Perception and Action of Nod Factors in Rhizobium-Legume Symbiosis

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STELLINGEN

1. Het waarnemen en doorgeven van de signalen die Nod factoren kunnen geven aan wortels van vlinderbloemige planten wordt niet beinvloed door nitraat.

   Dit proefschrift

2. De plaats waar in de cortex van wortels van vlinderbloemige planten, celdelingen worden geinduceerd die leiden tot knolvorming, wordt mede bepaald door ethyleen.

   Dit proefschrift

3. Het produkt van het *sym2*^{A} gen is betrokken bij de herkenning van Nod factoren en controleert de infectie van *Rhizobium* bacteriën in de epidermis

   Dit proefschrift

4. Zolang de moleculaire reacties op Nod factoren in de wortelhaar zoals Calcium spiking en membraandepolarisatie niet gekoppeld kunnen worden aan de morfologische reacties kan niet geconcludeerd worden dat deze processen een rol spelen in de signaal transductie van Nod factoren.

   Ehrhardt et al. (1992) Science 256, 998-1000; (1996) Cell 85, 673-681; Felle (1994) Plant J. 7, 495-499; Kurkdjian (1995) Plant Physiol 107, 783-790

5. Uit de homologie tussen het pollenspecifieke eiwit PO22 en het noduline ENOD8 leiden Wu et al. ten onrechte af dat beide eiwitten een vergelijkbare functie hebben bij respectievelijk pollenbuis- en infectiedraadgroei.

   Wu et al. (1996) Plant Mol. Biol. 32, 1205-1207;
   Dickstein et al. (1993) Mol. Plant-Microbe Interact. 6, 715-721

6. De resistentie van sommige mensen tegen HIV-1 infectie ten gevolge van een genetisch defect in het HIV-1 coreceptor gen CKR-5, zonder daarbij een fenotype te vertonen, geeft aan hoe flexibel de natuur is.

   Liu et al. (1996) Cell 86, 367-377

7. Galbraith en Pandey scoren de TNF-α allelen van mensen met de huidziekte alopecia areata ten onrechte als T1 (A^{-308}) en T2 (G^{-308}).

Galbraith en Pandey (1995) Hum. Genet. 96, 433-436

8. Uit de bewijzen voor fossiel leven op Mars mag het Arbeidsburo niet afleiden dat er buitenaardse levensvormen zijn die hier willen werken en bovendien Engels spreken.

Arbeidsvoorziening, de folder "Working in the Netherlands: the rules for aliens"

9. Het invoeren van Friese alternatieven voor algemeen geaccepteerde woorden, met het "akwadukt" over de A32 als schrijnend voorbeeld, dreigt de Friese taal belachelijk te maken.

10. Stellingen bij een proefschrift wijzen vaak op niets meer dan andermans fouten en behoren daarom niet meer verplicht gesteld te worden voor het promoveren.

Stellingen behorende bij het proefschrift:
"Perception and action of Nod factors in Rhizobium-legume interaction"
door Renze Heidstra, te verdedigen op 28 februari 1997.

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Outline

Rhizobium bacteria are able to invade the roots of their leguminous hosts and trigger the formation of a new organ, the root nodule. In these nodules the bacteria are hosted in the proper environment for fixing atmospheric nitrogen into ammonia, making plant growth independent of nitrogen compounds from the soil. The interaction between the bacterium and the host plant starts with a signal exchange, when flavonoids excreted by the plant induce the transcription of bacterial nodulation (*nod*) genes. Induction of these nod genes leads to the synthesis of specific lipo-oligosaccharides (Nod factors) required for inducing various root responses like root hair deformation, infection thread and nodule primordia formation.

The aim of the research described in this thesis is to contribute to the insight in the mechanism by which Nod factors initiate root nodule formation. To address this issue it is essential to have a plant system in which both the morphological and molecular changes induced by Nod factors can be studied. We adapted a root hair deformation assay for *Vicia sativa* (vetch), growing the seedlings in Fåhraeus slides, to study the activity and fate of the various Nod factors secreted by *Rhizobium leguminosarum* bv *viciae* in a fast, simple and semi-quantitative way (Chapter 2). Root hair deformation in this assay is limited to root hairs in a specific developmental stage and involves a reinitiation of tip growth which is visible within 3 hours after Nod factor application.

In an attempt to identify genes the expression of which is induced in an immediate respons to Nod factors we used the differential RNA display method to compare the pattern of cDNAs expressed in the zone of the vetch root where root hair deformation occurs at 0, 1 and 3 hours after Nod factor addition. Surprisingly, the sequence of one of the isolated clones already expressed 1 hour after Nod factor application coded for leghemoglobin. This gene was used to show that gene expression and root hair deformation are not coupled and to investigate how NH_4NO_3 might block root hair deformation (Chapter 3).

Root hair deformation induced by Nod factors involves a reinitiation of growth in the existing root hair tip, a mechanism reminiscent of ethylene induced tip growth leading to the formation of root hairs in the epidermis. Since ethylene is a potent inhibitor of cortical cell division there seems to be a paradox in its action during the initial interaction between rhizobia and its host. We showed that ethylene is not invoved in the root hair deformation process, but it is one of the factors involved in giving positional information determining where nodule primordia can be induced (Chapter 4).

How Nod factors are perceived and induce the various responses in the root is poorly understood. Considering that Nod factors are active at very low concentrations it is likely that they are recognized by receptors. Besides a biochemical approach to search for such receptors it will be very important to isolate and analyse host mutants disturbed in the early stages of the interaction with *Rhizobium*. We chose to focus on the $sym2^A$ gene originating from Afghanistan pea since the presence of this gene puts more stringent structural demands on the Nod facotrs secreted by *Rhizobium leguminosarum* bv *viciae*. After detailed analysis of the phenotype conferred by $sym2^A$ we propose that Sym2 controls the infection process in the epidermis, possibly representing a Nod factor receptor (Chapter 5).

CHAPTER 1

Nod factor-induced host responses and mechanisms of Nod factor perception

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Nod factor-induced host responses and mechanisms of Nod factor perception

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SUMMARY

Soil bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* are able to invade the roots of their leguminous host plants, where they trigger the formation of a new organ, the root nodule. At least two steps of signal exchange between the bacterium and the host plant are involved in starting the interaction. In the first step, flavonoids excreted by the plant induce the transcription of bacterial nodulation genes (*nod* genes). The induction of these *nod* genes leads to the synthesis of specific lipo-oligosaccharides (called Nod factors) that can induce various root responses, e.g. root hair deformation, depolarization of the root hair membrane potential, induction of nodulin gene expression, and formation of nodule primordia.

We will focus on the various nod factor-induced plant responses. We will discuss the ways these responses can be used to improve our knowledge of the mechanism of Nod factor perception. Furthermore, plant genes, in particular the *sym2* allele from Afghanistan pea, encoding proteins that probably are involved in Nod factor recognition, are discussed. Based on the Nod factor-induced plant responses and the phenotype displayed by *sym2* peas, a model will be presented of how Nod factors may be perceived.

Key words: Nodulation, *Rhizobium*, Nod factor, *sym2*, receptor.

