011ΔnodF-GFP* mutant on wild-type *M. truncatula* plants and incompatible Rh strains on *SYM2*-peas, and *LYK3* is therefore a putative Nod factor entry receptor.

*LYK3* is highly homologous to *LYK4*, especially in the extracellular domain (fig. S2). The first *LYK3* hairpin construct targeted a region of this extracellular domain, by which it is possible that *LYK4* was also knocked down. Although we did not detect *LYK4* expression in roots by RT-PCR, we identified a single clone in a root cDNA library. Therefore, we also made a *LYK4* hairpin construct from a region that shows <20% homology to *LYK3*. *LYK4* knockdown roots (n = 10) inoculated with *Sm 2011ΔnodF-GFP* did not show a clear effect on nodulation (averaging 5 nodules per root), but did affect infection thread morphology. Seventy percent of the tubular infection threads contained sac-like structures (averaging 11 structures per root) along their length, similar to what was observed for the *Sm 2011 nodL* mutant. These data indicate that *LYK4* also plays a role in infection thread formation. However, knock-out mutants such as those obtained by target-ed induced local lesions in genomes (TILLING) (21) are needed to elucidate the contribution of the individual genes.

Expression analysis by RT-PCR and electronic Northem based on EST data revealed that *LYK3* is exclusively expressed in root tissue (Figs. 2 and 3A). *LYK1* is specifically active in shoots, whereas *LYK5* and *LYK7* are expressed in both roots and shoots. In addition, screening of available EST sequences revealed four LYSks, indicating the existence of a rather large gene family. Some members of this family, such as LYSK3/4, have a specific function in the rhizobial infection process, and it is possible that other LYSks present in roots also contribute to the Nod factor–induced infection process. However, some LYSks, such as those specifically present in shoots, most likely have a nonsymbiotic function. The involvement of certain *LYK* genes in nonsymbiotic processes is further supported by the fact that LYSks are also present in nonlegume species. For example, *Arabidopsis* contains five such genes, of which *AtLYK3* is most homologous to the *LYK* genes.

Two models for Nod factor perception have been proposed. One model includes a two-receptor mechanism with a Nod factor signaling receptor that triggers all responses and an

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We show that knockdown of LYK3/4 causes an almost complete block of infection thread formation when plants are inoculated with an Sm 2011ΔnodFE mutant. Interaction with wild-type Sm leads to the formation of infection threads, but these frequently lose their polar mode of growth. This shows that, in the knockdown lines, an entry mechanism is still active that has a higher affinity for the wild-type C16:2 acyl chain–containing nod factors than for the C18:1 Nod factors produced by Sm 2011ΔnodFE. This could be due to the presence of multiple entry receptors. Alternatively, residual LYK3/4 protein could be present in the knockdown lines, which could be sufficient to allow wild-type bacteria to infect.

The hypothesis that LYK3/4 is a Nod factor receptor implies that the extracellular domain of LYK3/4, containing two LysM domains, specifically controls bacterial entry. A two-receptor model in which the NFP protein represents a Nod factor entry receptor that controls infection phenotype does not exclude factors, depending on their structure (4). Highly conserved amino acids are shown in bold.

References and Notes
16. BAC clones MH1-015B03, MH1-041N12, MH1-003I09, and MH1-052O10 were shotgun-sequenced with BAC MtH2-033L22 (accession no. AC123570) cloned into A. tumefaciens. BAC clones MH1-003I09 and MtH1-052O10 were shotgun-sequenced with 8× coverage. The sequence between MH1-015B03 and MH1-041N12 was determined with a PCR-based strategy that used MH1-022(24)A/BAC clones MH1-003I09 and MH1-052O10 formed a contig with BAC MH1-038L22 (accession no. AC123570) that was sequenced at Oklahoma University.
27. The consensus sequence was predicted by Pfam, available at www.sanger.ac.uk/Software/Pfam/search.shtml.
28. We thank Greenomics (Plant Research International, Wageningen) for sequencing the BAC clones and M. Twumasi, and I. Melis, who participated as undergraduate students in this project. Supported by the Netherlands Organization for Scientific Research (NWO), the European Union’s FP5 program (grant no. QLC-CT2000-00676), and the Dutch Graduate School of Experimental Plant Sciences.

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References
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