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Sym2 of Pea Is Involved in a Nodulation Factor-Perception Mechanism That Controls the Infection Process in the Epidermis[1]

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In pea (*Pisum sativum*) up to 50 nodulation mutants are known, several of which are affected in the early steps of the symbiotic interaction with *Rhizobium* sp. bacteria. Here we describe the role of the *sym2* gene in nodulation (Nod) factor perception. Our experiments show that the *sym2*^{A} allele from the wild pea variety Afghanistan confers an arrest in infection-thread growth if the *Rhizobium leguminosarum* bv *viciae* strain does not produce Nod factors with a NodX-mediated acetylation at their reducing end. Since the induction of the early nodulin gene *ENOD12* in the epidermis and the formation of a nodule primordium in the inner cortex were not affected, we conclude that more than one Nod factor-perception mechanism is active. Furthermore, we show that *sym2*^{A}-mediated control of infection-thread growth was affected by the bacterial nodulation gene *nodO*.

Rhizobium bacteria have the ability to induce a developmental process in the root of leguminous plants that results in the formation of a new organ, the root nodule. These new organs create the environment in which the bacteria fix nitrogen to ammonia, which can subsequently be utilized by the plant.

The symbiotic interaction of *Rhizobium* sp. bacteria and leguminous plants is set in motion by the exchange of signal molecules. Plant-excreted flavonoids induce the expression of bacterial nodulation (*nod*) genes, which are responsible for the synthesis of specific lipochitin oligosaccharides, named Nod factors (Lerouge et al., 1990; Spaink et al., 1991). Nod factors consist of a tetra- or pentameric *N*-acetylglucosamine backbone with a fatty acyl chain at the nonreducing terminal sugar moiety. Substituents at the terminal sugar residues and the structure of the acyl chain determine the differences in biological activity and host specificity (for review, see Carlson et al., 1994).

The role of Nod factor structure in host specificity is exemplified as follows: alfalfa (*Medicago sativa*) belongs to the cross-inoculation group that can be nodulated by *Rhizobium meliloti*, which produces Nod factors with a sulfate group at the reducing sugar (Lerouge et al., 1990). In contrast, pea (*Pisum sativum*) is nodulated by *Rhizobium leguminosarum* bv *viciae*, which produces Nod factors that lack a substitution at that position (Spaink et al., 1991). When the host-specificity genes *nodH*, *nodP*, and *nodQ*, which are responsible for the sulfation of the Nod factors in *R. meliloti*, are introduced into *R. leguminosarum* bv *viciae* these bacteria can now induce noninfected, nodule-like structures on alfalfa but concomitantly lose their ability to nodulate pea and vetch (Faucher et al., 1989).

Nod factors are responsible for the induction of a series of responses in the host, such as depolarization of the root hair plasma membrane (Ehrhardt et al., 1992; Felle et al., 1995; Kurkdjian, 1995), alkalinization of root hair cells (Felle et al., 1996), oscillation of the free cytoplasmic calcium concentration in root hairs (Ehrhardt et al., 1996), induction of root hair deformation (Lerouge et al., 1990; Spaink et al., 1991; Heidstra et al., 1994), induction of early nodulin (*ENOD*) genes (Horvath et al., 1993; Journet et al., 1994), and mitotic reactivation of cortical cells (Spaink et al., 1991). The latter is the beginning of the formation of primordia which, upon infection by rhizobia, develop into root nodules. Since Nod factors induce the responses at concentrations as low as 10^{-12} M, it has been proposed that they are recognized by host receptors (for review, see Geurts and Franssen, 1996).

Our current understanding of the mechanism of Nod factor perception is incomplete and based only on experiments with bacterial mutants and purified Nod factors. *Rhizobium* sp.-induced responses in the epidermis of alfalfa demand different structural features of Nod factors. Infection thread formation requires sulfated Nod factors with a C16:2 acyl group and a substitution of an acetyl group at the nonreducing terminal sugar, whereas the specific structure of the acyl chain and the acetyl substitution are not important for root hair deformation. Therefore, it was proposed that more than one Nod factor-perception mechanism is active in the epidermis (Ardourel et al., 1994). Like root hair deformation, the induction of *ENOD12* in alfalfa

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Abbreviation: X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

and pea does not require a highly unsaturated acyl chain (Horvath et al., 1993; Journet et al., 1994).

The complexity of Nod factor perception is further illustrated by the fact that, during the interaction of *R. leguminosarum* bv *viciae* with its host plants, structural deficiencies of Nod factors due to a *nodE* mutation can be compensated by a protein, NodO. An inactive *nodE* will lead to the production of Nod factors that are mainly acylated with vaccenic acid (C18:1), whereas normally also Nod factors with a highly unsaturated C18:4 acyl chain are produced (Spaink et al., 1991, 1995). NodO is a secreted bacterial protein that is not involved in Nod factor production or secretion but might form an ion channel in the plant plasma membrane (Sutton et al., 1994). Furthermore, it is proposed that plant-encoded chitinolytic enzymes contribute to the biological activity of Nod factors. Different decorations at the reducing terminal sugar of the Nod factor can protect the molecule against degradation to a greater or lesser extent, and it has been suggested that such host-specific substitutions might protect the Nod factor from degradation by enzymes from the host plant (Firmin et al., 1993; Staehelin et al., 1994).

To unravel the molecular mechanisms by which the host perceives Nod factors, it is important to have host mutants that are disturbed in such a mechanism. The characterization of such mutants and corresponding genes will improve our understanding of Nod factor perception. In pea several mutants have been identified as being affected in the early steps of the symbiotic interaction with *Rhizobium* bacteria. For *sym2* an allele has been identified in the wild pea variety Afghanistan, $sym2^A$, which only allows nodulation by specific *R. leguminosarum* bv *viciae* strains (Lie, 1984). A single bacterial nodulation gene, *nodX*, was shown to confer the ability to nodulate plants harboring this $sym2^A$ allele from cv Afghanistan (Lie, 1984; Firmin et al., 1993; Kozik et al., 1995). *nodX* encodes an acetyl transferase that specifically acetylates the reducing terminal sugar moiety of pentameric Nod factors (Firmin et al., 1993). Hence, there is a correlation between the presence of the $sym2^A$ allele in the pea genome and a specific Nod factor structure. Therefore, it was proposed that *sym2* is involved in Nod factor perception (Firmin et al., 1993; Kozik et al., 1995).