INTRODUCTION

Soil bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (here collectively called rhizobia) are able to invade the roots of their leguminous host plants and trigger the formation of a new organ, the root nodule. The interaction of rhizobia and legumes starts with signal exchange, followed by attachment of the rhizobia to the plant root hairs. The root hairs deform, and the bacteria invade the plant by a newly formed infection thread growing through the root hairs into the cortex. Simultaneously, cortical cells are mitotically activated, giving rise to the nodule primordium. Infection threads grow towards the primordium and the bacteria, surrounded by a plant-derived peribacteroid membrane, are released into the cytoplasm of the host cells. The nodule primordium then develops into a nodule, while the bacteria differentiate into their endosymbiotic form, the bacteroids. These bacteroids are able to fix nitrogen into ammonia, which can subsequently be utilized by the plant. All steps of nodule development involve the expression of nodule-specific plant genes, the so-called nodulin genes (Van Kammen, 1984).

At least two steps of signal exchange between bacterium and host plant are involved in starting the interaction (Fig. 1). In the first step, flavonoids (Table 1) excreted by the plant induce the transcription of bacterial nodulation genes (*nod* genes). This process involves the constitutively expressed *nodD* that can bind to specific flavonoids (Goethals, Van Montagu & Holsters, 1992) which turns this protein into a transcriptional activator of the other *nod* genes (Fisher & Long, 1992). The proteins encoded by these genes are involved in the synthesis of specific lipo-oligosaccharides (called Nod factors, Table 1) that can induce various responses in the root, e.g. root hair deformation, depolarization of the root hair membrane potential, induction of nodulin gene expression, and formation of nodule primordia.

The host-specific aspect of this symbiotic interaction between rhizobia and legumes is very pronounced, e.g. *Rhizobium leguminosarum* (*R.l.*) bv. *viciae* can only nodulate plants of the genera *Pisum*, *Vicia*, *Lathyrus* and *Lens*, whereas the closely related *R.l.* bv. *trifolii* nodulates plants of the genus *Trifolium*. This strict host specificity had led to the definition of cross-inoculation groups in which the host plants are classified according to the bacterial species that can nodulate (Table 2). Both bacteria and their host plants are involved in determining

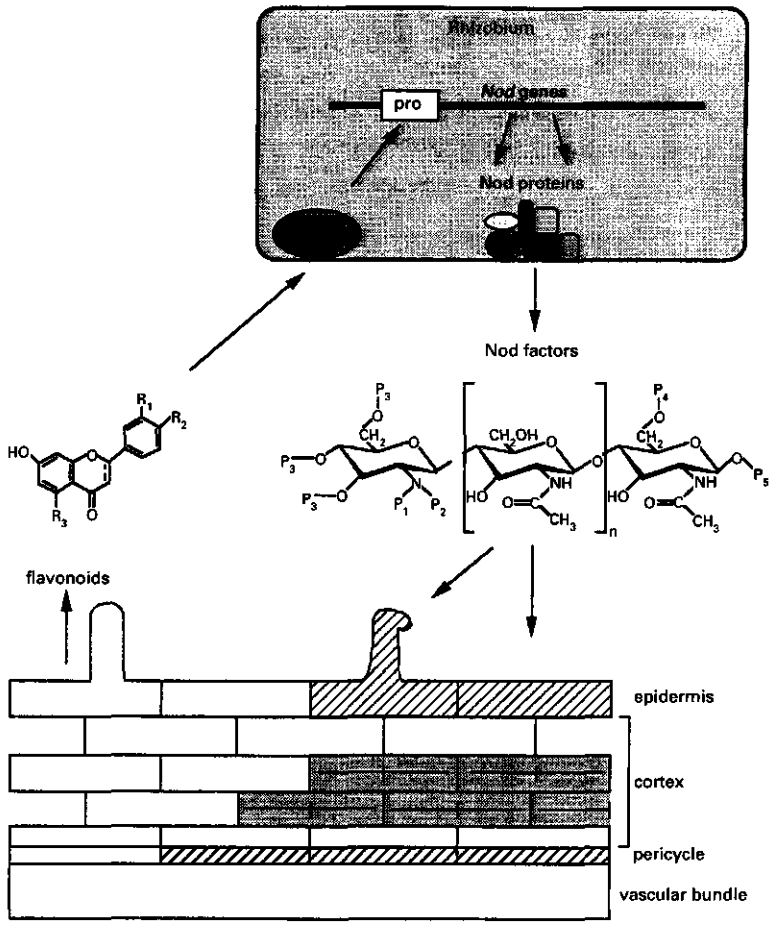

Figure 1. Early events in the legume-*Rhizobium* interaction. Flavonoids excreted by the plant induce the transcription of bacterial nodulation genes (*nod* genes). This process involves the constitutively expressed *nodD* that can bind to specific flavonoids which turns this protein into a transcriptional activator of the other *nod* genes. The Nod proteins are involved in the synthesis of Nod factors that can induce various responses in the root, e.g. root hair deformation, induction of nodulin gene expression, and formation of nodule primordia. These responses can be divided into three classes based on the root tissue where the response is induced; i.e. epidermis, cortex and pericycle. See Table 1 for the various substitutions on flavonoids and Nod factors.

host specificity, the bacteria by producing specific Nod factors and the host plants by expressing genes for recognition of these Nod factors.

In the following section, we will focus on the various Nod factor-induced plant responses. We will discuss how these responses can be used to improve our knowledge of the mechanism of Nod factor perception. Furthermore, we will discuss which of the already identified host genes required for normal nodulation might encode a Nod factor receptor.

NOD FACTOR STRUCTURE

The structure of the major Nod factor produced by *R. meliloti* was the first to be elucidated (Lerouge *et al.*, 1990). At present, the structure of Nod factors produced by most other rhizobia has been determined (Fisher & Long, 1992; Spaink, 1992; Dénarié & Cullimore, 1993; Carlson, Price & Stacey, 1995), showing that all Nod factors consist of a β-1,4-linked *N*-acetyl-D-glucosamine backbone varying in length between three and six sugar units. The non-reducing terminal sugar moiety is substituted on the C-2 position with a fatty acid, the structure of which is variable. Depending on the species, additional substitutions on the terminal sugar residues are present (Table 1).

Nod factor nomenclature is based on the similarities between all published Nod factors (Spaink, 1992). In this nomenclature, the species indication (e.g. Rlv for *R. leguminosarum* bv. *viciae*) is followed by a roman numeral referring to the number of

Table 1. *Examples of flavonoid and Nod factor structures*

Flavonoid structures

Compounds	R_1	R_2	R_3	Double bond	Activates NodD of*
Naringinin	—H	—OH	—OH	No	*Rhizobium leguminosarum* bv. *viciae* bv. *trifolii* *Rhizobium* sp. NGR234
Hesperitin	—OH	—OCH_3	—OH	No	*Rhizobium leguminosarum* bv. *viciae* *Rhizobium* sp. NGR234
Luteolin	—OH	—OH	—OH	Yes	*Rhizobium leguminosarum* bv. *viciae* bv. *trifolii* *Rhizobium* sp. NGR234 *Rhizobium meliloti*

Nod factor structures

Species	P_1	P_2	P_3	P_4	P_5	*n*	References
Rhizobium leguminosarum bv. *viciae*	—H	—C18:4 —C18:1	—$COCH_3$(O-6)†	—H —$COCH^3$‡	—H	2,3	Spaink *et al.* (1991) Firmin *et al.* (1993)
bv. *trifolii*	—H	—C18:0 —C18:1 —C18:3 —C20:1 —C20:2 —C20:3 —C20:4	—$COCH_3$ (O-6)	—H	—H	3,4,5	Spaink *et al.* (1995)
Rhizobium meliloti	—H	—C16:2 —C16:3	—$COCH_3$ (O-6) —H	—SO^3H	—H	1,2,3	Lerouge *et al.* (1990) Schultze *et al.* (1992)
Rhizobium loti	Me	—C18:0 —C18:1	—H Cb§ (O-4)‖	4-O-acetyl-fucosyl	—H	3	López-Lara *et al.* (1995)
Rhizobium tropici	Me	—C18:1	—H	—SO_3H	—H	3	Poupot, Martinez-Romero & Promé, (1993)
Rhizobium fredii	—H	—C18:1	—H	fucosyl 2-*O*-methyl-fucosyl	—H	1,2,3	Bec-Ferté *et al.* (1994)
Rhizobium sp NGR234	Me	—C18:1 —C16:0	Cb (O-3/O-4) —H	sulphated/acetylated 2-*O*-methyl-fucosyl	—H	3	Price *et al.* (1992)
Bradyrhizobium japonicum	—H	—C18:1 —C16:1 —C16:0	—$COCH_3$ (O-6) —H	2-*O*-methyl-fucosyl	—H	3	Sanjuan *et al.* (1992) Carlson *et al.* (1993)
Bradyrhizobium elkanii	—H Me	—C18:1	—$COCH_3$ (O-6), —H Cb¶	fucosyl 2-*O*-methyl-fucosyl	—H Gro**	2,3	Carlson *et al.* (1993)
Azorhizobium caulinodans strain ORS571	Me	—C18:1 —C18:0	Cb (O-6) —H	D-arabinosyl —H	—H	2,3	Mergaert *et al.* (1993)

* For review on *nod* gene activation see Dénarié, Debellé & Rosenberg (1992).
† O-n indicates the position of the substitution on the N-acylglucosamine residue.
‡ This substitution is present in Nod factors of *R.l.* bv. *viciae* strain TOM.
§ Cb indicates carbamyl group.
‖ A second carbamyl substitution is present in Nod factors of *R. loti* strain NPZ2037.
¶ The position of the carbamyl group could be O-3, O-4 or O-6.
** Gro indicates glyceryl group.

glucosamine units, a term in parentheses indicating substitutions on the non-reducing terminal sugar residue, the length of the acyl chain and degree of unsaturation, and substitutions on the reducing terminal sugar residue (e.g. NodRlv-V(Ac, C18:4,Ac)).

Genetic and biochemical studies have shown that the synthesis of the Nod factor backbone is catalysed by NodA, NodB and NodC. NodC has homology to chitin synthases and therefore it is most likely that it is the enzyme that catalyses the synthesis of the chitin oligomer (Geremia *et al.*, 1994). The chitin core is then modified by the action of NodB, which de-*N*-acetylates the terminal non-reducing sugar residue (John *et al.*, 1993). Subsequently, NodA transfers a fatty acid from an acyl carrier protein to

Table 2. *Examples of cross-inoculation groups*

Bacterial species	Host plants
Rhizobium leguminosarum	
biovar. *viciae*	*Vicia, Pisum, Lathyrus, Lens*
biovar. *trifolii*	*Trifolium*
Rhizobium meliloti	*Medicago, Melilotus, Trigonella*
Rhizobium etli	*Phaseolus*
Rhizobium loti	*Lotus*
Rhizobium tropici	Broad host range, e.g. *Phaseolus, Leuceana*
Rhizobium fredii	Broad host range, e.g. *Phaseolus, Glycine*
Rhizobium sp. NGR234	Broad host range, over 75 genera including the non-legume *Parasponia*
Bradyrhizobium japonicum	*Glycine, Macroptilium*
Azorhizobium caulinodans	*Sesbania*

Table 3. *Function of nod gene products involved in Nod factor biosynthesis*

nod gene	Function	Evidence	Reference
nodM	Glucosamine synthase	Sequence homology, complementation studies	Downie *et al.* (1990); Baev *et al.* (1991); Marie *et al.* (1992)
nodA	*N*-acetyltransferase	*in vitro* studies	Röhrig *et al.* (1994); Atkinson *et al.* (1994)
nodB	de-*N*-acetylase	Sequence homology; *in vitro* and *in vivo* studies	Kafetzopoulos *et al.* (1993); John *et al.* (1993); Spaink *et al.* (1994)
nodC	UDP-GlcNac transferase	Sequence homology; *in vitro* and *in vivo* studies	Bulawa & Wasco (1991); Atkinson & Long (1992); Debellé, Rosenberg & Dénarié (1992); Geremia *et al.* (1994); Spaink *et al.* (1994)
nodF	Acyl carrier protein	Sequence homology; required for production of poly-unsaturated fatty acyl moyeties	Shearman *et al.* (1986); Geiger, Spaink & Kennedy (1991); Ritsema *et al.* (1994)
nodE	β-ketoacyl synthase	Sequence homology; involved in production of poly-unsaturated fatty acyl moyeties	Bibb *et al* (1989); Sherman *et al.* (1989); Spaink *et al.*, (1991); Demont *et al.*, (1993); Geiger *et al.* (1994); Spaink *et al.* (1995)
nodL	*O*-acetyltransferase	Sequence homology; mutant studies; *in vitro* studies	Downie (1989); Spaink *et al.* (1991); Bloemberg *et al.* (1994)
nodP	ATP-sulphurylase	Sequence homology; complementation studies; *in vitro* studies	Schwedock & Long, (1990); Schwedock & Long (1994)
nodQ	ATP-sulphurylase subunit, APS kinase	Sequence homology; Complementation studies; *in vitro* studies	Schwedock & Long (1990); Schwedock & Long (1994)
nodH	Sulphotransferase	Sequence homology; mutant studies; *in vitro* studies	Roche *et al.* (1991*a*,*b*) Schultze *et al.* (1995)
nodX	*O*-acetyltransferase	Sequence homology; mutant studies	Clark, Beltrame & Manning (1991); Firmin *et al.* (1993)

this position (Röhrig *et al.*, 1994). The terminal sugar residues are modified by the action of other Nod proteins that synthesize or add various substituents. Table 3 shows a list of presently known *nod* genes and their functions. Two examples will illustrate that these substitutions, together with the structure of the fatty acid chain, determine host specificity.

In *R.l.* bv. *viciae*, NodE and NodX (strain TOM) mediate host specificity. NodE is homologous to a family of β-ketoacyl synthases (Bibb *et al.*, 1989). In case of *R.l.* bvs *trifolii* and *viciae*, NodE is the main determinant of host specificity (Spaink *et al.*, 1989). In *R.l.* bv. *viciae*, NodE is involved in the biosynthesis of a highly unsaturated C18:4 fatty acyl moiety (Spaink *et al.*, 1991; Geiger *et al.*, 1994), whereas in *R.l.* bv. *trifolii*, NodE activity results in the production of Nod factors containing a C20:4, C20:3, C20:2 or C18:3 fatty acid chain (Spaink *et al.*, 1995).