We named the allele in cultivated peas, homologous to $sym2^A$, $sym2^C$. Which of the two *sym2* alleles is dominant in heterozygous plants is, surprisingly, determined by the *R. leguminosarum* bv *viciae* strain used as inoculum. For example, the *R. leguminosarum* bv *viciae* $nodX^-$ strains 248 and PF_2 form nodules on heterozygotic $sym2^A sym2^C$ plants, whereas a similar $nodX^-$ strain PRE does not (Lie, 1984; Kozik et al., 1995). Strikingly, *R. leguminosarum* bv *viciae* strains 248 and PF_2 produce significantly higher amounts of Nod factors than does strain PRE. However, this quantitative difference in Nod factor production appears not to be responsible for the alternating dominant/recessive nature of $sym2^A$, since the introduction of the transcriptional activator *nodD* of *R. leguminosarum* bv *viciae* strain 248 into strain PRE, leading to an increase of Nod factor production, did not change the dominant nature of $sym2^A$ in heterozygous $sym2^A sym2^C$ plants (Kozik et al., 1995).

Here we report the role of $sym2^A$ in Nod factor perception. Our experiments show that *R. leguminosarum* bv *viciae* strains lacking *nodX* are specifically arrested in the infection process in their interaction with $sym2^A$-harboring peas. Furthermore, we show that $sym2^A$-mediated control of infection-thread growth is affected by *nodO*. By analyzing the efficiency of Nod factor degradation we show that $Sym2^A$ does not strongly enhance Nod factor degradation.

MATERIALS AND METHODS

Plant Material and Bacterial Strains

For all experiments we used the near-isogenic pea (*Pisum sativum* L.) lines cv Rondo-$sym2^C sym2^C$ and Rondo-$sym2^A sym2^A$, which were designated by Kozik et al. (1995) as line A.5.4.3. The backcross line contains an introgressed region of cv Afghanistan around $sym2^A$, as described by Kozik et al. (1995). Nodulation assays were performed in modified Leonard jars, as described by Lie et al. (1988).

All of the *Rhizobium* sp. strains used in the experiments were derivatives of *Rhizobium leguminosarum* bv *viciae* strain 248 (Josey et al., 1979). The bacterial strains and plasmids used in this study are listed Table I. The plasmids pMP225, pIJ1089, pXLGD4, pMW1071, and pMW2102 were transferred to *R. leguminosarum* bv *viciae* strains using triparental mating with pRK2013 as a helper plasmid (Ditta et al., 1980). The *R. leguminosarum* bv *viciae* strains 248*nodO*$^-$, 248*rhiA*$^-$, and 248*rhiR*$^-$ were made by crossing derivatives of pRL1JI carrying a *Tn*5 in *nodO*, *rhiA*, or *rhiR* (Economou et al., 1990; Cubo et al., 1992) as described by Beringer et al. (1978). Selection of *trans*-conjugants was done on B^- medium with the appropriate antibiotics (Spaink et al., 1989).

Spot Inoculation

Sterilized pea seeds (15 min in commercial bleach, 15 min in 7% H_2O_2) were germinated at 18°C for 5 d on 1.5% agar plates. The seedlings were transferred to square Petri dishes containing Fåhraeus medium (Fåhraeus, 1957) plus 1.5% agar. The plates had a hole in the rim, which allowed the stem of the plant to grow out while the roots grew on sterile medium in the dark. The cotyledons were covered by sterile cotton. Normally, the plants were grown at 18°C with a 16-h light period for 5 to 7 d; however, for the temperature-shift experiments the plants were grown at 26°C. The lateral roots were spot-inoculated at the just-emerging root hairs with 0.2 μL of bacterial culture (A_{600} = 0.5). The position was marked in the agar using sterile ink.

For the quantification of infection threads, *lacZ*-containing *R. leguminosarum* bv *viciae* strains were used. β-Galactosidase activity was assayed as described by Boivin et al. (1990) using 5-bromo-4-chloro-3-indolyl-β-D-galactoside as a substrate. Blue staining of bacteria was visible within 24 h using ×10 magnification. Cell divisions were quantified after the root segments were bleached for 15 min in commercial bleach.

Table I. *R. leguminosarum* bv *viciae* strains and plasmids used in this study

Rhizobium	Relevant Characteristics	Reference
Strain		
248	*R. leguminosarum* bv *viciae* strain containing pRL1JI	Josey et al. (1979)
248*nodX*	248 carrying pMW1071 or pMW2102	Kozik et al. (1995); this study
248^c	(1391) strain 248-Rif^R cured of its Sym plasmid pRL1JI	Schlaman et al. (1992)
248^c.pMP225	1391 carrying pMP225	This study
248^c.pIJ1089	1391 carrying pIJ1089	This study
248*nodO*$^-$	1391/pRL1JI*nodO*$_{94}$::*Tn*5	This study
248*rhiA*$^-$	1391/pRL1JI*rhiA*$_4$::*Tn*5	This study
248*rhiR*$^-$	1391/pRL1JI*rhiR*$_1$::*Tn*5	This study
248*nodO*$^-$*nodX*	1391/pRL1JI*nodO*$_{94}$::*Tn*5 carrying pMW1071	This study
248*lacZ*	248 carrying pXLGD4	This study
248*lacZnodX*	248 carrying pMW2102 and pXLGD4	This study
248*lacZnodO*$^-$	1391/pRL1JI*nodO*$_{94}$::*Tn*5 carrying pXLGD4	This study
Plasmid		
pRK2013	Helper plasmid	Ditta et al. (1980)
pMW1071	*nodX* of strain TOM cloned in pMP1070	Kozik et al. (1995)
pMP2733	incW, cloning vector	Spaink et al. (1994)
pMW2102	*nodX* of *R. leguminosarum* bv *viciae* strain TOM cloned in pMP2733	This study
pMP225	*nodABCIJDFELMNT* of pRL1JI	Spaink et al. (1989)
pIJ1089	*nodABCIJDFELMNTO rhi*ABCR *nifH* of pR11JI	Downie et al. (1983)
pXLGD4	*hemA:lacZ* fusion in pGD499	Leong et al. (1985)

Plastic Embedding and Sectioning

The spot-inoculated root segment was fixed for 1 to 2 h in 0.5% glutaraldehyde plus 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 6.85, washed four times for 15 min with phosphate buffer, washed two times for 15 min with water, and dehydrated in an ethanol series. Plastic infiltration was done according to the protocol of Kulzer Histo-Technik 8100 (Wehrheim, Germany).