In *R.l.* bv. *viciae* strain TOM, NodX determines the extended host range of this strain to pea lines containing the *sym2* allele originating from the wild pea variety Afghanistan (Lie, 1984; Davis, Evans & Johnston, 1988; Kozik *et al.*, 1995). Transfer of *nodX* into *R.l.* bv. *viciae* strains, in which it is normally absent, results in the production of Nod factors with an additional *O*-acetyl group at the

reducing sugar moiety of the pentameric NodRlv factor, suggesting that NodX is responsible for this *O*-acetylation (Firmin *et al.*, 1993).

In *R. meliloti*, NodH and NodPQ are the major host range determinants (Roche *et al.*, 1991*a*). NodPQ proteins have been shown to be enzymes that generate active forms of sulphate (Schwedock & Long, 1990; Roche *et al.*, 1991*a*). NodH is homologous to sulphotransferases and was shown to have *in vitro* sulphotransferase activity (Schultze *et al.*, 1995). Therefore, these enzymes are probably directly involved in catalysing the sulphation of NodRm factors (Roche *et al.*, 1991*b*; Fisher & Long, 1992; Schultze *et al.*, 1995). *R. meliloti nodH*$^{-}$ mutants have lost the ability to elicit root hair curling, infection thread formation and nodule formation on their host alfalfa, but have acquired the ability to nodulate the non-host vetch (Debellé *et al.*, 1986; Horvath *et al.*, 1986; Faucher *et al.*, 1989). Moreover, mutants defective in *nodPQ* exhibit an extended host range; they still infect and nodulate alfalfa, but they are also able to nodulate vetch (Cervantes *et al.*, 1989; Faucher *et al.*, 1989). This mutant produces a mixture of sulphated and non-sulphated Nod factor owing to a set of *nodPQ*-like genes located on an other megaplasmid present in the bacterium (Schwedock & Long, 1992).

NOD FACTOR-INDUCED RESPONSES

The early plant responses induced by rhizobia can be divided into three classes based on the root tissue where the response is induced i.e. epidermis, cortex and pericycle. Responses in each tissue shall be described. We shall discuss whether Nod factors are essential or even sufficient to induce these responses and whether the induced responses can be used as assays to determine Nod factor structure-function relationships. The Nod factor-induced plant responses used as assays are listed in Table 4.

Epidermis

When rhizobia colonize legume roots they attach to tips of root hairs and the root hairs deform and curl. The bacteria become entrapped in the curls and a local hydrolysis of the plant cell wall takes place in the curled region (Callaham & Torrey, 1981; Van Spronsen *et al.*, 1994). Subsequently, the plasma membrane invaginates and new plant cell wall material is deposited (Brewin, 1991; Kijne, 1992) resulting in the formation of a tubular structure, the infection thread, by which the bacteria enter the plant.

Membrane potential depolarization. The earliest responses observed after application of Nod factors to legume roots are depolarization of the root-hair plasma membrane (Ehrhardt, Atkinson & Long, 1992; Felle *et al.*, 1995; Kurkdjian, 1995), changes in the calcium and proton flux, rearrangements of the actin filaments (Allen *et al.*, 1994) and increased cytoplasmic streaming (Heidstra *et al.*, 1994). These changes occur within 5–30 min after Nod factor application and might be part of a series of events leading to root-hair deformation, since Nod factor-induced deformation first becomes apparent after 1 h (Heidstra *et al.*, 1994).

Application of NodRm-IV(C16:2,S) at concentrations as low as 10^{-10} M to alfalfa roots causes a depolarization of the membrane potential in alfalfa root hairs (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995;

Table 4. *Nod factor-induced plant responses used as assays*

Assay	Test plant	Reference
Epidermis		
Membrane potential depolarization	Alfalfa	Ehrhard *et al.* (1992); Felle *et al.* (1995); Kurkdjian (1995)
Root hair deformation	Vetch, alfalfa, soybean *Vigna, Sesbania, Macroptilium*	Lerouge *et al.* (1990); Spaink *et al.* (1991); Price *et al.* (1992); Sanjuan *et al.* (1992); Schultze *et al.* (1992); Mergeart *et al.* (1993); Relic *et al.* (1993); Heidstra *et al.* (1994)
Gene expression (*ENOD5, ENOD12, Mtrip1*)	Vetch, pea alfalfa	Horvath *et al.* (1993); Journet *et al.* (1994); Cook *et al.* (1994); Vijn *et al.* (1995*a*)
Infection	Pea, alfalfa	Ardourel *et al.* (1994); Geurts, Hadri & Heidstra (unpublished)
Cortex		
Pre-infection thread formation	Vetch	Van Brussel *et al.* (1992)
Primordium formation	Vetch, pea, alfalfa, soybean, *Sesbania, Macroptilium*	Spaink *et al.* (1991); Truchet *et al.* (1991); Mergaert *et al.* (1993); Relic *et al.* (1993); Stokkermans & Peters (1994); Vijn *et al.* (1995*b*); Lopez-Lara *et al.* (1995)
Gene expression (*ENOD12, ENOD40*)	Vetch	Vijn *et al.* (1995*a*, *b*)
Pericycle		
Gene expression (*ENOD40*)	Vetch, alfalfa	Vijn *et al.* (1995*b*); Yang (pers. comm.)

Kurkdjian, 1995). It was shown that the induction of depolarization depends on the differentiation stage of the epidermal cells, but occurs in trichoblasts as well as atrichoblasts. Root hairs just bulging out from the epidermal cells were sensitive to Nod factors, whereas neither epidermal cells that had not yet formed root hairs nor the old root hairs responded to Nod factor (Kurkdjian, 1995). However, atrichoblasts in the zone containing root hairs reacted to Nod factors (Felle *et al.*, 1995). Tomato root hairs treated with Nod factors failed to exhibit significant changes in membrane potential, therefore it is unlikely that Nod factors are recognized by root hairs of all plant species (Ehrhardt *et al.*, 1992). Membrane potential depolarization induced by Nod factors displays a characteristic often seen in animal cells responding to external signals; root hairs become desensitized to polarization after a first dose of Nod factors (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995; Kurkdjian, 1995).

The membrane potential depolarization studies showed that the electrical response was Nod factor concentration-dependent (Felle *et al.*, 1995; Kurkdjian, 1995). Therefore depolarization can be used as a semi-quantitative assay to determine the structural requirements of Nod factors for this response.

Root hair deformation. Root hair deformation is induced by purified Nod factors at concentrations as low as 10^{-12} M (Lerouge *et al.*, 1990; Spaink *et al.*, 1991; Price *et al.*, 1992; Sanjuan *et al.*, 1992; Schultze *et al.*, 1992; Mergeart *et al.*, 1993; Heidstra *et al.*, 1994). In general, however, to obtain root-hair curling rhizobia are required (Relic *et al.*, 1993).

For vetch (*Vicia sativa*), a fast and simple semi-quantitative root hair deformation assay has been developed that has allowed the characterization of the root hair deformation process in more detail. In this system, root hair deformation is induced in a small susceptible zone (± 2 mm) of the root encompassing young root hairs that have almost reached their mature size (Heidstra *et al.*, 1994). Deformation starts with a swelling of the root hair tips, which is already apparent within 1 h after application of Nod factors. Subsequently, new tip growth is initiated at the swollen tips, resulting within 3 h in clearly deformed root hairs. Incubation for 10 min in the presence of 10^{-11} M NodRlv factor is sufficient to induce the deformation process (Heidstra *et al.*, 1994). It was shown that root hair deformation can be inhibited by actinomycin D and cyclohexamide, suggesting that both DNA-dependent RNA and protein synthesis are prerequisites for root hair deformation (Vijn *et al.*, 1995*a*). The semi-quantitative nature of the root hair deformation assay for vetch makes it suitable for the study of the relation between Nod factor structure and ability to induce deformation.

Infection. Purified Nod factors alone are not sufficient to induce infection thread formation. However, they are essential for the induction of the formation of these structures since rhizobia carrying mutations in certain *nod* genes involved in Nod factor synthesis lose the ability to infect their host plant, while they remain able to deform root hairs (see below). In addition, other bacterial signals or the physical presence of bacteria seem to be required for the formation of infection threads. Mutations in rhizobial genes which alter exopolysaccharide synthesis can render bacteria unable to induce infection threads (Dylan *et al.*, 1986; Niehaus, Kapp & Pühler, 1993). In addition, pre-treatment of clover roots with lipopolysaccharides of *R.l.* bv. *trifolii* improves the efficiency of infection thread formation, whereas pre-treatment with lipopolysaccharides from a heterologous strain leads to an increase in aborted infections (Dazzo *et al.*, 1991). Thus, interaction with bacterial surface compounds plays an important role in the infection process, and these compounds might be the additional signal molecules required to elicit infection thread formation.

Analysing infection thread formation by rhizobia carrying mutations in *nod* genes can help to determine what structural requirements are essential to enable Nod factors to induce this process. However, since these studies cannot be done with purified Nod factors, infection thread formation is a qualitative assay.

Gene expression. In the epidermis, the plant genes *ENOD5, ENOD12* and *Mtrip1* are induced within a few hours after application of Nod factors at concentrations between 10^{-9} and 10^{-12} M (Horvath *et al.*, 1993; Journet *et al.*, 1994; Cook *et al.*, 1995; Vijn *et al.*, 1995*a*). *Mtrip1* is not expressed during other steps of nodulation, whereas *ENOD5* and *ENOD12* are also expressed during infection and nodule development. *ENOD5* and *ENOD12* encode proline-rich proteins which possibly represent components of the infection thread wall, since cells containing an infection thread express these genes (Scheres *et al.*, 1990*a*,*b*). *Mtrip1* encodes a peroxidase which might facilitate infection, perhaps by contributing to repair of the cell wall at the site of infection or to the formation of novel cell wall structures, e.g. those associated with susceptible root hairs (Cook *et al.*, 1995).

In the epidermis of alfalfa, the induction of *ENOD12* and *Mtrip1* expression occurs within 3 h in a relatively broad zone of the root, starting just above the root tip, where the root hairs have not yet emerged, and extending to the region containing mature root hairs (Pichon *et al.*, 1992; Cook *et al.*, 1995). Cytological studies have shown that Nod factors elicit the expression of these genes in all epidermal cells, suggesting that cells lacking a root hair (atrichoblasts) also respond to Nod factors. This

result is consistent with the depolarization response that is also observed in both trichoblasts and atrichoblasts (Felle *et al.*, 1995). However, depolarization is observed during a shorter developmental window, since in epidermal cells that had not yet developed a root hair no depolarization was induced (Felle *et al.*, 1995; Kurkdjian, 1995).

Gene expression is restricted to the epidermis, since the *ENOD12* and *Mtrip* genes are not even expressed in the hypodermal cell layer (Journet *et al.*, 1994; Cook *et al.*, 1995). In *Egeria* and *Arabidopsis* the epidermis was shown to be symplastically isolated from the hypodermal layer (Erwee & Goodwin, 1985; Duckett *et al.*, 1994). If this is also the case in legumes it might explain why the response is restricted to the epidermis. In addition, symplastic communication between root epidermal cells in *Arabidopsis* becomes restricted as they differentiate into trichoblasts and atrichoblasts (Duckett *et al.*, 1994). Since it is not known at which stage of development in legumes epidermal cells become symplastically isolated it remains unclear whether both trichoblasts and atrichoblasts recognize Nod factors.

In vetch, *ENOD12* and *ENOD5* are first detectable 8 h after addition of Nod factors, which is ample time after root hair deformation (Vijn *et al.*, 1995*a*), showing that these genes cannot be involved in the deformation process. Furthermore, *de novo* protein synthesis is required for their activation. Thus, signal transduction pathways that lead to the activation of these genes are probably very complex. Therefore it will be useful to obtain additional marker genes that are induced soon, preferably within 1 h, after Nod factor application in order to study which signal transduction pathways are activated.

Gene expression induced by Nod factors has been studied by Northern analysis and RT-PCR as well as in transgenic plants containing nodulin promotor-β-glucuronidase (GUS) constructs. So far, studies with transgenic plants have only been done in alfalfa, which is a tetraploid (Journet *et al.*, 1994). For this reason it has been hard to obtain quantitative data. The use of a homogenous population of transformed diploid plants can improve this assay. Since the methods mentioned are quantitative, Nod factor-induced gene expression can be used as a quantitative assay to determine the Nod factor structure required to induce gene expression.

Root cortex

Concomitant with infection thread formation, cortical cells are mitotically re-activated by *Rhizobium*, forming the nodule primordium. Infection threads grow towards this primordium and, once there, bacteria are released into the cytoplasm of the host cells. Which root cortical cells will divide is determined by the plant. In temperate legumes, e.g. pea, vetch and alfalfa, it is the inner cortical cells, and especially those located opposite protoxylem poles (Kijne, 1992). Hence, the infection threads must traverse the outer cortex to reach these cells. Prior to infection thread penetration, cytological rearrangements occur in the outer cortical cells; the nuclei move to the centre of these cells, and the microtubules and the cytoplasm rearrange to form a radially oriented conical structure, the cytoplasmic bridge, which resembles a preprophase band (Kijne, 1992). The infection threads traverse the cortical cells through the radially aligned cytoplasmic bridges, which are therefore called pre-infection threads (Van Brussel *et al.*, 1992). Infection threads grow to and enter the primordium, where they ramify, and infect cells at the base of the primordium. At the same time, cells at the distal part of the primordium continued to divide and form a zone of small cells rich in cytoplasm, constituting the apical nodule meristem. The nodule meristem differentiates during the complete nodule life time into infected and uninfected cells as well as into cells of the peripheral tissues. In consequence, these nodules have an indeterminate development and represent the indeterminate nodule type.

In tropical legumes, e.g. soybean, the outer cortical cells form the nodule primordia. Inner cortical cells between the primordium and the nearby protoxylem pole are also activated to divide and to form the connecting vascular bundle. The infection threads directly invade the nodule primordium after they have penetrated the root hairs (Newcomb, Sippel & Peterson, 1979; Calvert *et al.*, 1984) Although transcellular cytoplasmic bridges are observed in the outer cortex upon application of Nod factors, these structures are not described as pre-infection threads because all the activated cortical cells go into cell division (Stokkermans & Peters, 1994). Cells at the periphery of the primordium remain mitotically active and form a spherical meristem (Newcomb *et al.*, 1979; Calvert *et al.*, 1984). Such a meristem ceases to divide about 10 d after inoculation (Newcomb *et al.*, 1979). Therefore, these nodules have a determinate growth pattern and are called determinate nodules.