Reverse Transcriptase-PCR to Quantify *ENOD12* Expression

Pea plants were cultured as described by Bisseling et al. (1978). Four-day-old seedlings were inoculated (1 mL of bacterial culture [A_{600} = 0.5] for each plant), and root hairs were harvested from 5-d-old seedlings (Gloudemans et al., 1989). Total RNA was isolated according to the method of Pawlowski et al. (1994) and then treated with DNaseI (Promega). cDNA was made from 2.5 μg of total RNA in a volume of 20 μL of 10 mM Tris-Cl, pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphate, 1 μg of oligo $d(T)_{12-18}$ (Pharmacia), 17 units of RNA guard (Pharmacia), and 20 units of avian myeloblastosis virus reverse transcriptase (Stratagene) for 10 min at room temperature, followed by 1 h at 42°C and 5 min at 95°C. The PCR reactions were performed with 1 μL of the cDNA solution in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μM deoxyribonucleotide triphosphates, 50 ng of each primer, and 1 unit of *Taq* polymerase (Boehringer Mannheim) in a total volume of 50 μL.

ENOD12 and ubiquitin were amplified using the following PCR program: 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C by using the primers PsENOD12-f (5′-TCACTAGTGTTGTTCCTTGC-3′) and PsENOD12A-r (5′-CCATAAGATGGTTTGTCACG-3′) to amplify only *PsENOD12A*, and UBIQ-f (5′-ATGCAGAT$^C/_T$TTTGTGAAGAC-3′) and UBIQ-r (5′-ACCACCACG$^G/_A$AGACGGAG-3′) to amplify ubiquitin. The amplified DNA samples were separated on a 1.6% agarose gel and, after alkaline blotting onto a nylon membrane (Hybond-N$^+$, Amersham), hybridized to ^{32}P-labeled PsENOD12A or ubiquitin DNA probes.

Degradation of Nod Factors

The Nod factor NodRlv-V(Ac,C18:4) was labeled with [^{14}C]acetate using the Nod factor-overproducing *R. leguminosarum* bv *viciae* strain 248^c.pIJ1089, as described by Heidstra et al. (1994). The specific activity of the Nod factor was about 10 mCi mmol^{-1}. Two 5-d-old pea seedlings were incubated in 4 mL of medium with 25,000 cpm (6×10^{-7} M) labeled Nod factor for 1, 3, 8, and 24 h. At each time point the medium and roots were collected and extracted with *n*-butanol. The extracts were analyzed by TLC (Silica Gel 60, Merck, Darmstadt, Germany), as described by Heidstra et al. (1994). The TLC plates were exposed to a phosphor screen and the amount of radioactivity was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

NodO Can Partially Compensate for the Absence of NodX-Mediated Nod Factors

Since the *R. leguminosarum* bv *viciae* strain used as inoculum determines which *sym2* allele is dominant, it is possible that those strains that can nodulate heterozygous Rondo-*sym2*^{A}*sym2*^{C} plants may have extra genes compared with those strains that cannot nodulate. To address this we analyzed the nodulation behavior of a pSym-cured derivative of *R. leguminosarum* bv *viciae* strain 248 containing large, cloned *nod* gene regions from the Sym plasmid pRL1JI (Fig. 1). The cured strain carrying pIJ1089 retained the characteristics of *R. leguminosarum* bv *viciae* strain 248 in

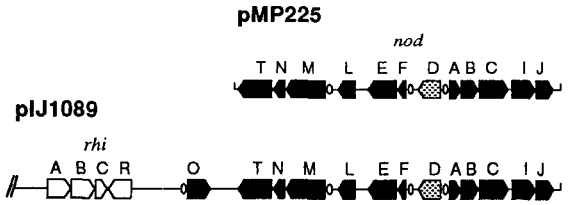

Figure 1. Map of the *nod-rhi* gene region of the *R. leguminosarum* bv *viciae* Sym-plasmid pRL1JI cloned in pMP225 (Spaink et al., 1987) and pIJ1089 (Downie et al., 1983). The *nod* genes are indicated as black arrows with the open circle indicating a *nod* box promoter. The constitutively expressed *nodD* is shown in gray and the *rhi* genes in white. pIJ1089 harbors, in addition to the *nod* genes present in pMP225, a region containing *nodO, rhiABC, rhiR,* and *nifH* (not shown).

that it nodulated heterozygous Rondo-$sym2^{A}sym2^{C}$ and homozygous cv Rondo-$sym2^{C}sym2^{C}$ plants (Table II). Although the equivalent strain carrying pMP225 did nodulate cv Rondo-$sym2^{C}sym2^{C}$ plants, it could not nodulate heterozygous Rondo-$sym2^{A}sym2^{C}$ plants. The major difference between pMP225 and pIJ1089 is that pIJ1089 is about 9 kb larger and the *nodO, rhiABC,* and *rhiR* operons are contained within the additional DNA. It follows that a gene or genes within this region of DNA determine whether the bacteria can nodulate heterozygous $sym2^{A}sym2^{C}$ plants.

To establish which of the known genes in the additional 9-kb region of pIJ1089 is required for nodulation of heterozygous $sym2^{A}sym2^{C}$ plants, we analyzed nodulation of *R. leguminosarum* bv *viciae* strain 248 derivatives carrying mutations in *nodO, rhiA,* or *rhiR*. The mutation of *rhiA* or *rhiR* did not significantly affect nodulation on any of the Rondo genotypes. However, mutation of *nodO* almost completely inhibited nodulation on the heterozygous $sym2^{A}sym2^{C}$ genotype, whereas nodulation of the $sym2^{C}sym2^{C}$ genotype was not affected (Table II). This demonstrates that *nodO* is essential for nodulation of heterozygous Rondo-$sym2^{A}sym2^{C}$ plants by *R. leguminosarum* bv *viciae* strain 248.

A role for *nodO* can also be seen in the nodulation of homozygous Rondo-$sym2^{A}sym2^{A}$ plants. *R. leguminosarum* bv *viciae* strain 248 carrying *nodO* can nodulate the homozygous $sym2^{A}$ plants at a low level (up to five nodules), but when *nodO* is absent, this nodulation is completely blocked. The introduction of the *nodX* gene in the *nodO* mutant strain enabled it to nodulate $sym2^{A}sym2^{A}$ and $sym2^{A}sym2^{C}$ peas as efficiently as *R. leguminosarum* bv *viciae* strain 248 carrying *nodX* (Table II). This demonstrates that in the compatible interaction with $sym2^{A}$-containing plants (i.e. when NodX-acetylated Nod factors are made) *nodO* is not essential for nodulation.