In temperate legumes, the mechanism determining which cortical cells are susceptible to Nod factors and will start to divide, is unknown. It has been postulated for decades that this susceptibility is conferred by an arrest in the G2 phase (Wipf & Cooper, 1938; Verma, 1992), but the use of cell phase specific genes as probes in *in situ* hybridization experiments has shown that this is not the case (Yang *et al.*, 1994). Instead, susceptible cortical cells are, like other cortical cells, arrested in G0/G1. Since the susceptibility of cortical cells is correlated with a position opposite protoxylem poles, it is possible that the stele somehow provides positional information to

the inner cortical cells determining their susceptibility. Over 20 y ago, Libbenga *et al.* (1973) found that an alcohol extract of the stele could induce cell divisions in explants of the pea root cortex in the presence of auxin and cytokinin. The so-called stele factor, responsible for this activity, is thought to be released from the protoxylem poles. This compound might then confer susceptibility to the cortical cells located opposite the protoxylem poles (Smit, van Brussel & Kijne, 1993).

During the mitotic re-activation of root cortical cells by rhizobia, genes that control the progression through the cell cycle, e.g. *cdc2* and *cyclin* genes, are induced in temperature legumes (Yang *et al.*, 1994). The pre-infection thread-forming outer cortical cells never divide, but the induced morphological changes are reminiscent of those in cells that are entering the cell cycle. By studying the *in situ* expression of the histone *H4* gene, which is specifically expressed only during the S phase, it was shown that pre-infection thread-forming cells indeed enter the cell cycle (Yang *et al.*, 1994). However, a mitotic cyclin gene that is expressed specifically during the G2 to M phase transition is not induced. Hence, the cells forming pre-infection threads enter the cell cycle and probably become arrested in the G2 phase. This shows that part of the infection process is derived from the process of cell cycling.

In addition to the cell-cycle specific genes, several nodulin genes are expressed in the primordia. *ENOD12, Gm93, ENOD40* and *MtPRP4* are expressed in all cells of the primordia (Scheres *et al.*, 1990*a*; Kouchi & Hata, 1993; Yang *et al.*, 1993; Asad *et al.*, 1994; Matvienko *et al.*, 1994; Wilson *et al.*, 1994), whereas *ENOD5* is expressed only in primordial cells containing rhizobia (Scheres *et al.*, 1990*b*).

Primordium formation and gene expression. Nod factors are able mitotically to re-activate cortical cells, as has been observed in studies of several temperate and tropical legumes (Spaink *et al.*, 1991; Truchet *et al.*, 1991; Relic *et al.*, 1993; López-Lara *et al.*, 1995). Pre-infection thread formation induced by Nod factors has so far only been studied in vetch (Van Brussel *et al.*, 1992). The induction of primordia and pre-infection threads demands concentrations of around 10^{-7} M Nod factors, much higher than the concentrations of 10^{-9}–10^{-12} M required to induce responses in the epidermis.

In vetch, the Nod factor-induced primordia never develop into genuine nodules. The early nodulin gene *ENOD2*, which is specifically expressed in the nodule parenchyma (Van de Wiel *et al.*, 1990), is not induced in these primordia, showing that the development of the primordia is blocked before differentiation into nodule tissues starts (Vijn *et al.*, 1995 *a*). This suggests that an additional signal is needed for differentiation of the vetch primordium into a nodule, which might be generated when infection by the bacteria takes place. On the other hand, in some legumes, purified Nod factors can induce complete nodules (Truchet *et al.*, 1991; Mergaert *et al.*, 1993; Stokkermans & Peters, 1994).

The early nodulin genes *ENOD12* and *ENOD40* are induced in the primordia induced by Nod factors in vetch, whereas *ENOD5* is not (Vijn *et al.*, 1995 *a*). In the epidermis, both *ENOD12* and *ENOD5* are activated. These results indicate that *ENOD5* can only be induced by Nod factors in cells which are in direct contact with the Nod factor, unlike *ENOD12* and *ENOD40* which are induced in primordial cells without direct contact with Nod factors.

Primordium formation and pre-infection thread formation can be used as assays to determine the structural requirements of the Nod factor for these processes. There are two ways of inducing nodule primordia with Nod factors: bathing the roots in Nod factors, or spot inoculation. Spot inoculation is considered a qualitative assay in which a high concentration of Nod factors (*c.* 10^{-4} M) is applied locally on the root (López-Lara *et al.*, 1995). In the bathing assay the number of induced primordia was found to be dependent on the concentration of Nod factors (Vijn *et al.*, 1995*a*); therefore this assay is considered to be semi-quantitative.

Pericycle

In the root pericycle, cytological changes do not take place during the early stages of the interaction with rhizobia. However, the early nodulin gene *ENOD40* is induced in the region of the pericycle opposite the dividing cortical cells both in legumes forming indeterminate nodules and in legumes forming determinate nodules, where the pericycle cells are not adjacent to the dividing cortical cells (Kouchi & Hata, 1993; Yang *et al.*, 1993; Asad *et al.*, 1994). Nod factors are sufficient to induce *ENOD40* expression in the root pericycle as well as in the dividing cortical cells. Interestingly, *ENOD40* gene expression in the pericycle can already be detected after a few hours and therefore precedes the first cortical cell division (Yang, pers. comm.).

Both cytokinin and compounds that block polar auxin transport (e.g. NPA, TIBA) are also able to induce the formation of nodule-like structures in which early nodulin genes are expressed (Cooper & Long, 1994; Hirsch *et al.*, 1989). Therefore it has been postulated that Nod factors cause a change in phytohormone balance that subsequently leads to cell division. Since *ENOD40* expression precedes cortical cell division it is possible that this nodulin is involved in changing the phytohormone balance (Yang, pers. comm.). This was examined in a tobacco protoplast system in which the correlation between efficiency of cell division and auxin concentration is monitored (Walden *et al.*, 1994). Tobacco proto-

plasts expressing a legume *ENOD40* gene under the control of the 35S promotor divide efficiently at high auxin concentration, whereas in the control protoplasts this level of auxin suppressed the ability of these cells to divide (Bisseling & Walden, pers. comm.). Thus, introduction of *ENOD40* in the non-legume tobacco changes the response to phytohormones.

ENDO40 cDNA clones have been isolated from several legumes, and sequence comparison showed that the legume *ENOD40* RNAs do not contain a long conserved ORF (Kouchi & Hata, 1993; Yang *et al.*, 1993; Asad *et al.*, 1994; Crespi *et al.*, 1994; Matvienko *et al.*, 1994; Vijn *et al.*, 1995*b*). Therefore, it has been postulated that the *ENOD40* RNA is the active compound (Crespi *et al.*, 1994; Matvienko *et al.*, 1994). However, all *ENOD40* sequences contain two small conserved regions one of which encodes a highly conserved small polypeptide of 12 or 13 amino acids (Vijn *et al.*, 1995*b*). Application of this peptide to tobacco protoplasts enables these cells to divide efficiently at high auxin concentrations (Bisseling, unpublished). Therefore, it cannot be ruled out that the active gene product of *ENOD40* is a peptide.

Expression of *ENOD40* in the pericycle can be used as an assay to determine the structural requirements of Nod factors for expression of this gene. *ENOD40* expression in the pericycle is induced with spot inoculation using a high concentration of Nod factor (*c.* 10^{-4} M; Yang, pers. comm.) and therefore is considered a qualitative assay.

NOD FACTOR STRUCTURE-FUNCTION RELATIONSHIP AND PERCEPTION MECHANISMS

Nod factors have the ability to induce various responses in the epidermis, cortex and pericycle. Qualitative and (semi-)quantitative assays have been developed based on these responses. Using these assays it is possible to address the following questions:

(1) Are Nod factors themselves or Nod factor-derived molecules active in inducing these responses?

(2) Are one or more receptors involved in Nod factor perception?

(3) Are second messengers generated in the epidermis or is the Nod factor itself perceived in the cortex and pericycle?

To answer these questions we will concentrate on the responses induced in vetch and alfalfa, since in these plants several Nod factor-induced responses have been studied.

Are Nod factors processed before perception?

To understand the mode of action of Nod factors it is important to determine whether the Nod factors as they are secreted by the bacteria are active, or whether they first have to be processed by the plant to become active. When Nod factors are added to roots they are rapidly degraded, and Nod factor derived molecules containing only two or three sugar residues are found in the growth medium and on the root. These molecules are probably generated by chitinases secreted by the plant. The fact that these degraded Nod factors are at least 1000-fold less active in the root hair deformation assay than Nod factors with four or five sugars (Heidstra *et al.*, 1994; Staehelin *et al.*, 1994*a*) indicates that these molecules are not involved in inducing root hair deformation. Therefore we conclude that the Nod factors as secreted by the bacteria are active in inducing the various responses.

Studies of degradation of Nod factors revealed an other interesting role for chitinases. The degradation rate of NodRlv-V(C18:4) and NodRlv-IV(C18:4) showed that the tetramer is more stable than the pentamer (Heidstra *et al.*, 1994). In the case of the Nod factors from *R. meliloti*, it was found that endochitinases from the host alfalfa rapidly degraded unsulphated and pentameric NodRm factors, but not the most active NodRm factor, NodRm-IV(C16:2,S) (Staehelin *et al.*, 1994*b*). These results suggest that structural modifications or length of the chitin backbone of the various Nod factors are involved in protecting them against rapid degradation by host chitinases.

Nod factor perception in the epidermis

The very low concentrations (10^{-12} M) of Nod factors inducing various responses in the epidermis indicate that a receptor might be involved in the perception of Nod factors. The various responses induced in the epidermis allow the determination of the structural requirements of the Nod factors for the different responses. Such studies can reveal whether one or more receptors are active in the epidermal cells. For induction of root hair deformation, *ENOD12* gene expression, root-hair membrane potential depolarization and infection thread formation, the Nod factor structure-function relationship has been determined.

For root hair deformation and activation of *ENOD12* expression the structure and unsaturation level of the fatty acyl group is not very important, although the presence of a fatty acyl group is required (Heidstra *et al.*, 1994; Journet *et al.*, 1994). The presence of the *O*-acetyl group at the non-reducing end of NodRlv and NodRm factors has only a marginal effect on their ability to induce deformation and *ENOD12* expression (Roche *et al.*, 1991*a*; Spaink *et al.*, 1991; Journet *et al.*, 1994).

On the other hand, for depolarization and infection thread formation, the structure of the fatty acyl group and the presence of the *O*-acetyl group at the

non-reducing end are important. The activity in depolarization of NodRm-IV(C18: 1,S), carrying a saturated acyl chain, was severely reduced compared with that of NodRm-IV (C16: 2,S), carrying the unsaturated acyl group specific for *R. meliloti* (Felle *et al.*, 1995). Although *O*-acetylation of the non-reducing end sugar had no effect on the maximum of depolarization, the response to the *O*-acetylated factor was initially faster. Furthermore, markedly fewer infection threads were initiated by *R. meliloti* mutated in *nodE* or *nodL* which produces Nod factors that do not contain the appropriate unsaturated fatty acid or the *O*-acetyl group, respectively (Ardourel *et al.*, 1994). A strain mutated both in *nodE* and *nodL*, secreting Nod factors lacking the *O*-acetyl group and containing an inappropriate fatty acid had completely lost the ability to induce infection threads (Adourel *et al.*, 1994).

Substitutions at the reducing end of the Nod factors can have a dramatic effect on the activity to induce the epidermal responses. For example, desulphation of the NodRm factors reduces at least 1000-fold their activity to induce root hair deformation, *ENOD12* expression and membrane potential depolarization on alfalfa (Journet *et al.*, 1994; Felle *et al.*, 1995). In addition, *R. meliloti* strains mutated in *nodH*, producing only non-sulphated NodRm factors, are unable to induce infection thread formation in alfalfa (Roche *et al.*, 1991 *a*).

These results indicate that all parts of the Nod factor are recognized in the epidermis but that the responses can be divided in two classes, deformation and gene expression versus depolarization and infection thread formation, each demanding different structural requirements. This led Ardourel *et al.* (1994) to propose the hypothesis that at least two different Nod factor receptors are present in the epidermis: a 'signalling' receptor and an 'uptake' receptor. The signalling receptor recognizes Nod factors even if the non-reducing end is modified and, upon binding, activates the induction of, for example, root hair deformation. The uptake receptor is activated only by the fully decorated Nod factor and, upon binding, infection thread formation is initiated. Alternatively a model involving a single receptor with different binding affinities for the different Nod factors can explain the various responses. In the latter model, Nod factors containing a modified non-reducing end may bind with low affinity, resulting in for example, root hair deformation, whereas fully decorated Nod factors bind with high affinity, resulting in induction of all responses including infection thread formation. Such a correlation between binding affinity of ligands and their ability to induce a response has been described in phytoalexin accumulation in soybean cotelydons (Schmidt & Ebel, 1987; Cheon & Hahn, 1991). Oligoglucosides with high binding affinity had a high elicitor activity, whereas those with a low binding affinity had a low elicitor activity. However, a single response was scored in this assay, whereas different responses are induced by Nod factors.

Thus, a one receptor model and a two receptor model can explain how Nod factors induce responses in the epidermis. Analysis of legume mutants disturbed in the early stages of the interaction with rhizobia might help to discriminate between these two hypotheses.

Nod factor perception in the cortex and pericycle

Exogenously applied Nod factors induce responses in the epidermis, i.e. the tissue in direct contact with Nod factors, but also in the cortex and the pericycle. The first question that can be asked is whether Nod factors are translocated to, and also recognized in, the cortex and the pericycle, or whether secondary messengers generated in the epidermis are responsible for the induction of responses in these tissues.