Sym2^{A}-Mediated Response

We attempted to examine the differences in Nod factor-induced responses in the incompatible interactions of *R. leguminosarum* bv *viciae* strains 248 and 248*nodO*$^{-}$ and the compatible interaction of strain 248*nodX* on Rondo-$sym2^{A}sym2^{A}$ plants. Root hair deformation and *ENOD12* induction are both responses in the epidermis, which do not demand stringent Nod factor structure requirements (Horvath et al., 1993; Journet et al., 1994). We found it extremely difficult to analyze root hair deformation in a quantitative manner in pea, whereas *ENOD12* expression could be quantified by reverse transcriptase-PCR studies. Four-day-old Rondo-$sym2^{A}sym2^{A}$ seedlings were inoculated with *R. leguminosarum* bv *viciae* strains 248, 248*nodO*$^{-}$, and 248*nodX*. The level of *ENOD12* mRNA in the root hairs was determined by reverse transcriptase-PCR after 24 h (Fig. 2). In spite of the inability of *R. leguminosarum* bv *viciae* strains 248 and 248*nodO*$^{-}$ to nodulate homozygous $sym2^{A}$ plants, they trigger *ENOD12* expression to a level similar to the compatible strain 248*nodX*.

We studied infection-thread formation and the induction of cortical cell divisions using a spot-inoculation assay. When a constitutively expressed β-galactosidase (*lacZ*) gene was introduced into the *Rhizobium* strains, it was possible to observe infection threads by staining for LacZ activity (Leong et al., 1985; Ardourel et al., 1994); while we were also able to examine cortical cell divisions in the same segment. Every experiment included at least 30 spot-inoculated roots, which were harvested after 10 d. Spot-inoculation of Rondo-$sym2^{A}sym2^{A}$ plants with *R. leguminosarum* bv *viciae* strain 248*nodX* resulted in more than 90% of the cases in formation of a nodule within 10 d, which implied that both infection thread and nodule primordium were formed. If Rondo-$sym2^{A}sym2^{A}$ was inoculated with *R. leguminosarum* bv *viciae* strain 248 or 248*nodO*$^{-}$, the formation of a nodule primordium in the inner cortex was induced by both in about 70% to 90% of the cases (Table III), but the cells never appeared to be infected. Figure 3 shows a cross-section of a Rondo-$sym2^{A}sym2^{A}$ root segment, spot-inoculated with *R. leguminosarum* bv *viciae* strain 248*nodO*$^{-}$. A nodule primordium was formed in the inner cortex, but there was neither differentiation into nodule tissues nor formation of a nodule meristem at the apex of the primordium. The primordia formed by *R. leguminosarum* bv *viciae* strain 248*nodO*$^{-}$ appeared to be smaller than those formed by strain 248 (data not shown). This might explain why a slightly reduced number of primordia was found in plants inoculated with *R. leguminosarum* bv *viciae* strain 248*nodO*$^{-}$.

Table II. *Nodulation behaviour of R. leguminosarum bv viciae strains on the CV Rondo-$sym2^{C}sym2^{C}$, the near-isogenic line Rondo-$sym2^{A}$ $sym2^{A}$, and the heterozygote Rondo-$sym2^{A}sym2^{C}$*

At least 10 plants were used in each inoculation. The number of nodules was determined 3 weeks after inoculation.

Rhizobium Strain	Plant		
	Rondo-$sym2^{C}sym2^{C}$	Rondo-$sym2^{A}sym2^{C}$	Rondo-$sym2^{A}$ $sym2^{A}$
248	40–60	40–60	0–5
248*nodX*	40–60	40–60	40–60
248^{c}	0	0	0
248^{c}.pMP225	40–60	0–5	0
248^{c}.pIJ1089	40–60	40–60	0–5
248*nodO*$^{-}$	40–60	0–5	0
248*rhiA*$^{-}$	40–60	40–60	0–5
248*rhiR*$^{-}$	40–60	40–60	0–5
248*nodO*$^{-}$*nodX*	40–60	40–60	40–60

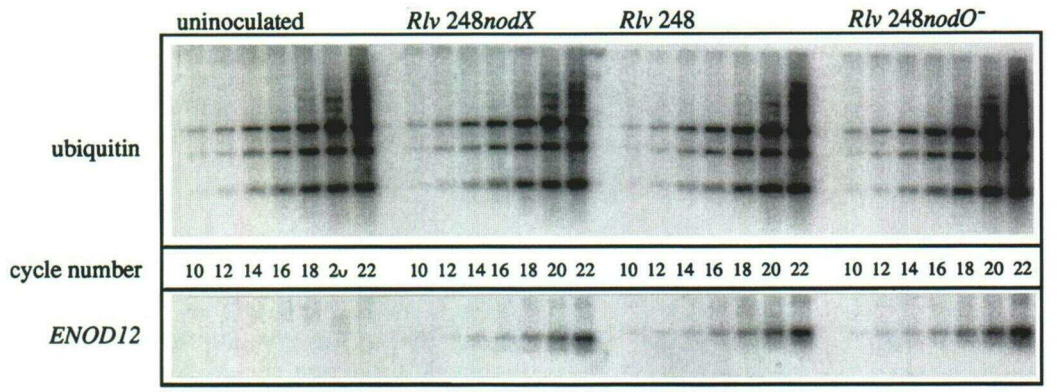

Figure 2. Induction of *ENOD12* expression in root hairs of Rondo-*sym2*^{A}*sym2*^{A}. *ENOD12* expression was analyzed by reverse transcriptase-PCR using total RNA isolated from root hairs collected 24 h after inoculation with *R. leguminosarum* bv *viciae* strain 248, 248*nodO*$^{-}$, *or 248nodX*. As a control, ubiquitin mRNA was amplified. Under the conditions used the amplification of ubiquitin mRNA is exponential between 12 and 16 cycles, whereas *ENOD12* amplification is exponential up to 22 cycles.

Infection-thread formation was only rarely found (less than 10% of the cases, Fig. 4), and these infection threads could be detected only in the epidermis and never grew into the inner cortical cell layers (Table III).

In summary, a similar level of *ENOD12* expression in the epidermis and an equal number of nodule primordia in the cortex were induced in the compatible and incompatible interaction. But, *R. leguminosarum* bv *viciae* strains lacking *nodX* formed a notably reduced number of infection threads, whereas formed infection threads were arrested in the epidermis.

Sym2^{A} Is Specifically Active during the First Days of the Interaction

Based on studies with *R. meliloti* mutants, it was proposed that a fully decorated Nod factor is required for infection events at the epidermis and that further growth of the infection thread through the cortex is less demanding in terms of Nod factor structure (Ardourel et al., 1994). During an incompatible interaction on *sym2*^{A} peas the growth of the infection thread is arrested in the epidermis. Therefore, we wondered whether *sym2*^{A} controls infection-thread growth only in the epidermis or also in the cortical cell layers. To locate the activity of *sym2*^{A} we made use of the temperature-sensitive nature of the phenotype of *sym2*^{A} peas (Kozik et al., 1995). At the permissive temperature (26°C) the number of nodules formed by *R. leguminosarum* bv *viciae* strains 248 and 248*nodO*$^{-}$ on Rondo-*sym2*^{A}*sym2*^{A} was markedly increased when compared with the nodulation efficiency of both strains at the nonpermissive temperature (18°C; Table IV). However, at the permissive temperature the *nodO* mutant nodulates less efficiently on *sym2*^{A}-harboring plants when compared with *R. leguminosarum* bv *viciae* strains 248 and 248*nodX*, which might be due to an infection process that is less efficient.

We determined when *sym2*^{A} is active in the nodule-formation process by growing plants for different periods

Table III. *Infection-thread and primordium formation in the cortex of spot-inoculated Rondo-sym2*^{A}*sym2*^{A}

Infection threads and primordia were scored 10 d after inoculation.

Rhizobium Strain	Spots	Infection Threads	Primordia
248*lacZnodX*	30	27 (90%)	27 (90%)
248*lacZ*	48	0 (0%)	41 (85%)
248*lacZnodO*$^{-}$	32	0 (0%)	22 (73%)

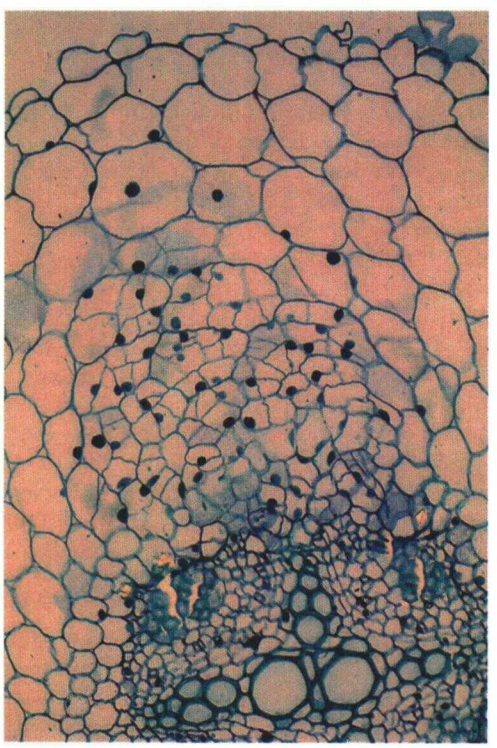

Figure 3. A cross-section (7 μm) of a Rondo-*sym2*^{A}*sym2*^{A} root segment, 10 d after spot inoculation with *R. leguminosarum* bv *viciae* strain 248*nodO*$^{-}$. A nodule primordium is formed in the inner cortex but the cells are not infected, since there is no infection thread formed.

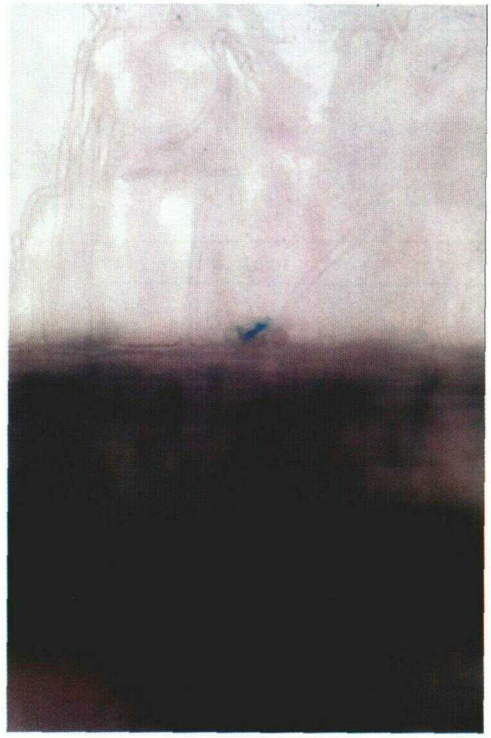

Figure 4. Infection-thread formation in the epidermis of Rondo-*sym2*^{A}*sym2*^{A} by *R. leguminosarum* bv *viciae* strain 248*lacZnodO*$^{-}$. Root segments were collected 10 d after spot inoculation with *R. leguminosarum* bv *viciae* 248*lacZnodO*$^{-}$, and infections were scored by staining the roots to detect β-galactosidase activity. Infection threads were rarely found in the epidermis and were never detected in the root cortex.

at the permissive temperature and then transferring them to the nonpermissive temperature. Rondo-*sym2*^{A}*sym2*^{A} roots were spot-inoculated with the *R. leguminosarum* bv *viciae* strains 248, 248*nodO*$^{-}$, and 248*nodX* and cultured for 1, 2, or 3 d at the permissive temperature (26°C) and, subsequently, the plants were cultured at the nonpermissive temperature (18°C). The formation of nodules was scored 10 d after spot inoculation. Every experiment included at least 20 spot-inoculated roots. A period of 3 d at 26°C postinoculation turned out to be sufficient to allow nodulation by *R. leguminosarum* bv *viciae* strain 248 with an efficiency similar to strain 248*nodX* (60–80%). *R. leguminosarum* bv *viciae* strain 248*nodO*$^{-}$ was also able to nodulate, but the number of successful infections was lower than in the compatible interaction with strain 248*nodX* (Table V).

To determine how far an infection thread develops within 3 d at 26°C, we spot inoculated Rondo-*sym2*^{A}*sym2*^{A} roots with *R. leguminosarum* bv *viciae* strains 248, 248*nodO*$^{-}$, and 248*nodX*, harboring the *lacZ* construct and stained for LacZ activity 3 d postinoculation. The experiment was performed at least 20 times with every bacterial strain, but in none of the cases could we detect an infection thread in the inner cortical cell layers. All formed infection threads were not beyond the root hairs.

These observations demonstrate that after 2 to 3 d *sym2*^{A} has little or no control over infection-thread development induced by strain *R. leguminosarum* bv *viciae* strain 248, even though the infection thread must still grow through the cortex to reach the cells of the nodule primordium. Together with the observation that, in the incompatible interaction infection-thread formation is not detectable or arrested in the outermost cell layers of the root, we have concluded that *sym2*^{A} is active in the first cell layer.

Sym2^{A} Does Not Enhance Nod Factor Degradation

In theory it is possible that *R. leguminosarum* bv *viciae* strains harboring *nodX* are able to nodulate *sym2*^{A}-containing plants, because the NodX modification might provide protection against Nod factor-degrading activity encoded by *sym2*^{A} (Firmin et al., 1993). This hypothesis implies that Nod factors that do not harbor an acetyl group at their reducing end are less stable on *sym2*^{A}-harboring peas when compared with *sym2*^{C}-harboring peas. This hypothesis was tested by comparing the degradation of the labeled pentameric Nod factor [^{14}C]NodRlv-V(Ac,C18:4) by the near-isogenic lines cv Rondo-*sym2*^{C}*sym2*^{C} and Rondo-*sym2*^{A}*sym2*^{A}. Two 5-d-old seedlings were incubated for 1, 3, 8, and 24 h, respectively, in the presence of radioactive Nod factor, after which the medium and the roots were collected. After extraction with *n*-butanol, the root and medium extracts were analyzed by TLC and the presence of Nod factor and its degradation products was determined by quantification of the radioactivity. The rate of Nod factor degradation of cv Rondo-*sym2*^{C}*sym2*^{C} was not significantly different from that of Rondo-*sym2*^{A}*sym2*^{A} (Fig. 5). Furthermore, the tetra-, tri-, and dimeric Nod factor derivatives were formed in similar amounts (data not shown). Thus, the presence of the *sym2*^{A} allele does not significantly enhance degradation of Nod factors lacking the NodX-mediated acetyl group. Comparison of the degradation activity of both genotypes of NodX-acetylated Nod factors showed similar results (data not shown), indicating that Sym2^{A} is unlikely to be involved in a general breakdown of Nod factors.

DISCUSSION

Here we show that Nod factors produced by *R. leguminosarum* bv *viciae* strains lacking *nodX* are perceived in the incompatible interaction with *sym2*^{A}-harboring peas, where they induce Nod factor-specific responses. *R. leguminosarum* bv *viciae* strains lacking or harboring *nodX* induce with a similar efficiency the expression of the early nodulin gene *ENOD12* in the epidermis and the formation of a nodule primordium in the inner cortex. The *sym2*^{A} allele appears to confer a very specific block in formation

Table IV. *Temperature-sensitive nodulation phenotype of Rondo-sym2*^{A}*sym2*^{A}

At least 10 plants were used in each inoculation and the number of nodules was determined 3 weeks after inoculation.

Rhizobium Strain	Nodules at 18°C	Nodules at 26°C
248*nodX*	40–60	40–60
248	0–5	40–60
248*nodO*$^{-}$	0	20–30

Table V. *Temperature-shift experiments with the spot-inoculated Rondo-sym2^{A}sym2^A*

Spot-inoculated plants were cultured for 1 to 3 d at 26°C and subsequently shifted to 18°C. The number of inoculations that led to nodule formation was scored 10 d after inoculation.

Rhizobium Strain	Days at 26°C					
	1		2		3	
	Spots	Nodules	Spots	Nodules	Spots	Nodules
248*nodX*	26	21 (80%)	23	20 (87%)	33	26 (79%)
248	20	0 (0%)	31	0 (0%)	31	20 (65%)
248*nodO*$^-$	21	0 (0%)	30	0 (0%)	33	5 (15%)

and growth of infection threads in the epidermis. When rhizobia produce the NodX-substituted Nod factors, infection-thread formation takes place efficiently on *sym2*^{A}-harboring plants.

NodO Stimulates Nod Factor-Induced Infection

The bacterial NodO protein can fully compensate for the lack of NodX-mediated substitution on Nod factors of *R. leguminosarum* bv *viciae* strains in the interaction with heterozygous *sym2*^{A}*sym2*^{C} plants, whereas only a partial compensation is achieved in the interaction with homozygous *sym2*^{A}*sym2*^{A} plants. Previously, it was shown that *nodO* can compensate for the absence of the highly unsaturated acyl chain (C18:4) in Nod factors from *R. leguminosarum* bv *viciae* strain 248*nodE*$^-$ (Economou et al., 1994). *R. leguminosarum* bv *viciae* strain 248*nodE*$^-$*nodO*$^-$ is seriously hampered in nodule formation on pea, whereas *R. leguminosarum* bv *viciae* strain 248*nodE*$^-$*nodO*$^+$ has similar abilities to induce nodule formation as the wild-type strain (Economou et al., 1994). These studies demonstrated that NodO can stimulate nodule formation, but it remained unclear which step(s) of nodulation were affected. Since the *sym2*^{A} allele confers inhibition of infection-thread growth, which can partially be overcome by *nodO*, it can be concluded that NodO at least stimulates the infection process. Therefore, it is plausible that in *R. leguminosarum* bv *viciae* *nodE*$^-$ mutants NodO also stimulates the infection process (Sutton et al., 1994).

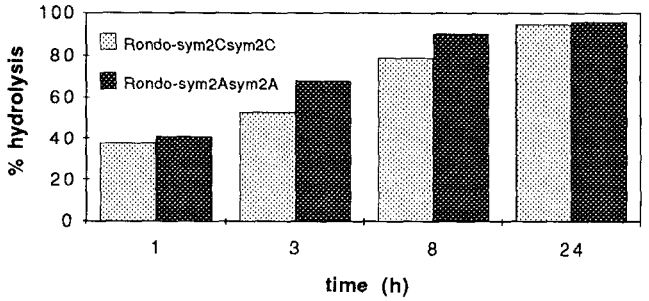

Figure 5. Degradation of [^{14}C]NodRlv-V(Ac,C18:4) by roots of cv Rondo-*sym2*^{C}*sym2*^{C} and Rondo-*sym2*^{A}*sym2*^{A}. Two 5-d-old pea seedlings were incubated in 4 mL of medium with 25,000 cpm (6×10^{-7} M) of labeled Nod factor for 1, 3, 8, and 24 h. The amount of radioactive pentameric Nod factor recovered from the medium and root extracts was determined and compared with the amount initially added. The data shown are the average of two experiments, and the data of the different experiments never varied more than 20%. The degradation rate of the Nod factor did not significantly differ in the presence of either of the roots.

The question remains how *nodO* can compensate for the Nod factor structure deficiency. NodO is a secreted protein that can integrate into artificial membranes, where it forms ion channels. It has been proposed that it could form ion channels in the host plasma membrane (Economou et al., 1994; Sutton et al., 1994), thereby, it could facilitate the effect of a nonoptimal Nod factor structure. Either the initial Nod factor induced signal transduction or the mechanisms by which infection-thread growth is sustained could be affected by NodO activity.

Infection Is Controlled in the Epidermis

Our studies on *sym2*^{A} show that the structural demands on Nod factors are more stringent for the formation of an infection thread than for triggering *ENOD12* expression in root hairs. When Rondo-*sym2*^{A}*sym2*^{A} is spot inoculated with the incompatible *R. leguminosarum* bv *viciae* strains 248 or 248*nodO*$^-$, infection-thread formation in the epidermis occurs only incidentally (<10%). Moreover, if infection occurs, the infection thread stops growing in the epidermis (Fig. 4). Furthermore, using the temperature-sensitive nature of the *sym2*^{A} phenotype, we showed that this gene has its effect only during the first days of the interaction. Taken together, these data strongly suggest that *sym2*^{A} is active in the epidermis, but it is unable to confer a block upon nodulation once the infection thread has reached the cortical cells.

Similar results have been obtained by using *R. meliloti* *nod* mutants (Ardourel et al., 1994). The *nodFE*, *nodL*, and *nodFEL* mutants produce modified Nod factors, which affects their capacity of inducing infection-thread formation. The mutant lacking NodF, NodE, and NodL is completely unable to trigger an infection on alfalfa, whereas it remains fully able to induce root hair deformation. The *nodFE* and *nodL* mutants form only a very few infection sites, but once infection-thread formation is initiated, they grow into the root cortex and become associated with nodule development (Ardourel et al., 1994). Hence, studies of plant genes and *Rhizobium* mutants show that infection is controlled in a more stringent way in the epidermis than in other layers of the root or root nodule.

How Does Sym2 Function?

The phenotype of *sym2*^{A}-harboring plants can be explained in two different ways. Sym2^A could act in a Nod

factor-activated signal transduction pathway, and since *sym2*^{A} plants require a Nod factor with a specific structure, Sym2^A might be a Nod factor receptor that by interaction with the NodX-modified Nod factor regulates infection-thread growth in the epidermis. Alternatively, *sym2*^{A} could encode a chitinolytic or other degradative enzyme that preferentially degrades Nod factors that lack the NodX-mediated acetylation (Firmin et al., 1993).

Our results show that the Nod factor-degradative activity of intact plant roots carrying *sym2*^{A} or *sym2*^{C} is similar when *R. leguminosarum* bv *viciae* Nod factors without a NodX-mediated modification are provided as a substrate. For both plants a similar pattern of breakdown products could be detected. This demonstrates that the modification of Nod factors by pea roots is not markedly affected by the presence of Sym2^A. However, since these experiments were performed with whole-root systems, it cannot be excluded that a putative *sym2*^{A}-encoded Nod factor-degradative enzyme is only active very locally at the infection site. However, there are two additional arguments against a role of Sym2^A in Nod factor degradation. If Nod factor-degrading activity controls strain-specific nodulation, it could be expected that an increased Nod factor production would lead to an enhanced number of successful infections. This is not the case, since the level of Nod factor synthesis in a strain lacking *nodX* does not influence the efficiency of nodulation (Kozik et al., 1995). Moreover, if Sym2^A encodes a Nod factor-degrading enzyme, a strict dominant character of *sym2*^{A} would be expected and this is not the case either, since nodulation by *R. leguminosarum* bv *viciae* strain 248 (harboring *nodO*) is as effective as by strain 248*nodX* on heterozygous *sym2*^{A}*sym2*^{C} plants. Although it cannot be completely excluded that Sym2^A is a Nod factor-degrading enzyme, we think that it is more probable that it functions in a Nod factor-perception mechanism.

The data obtained with *R. meliloti* mutants, which showed that Nod factor-induced epidermal responses can be uncoupled have led to the hypothesis that there are at least two Nod factor receptors in the epidermis: a signaling receptor and an entry receptor. The proposed entry receptor recognizes only Nod factors with appropriate decorations and induces the formation of an infection site and initial ingestion of bacteria, whereas infection-thread growth and root hair deformation are controlled by the signaling receptor that is less selective in Nod factor structure (Ardourel et al., 1994). The fact that the *nodX-sym2*^{A} system shows selective effects of Nod factor structure on infection-thread growth, as opposed to *ENOD12* induction, supports the general model that the plant may have more than one Nod factor receptor.

Since *sym2*^{A} controls infection and requires specific Nod factors, at first sight it seems plausible that in pea *sym2*^{A} encodes the postulated entry receptor. However, there are some differences between Sym2^A and the proposed alfalfa entry receptor. The entry receptor is supposed to be specifically involved in the initiation of infection. This receptor does not seem to have a role in infection-thread growth, since the few infection threads formed by *R. meliloti nodFE*$^-$ or *nodL*$^-$ mutants do not come aborted (Ardourel et al., 1994). Similarly, the number of infections of incompatible *R. leguminosarum* bv *viciae* strains on *sym2*^{A}*sym2*^{A} plants are markedly reduced. However, the few infection threads that are formed become mostly arrested. Therefore, Sym2^A seems to be a negative regulator of infection-thread growth. Whether the markedly reduced number of infections in the Sym2^A system is due to a block in the initiation of infection sites or a rapid block of growth after initiation cannot be concluded from our data. Therefore, it is not yet clear whether Sym2^A has, in addition to its negative effect on infection thread growth, the properties of the proposed entry receptor.

How *sym2*^{A} controls infection-thread formation remains unclear. The relation between *nodX* and *sym2*^{A} suggests a ligand (Nod factor) receptor interaction. However, recent studies of resistance genes and the corresponding avirulence genes show that such a direct interaction is not always the case (e.g. Cf-9 and Avr9, Kooman-Gersmann et al., 1996). Cloning and characterization of *sym2* will be essential to elucidate its role in Nod factor perception and transduction.

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LITERATURE CITED

Ardourel M, Demont N, Debellé F, Maillet F, De Billy F, Promé J-C, Dénarié J Truchet G (1994) *Rhizobium meliloti* lipooligosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of symbiotic developmental responses. Plant Cell **6:** 1357–1374

Beringer JE, Hoggan SA, Johnston AWB (1978) Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. J Gen Microbiol **104:** 201–207

Bisseling T, Van den Bos RC, Van Kammen A (1978) The effect of ammonium nitrate on the synthesis of nitrogenase and the concentration of leghemoglobin in pea root nodules induced by *Rhizobium leguminosarum*. Biochim Biophys Acta **539:** 1–11

Boivin C, Camut S, Malpica CA, Truchet G, Rosenberg C (1990) *Rhizobium meliloti* genes encoding catabolism of trigoneline are induced under symbiotic conditions. Plant Cell **2:** 1157–1170

Carlson RW, Price NPJ, Stacey G (1994) The biosynthesis of Rhizobial lipo-oligosaccharide nodulation signal molecules. Mol Plant-Microbe Interact **6:** 684–695

Cubo T, Economou A, Murphy G, Johnston AWB, Downie JA (1994) Molecular characterisation and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *R. leguminosarum* bv *viciae*. J Bacteriol **174:** 4026–4035

Ditta G, Stanfield S, Corbin D, Helsinki DR (1980) Broad host-range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA **77:** 7347–7351

Downie JA, Ma QS, Knight CD, Hombrecher G, Johnston AWB (1983) Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between the nitrogenase genes and the *nifA*-like gene. EMBO J **2:** 947–952

Economou A, Davies AE, Johnston AWB, Downie JA (1994) The *Rhizobium leguminosarum* biovar *viciae nodO* gene can enable a *nodE* mutant of *Rhizobium legominosarum* biovar *trifolii* to nodulate vetch. Microbiology **140:** 2341–2347

Economou A, Hamilton WDO, Johnston AWB, Downie JA (1990) The *Rhizobium* nodulation gene *nodO* encodes a Ca^{2+}-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. EMBO J **9:** 349–354

Ehrhardt DW, Atkinson EM, Long SR (1992) Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science **256:** 998–1000

Ehrhardt DW, Wais R, Long SR (1996) Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. Cell **85:** 673–681

Fåhraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass technique. J Gen Microbiol **16:** 374–381

Faucher C, Camut S, Dénarié J, Truchet G (1989) The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as avirulence genes in *R. leguminosarum* bv. *viciae* and determine changes in the production of plant-specific extracellular signals. Mol. Plant-Microbe Interact **6:** 291–300

Felle HH, Kondorosi E, Kondorosi A, Schultze M (1995) Nod signal-induced plasma membrane potential changes in alfalfa root hairs are differentially sensitive to structural modifications of the lipochitooligosaccharide. Plant J **7:** 939–947

Felle HH, Konodrosi E, Kondorosi A, Schulze M (1996) Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. Plant J **10:** 295–301

Firmin JL, Wilson KE, Carlson RW, Davies AE, Downie JA (1993) Resistance to nodulation of c.v. Afghanistan peas is overcome by *nodX*, which mediates an *O*-acetylation of the *Rhizobium leguminosarum* lipo-oligosaccharide nodulation factor. Mol Microbiol **10:** 351–360

Geurts R, Franssen H (1996) Signal transduction in *Rhizobium*-induced nodule formation. Plant Physiol **112:** 447–453

Gloudemans T, Bhuvaneswari TV, Moerman M, Van Brussel AAN, Kammen A, Bisseling T (1989) Involvement of *Rhizobium leguminosarum* nodulation genes in gene expression in pea root hairs. Plant Mol Biol **12:** 157–167

Heidstra R, Geurts R, Franssen H, Spaink HP, Van Kammen A, Bisseling T (1994) Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. Plant Physiol **105:** 787–797

Horvath B, Heidstra R, Lados M, Moerman M, Spaink HP, Promé J-C, Van Kammen A, Bisseling T (1993) Lipo-oligosaccharides of *Rhizobium* induce infection-related early nodulin gene expression in pea root hairs. Plant J **4:** 727–733

Josey DP, Beylon JL, Johnston AWB, Beringer JE (1979) Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J Appl Bacteriol **46:** 343–350

Journet EP, Pichon M, Dedieu A, De Billy F, Truchet G, Barker DG (1994) *Rhizobium meliloti* Nod factors elicit cell-specific transcription of the *ENOD12* gene in transgenic alfalfa. Plant J **6:** 241–249

Kooman-Gersmann M, Honee G, Bonnema G, De Wit PJGM (1996) A high affinity binding site for the AVR9 peptide elicitor of *Cladosporium fulvum* is present on plasma membranes of tomato and other solanceous plants. Plant Cell **8:** 929–938

Kozik A, Heidstra R, Horvath B, Kulikova O, Tikhonovich I, Ellis THN, Van Kammen A, Bisseling T (1995) Pea lines carrying *sym1* or *sym2* can be nodulated by *Rhizobium* strains containing *nodX; sym1* and *sym2* are allelic. Plant Sci **108:** 41–49

Kurkdjian AC (1995) Role of the differentiation of root epidermal cells in Nod factor (from *Rhizobium meliloti*)-induced root hair depolarization of *Medicago sativa*. Plant Physiol **107:** 783–790

Leong SA, Williams PH, Ditta GS (1985) Analysis of the 5′ regulatory region of the gene for δ-aminolevulinic acid synthetase of *Rhizobium meliloti*. Nucleic Acids Res **13:** 5965–5976

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé 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

Lie TA (1984) Host genes in *Pisum sativum* L. conferring resistance to European *Rhizobium leguminosarum* strains. Plant Soil **82:** 415–425

Lie TA, Pijnenborg J, Timmermans PCJM (1988) Analysis of the host genes controlling the legume-*Rhizobium* symbiosis: some technical problems and pitfalls. *In* DP Beck, LA Materon, Nitrogen Fixation by Legumes in Mediterranean Agriculture. Nyhoff, Dordrecht, The Netherlands, pp 93–100

Pawlowski K, Kunze R, De Vries S, Bisseling T (1994) Isolation of total, poly(A) and polysomal RNA from plant tissues. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1–13

Schlaman HRM, Lugtenberg BJJ, Okker RJH (1992) The NodD protein does not bind to the promoters of inducible nodulation genes in extracts of bacteroids of *Rhizobium leguminosarum* biovar *viciae*. J Bacteriol **174:** 6109–6116

Spaink HP, Bloemberg GV, Van Brussel AAN, Lugtenberg BJJ, Van der Drift KMGM, Haverkamp, J, Thomas-Oats JE (1995) Host specificity of *Rhizobium leguminosarum* is determined by hydrophobicity of highly unsaturated fatty acyl moieties of the nodulation factor. Mol Plant-Microbe Interact **8:** 155–164

Spaink HP, Sheeley DM, Van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature **354:** 125–130

Spaink HP, Weinman J, Djordjevic MA, Wijfelman CA, Okker RJH, Lugtenberg BJJ (1989) Genetic analysis and cellular localization of the *Rhizobium* host specificity-determining NodE protein. EMBO J **8:** 2811–2818

Spaink HP, Wijfjes AHM, Van der Drift KMGM, Haverkamp J, Thomas-Oates JE, Lugtenberg BJJ (1994) Structural identification of metabolites produced by the NodB and NodC proteins of *Rhizobium leguminosarum*. Mol Microbiol **13:** 821–831

Staehelin C, Schultze M, Kondorosi E, Mellor RB, Boller T, Konodorosi A (1994) Structural modifications in *Rhizobium meliloti* Nod factors influence their stability against hydrolysis by root chitinases. Plant J **5:** 319–330

Sutton JM, Lea EJA, Downie JA (1994) The nodulation-signaling protein *nodO* from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. Proc Natl Acad Sci USA **91:** 9990–9994