Since temperate legumes forming indeterminate nodules require nod factors substituted with a highly unsaturated fatty acyl group for the induction of cell division in the inner cortex, whereas Nod factors of rhizobia nodulating tropical legumes forming determinate nodules generally contain a C18: 1 acyl group, it is possible that the highly unsaturated fatty acyl group might be required for transporting the Nod factor to the inner layers. However, in the indeterminate primordia, *ENOD12* expression is induced and *ENOD5* expression is not, whereas in the epidermis both genes are activated. In *Rhizobium*-induced primordia, *ENOD12* is expressed in all cells but *ENOD5* is only induced in cells infected by the bacteria (Vijn *et al.*, 1995*b*). During the infection process and in the pre-fixation zone of the nodule the bacterial *nod* genes are still expressed (Sharma & Signer, 1990; Schlaman *et al.*, 1991), suggesting that the Nod factors are still produced. These results indicate that *ENOD5* is only induced in cells which are in direct contact with the Nod factor, unlike *ENOD12* which is induced in primordial cells without direct contact with Nod factors. The lack of *ENOD5* expression in Nod factor-induced primordia suggests that exogeneously applied Nod factors do not reach the inner cortical cells. This implies that mitotic reactivation of cortical cells, as well as *ENOD12* expression in primordia is induced by a diffusible secondary signal, probably generated in the epidermis by Nod factors. In addition, *ENOD40* expression in the pericycle can be induced by chitin fragments (Yang, pers. comm.) also indicating that the unsaturated fatty acyl group is not required for transport to the inner layers.

Experiments using labelled Nod factors tracking these molecules after application to legume roots must be performed to show whether Nod factors are perceived only in the epidermis or whether they are also either completely or in part transported to the

inner layers. Alternatively, labelled Nod factors or Nod factor components may be introduced into these tissues in order to determine their activity to induce responses normally induced by Nod factors applied to the outside of the root.

GENETIC APPROACH TOWARDS NOD FACTOR PERCEPTION

As described above, based on the nodulation phenotype of *R. meliloti* mutants, two models concerning Nod factor perception in the epidermis can be postulated.
(1) One receptor binding the different Nod factors with different affinities which determines the type of response that is induced.
(2) Two receptors are present, a signalling receptor induces root hair deformation and gene expression and an uptake receptor induces infection thread formation.

To distinguish between these hypotheses we decided to analyse legume mutants that are disturbed in the early stages of the interaction with *Rhizobium*. Such mutants have been isolated for several legumes, but this approach was especially successful for pea for which about 30 genes involved in nodulation, the so-called *sym* genes, have been identified (LaRue & Weeden, 1994). We focused our studies on the *sym2* gene of Afghanistan pea (Geurts, Hadri & Heidstra, unpublished) since the presence of this gene puts more stringent structural demands on the Nod factors secreted by *R.l.* bv. *viciae*.

sym2

The *sym2* allele was first identified in Afghanistan pea and later in several other wild pea varieties including Iran pea, in which this allele was first named *sym1* (Lie, 1971*a*,*b*; 1978; Kozik *et al.*, 1995). Although most of the literature refers to the *sym2* allele as recessive, *sym2* is in fact dominant (see below). The *sym2* allele is responsible for the inability of European *R.l.* bv. *viciae* strains (e.g. strain PRE and 248) to nodulate Afghanistan pea. However, certain strains from the Middle East, like strain TOM, can nodulate *sym2*-containing peas (Lie, 1978; 1984). The extended host range of strain TOM is due to *nodX*, a gene that does not occur in European *R.l.* bv. *viciae* strains (Davis *et al.*, 1988). NodX is an acetyl transferase adding an *O*-acetyl substitution to the reducing terminal sugar residue of the pentameric NodRlv factors (Firmin *et al.*, 1993). Introduction of *nodX* into, e.g., *R.l.* bv. *viciae* strain 248 results in the production of two additional Nod factors enabling this strain to nodulate *sym2*-containing peas (Table 5; Kozik *et al.*, 1995). This suggests that *sym2* is involved in recognition of these NodX modified Nod factors (Heidstra *et al.*, 1993).

Alternatively, *sym2* might encode a chitinase which degrades NodRlv factors lacking the NodX modification, whereas NodRlv factors carrying this modification are more resistant to degradation. This hypothesis seems very unlikely since the rate of degradation of NodRlv-V(Ac,C18:4) on peas with or without *sym2* is equal (Geurts, Hadri & Heidstra, unpublished). Therefore, it is more probable that *sym2* encodes a receptor of NodX-modified NodRlv factors. Hence, the nodulation phenotype of *sym2*-containing peas might provide clues on the number of Nod factor receptors active in the epidermis.

The interaction between *R.l.* bv. *viciae* strains with or without *nodX*, and peas containing *sym2* results in a similar induction of root hair deformation, cortical cell division and the activation of *ENOD12*. Only the activation of *ENOD5* is reduced compared with that in peas lacking *sym2*. However, the number of infections on *sym2* peas was severely reduced in comparison with that of infections on peas without *sym2*, as was shown by using *R.l.* bv. *viciae* strain 248 containing a constitutively expressed *lacZ* gene. The few infections that took place were aborted in the epidermis (Geurts, Hadri & Heidstra, unpublished). In this respect, there is a striking similarity between the responses induced by the *R. meliloti nodFEL* mutants on alfalfa and those induced by *R.l.* bv. *viciae* $nodX^-$ strains on *sym2* peas. So, it is possible that *sym2* encodes either a Nod factor receptor with similar characteristics to those proposed for the uptake receptor (hypothesis 2), or a Nod factor receptor with different binding affinities for NodRlv factors with or without the NodX modification (hypothesis 1). Both hypotheses imply that the Sym2 receptor induces the infection process upon binding NodX-modified Nod factors. However, analyses of the phenotype of *sym2* peas (see below) indicate that the Sym2 receptor is involved in the repression of infection thread formation.

Peas containing *sym2* show a temperature-sensitive nodulation phenotype. *R.l.* bv. *viciae* strain 248 does not nodulate *sym2*-containing peas at 18 °C (incompatible) but it does at 26 °C (compatible) (Lie, 1971*b*; Kozik *et al.*, 1995). Using this temperature-sensitive nature of *sym2* it was determined when during the interaction *sym2* is active. Growth for 2 to 3 d at 26 °C followed by continuous growth at 18 °C is sufficient to induce nodulation (Lie *et al.*, 1976). During this time infection threads have been formed and most of them have just passed the epidermis (Geurts, Hadri & Heidstra, unpublished). These results suggest that Sym2 is only active during the first 2 d of the interaction. Sym2 probably functions in the epidermis since in the incompatible interaction the infection threads continue to grow once the infection thread has passed the epidermis. After 2 d the infection threads still have to traverse the outer cortical cells and subsequently even

Table 5. *Nodulation phenotype shows that Sym2^A is a dominant allele*

R.l. bv. *viciae* strain	Plant genotype		
	Sym2^{A}Sym2^A	§Sym2^{A}Sym2^C	Sym2^{C}Sym2^C
TOM	+	+	+
*TOM-X$^-$	0	0–5	+
PRE	0	0–5	+
†PRE-X	+	+	+
248	0–5	+	+
†248-X	+	+	+
‡248-O$^-$	0	0–5	+

Nodulation was scored 3 wk after inoculation; +, 40–60 nodules.

* strain TOM-X$^-$ = TOMnodX$_{135}$::Tn3 (Davis *et al.*, 1988).

† strain PRE-X and 248-X contain pMW1071, expressing nodX from strain TOM (Kozik *et al.*, 1995).

‡ strain 248-O$^-$ = 1387.pRL1nodO$_{84}$::Tn5 (Economou *et al.*, 1994), where strain 1387 = 248 cured of its Sym plasmid (Priem & Wijffelman, 1984).

§ Sym2^A = Sym2 allele of Afghanistan pea; Sym2^C = Sym2 allele of cultivated pea (here cv. Rondo).

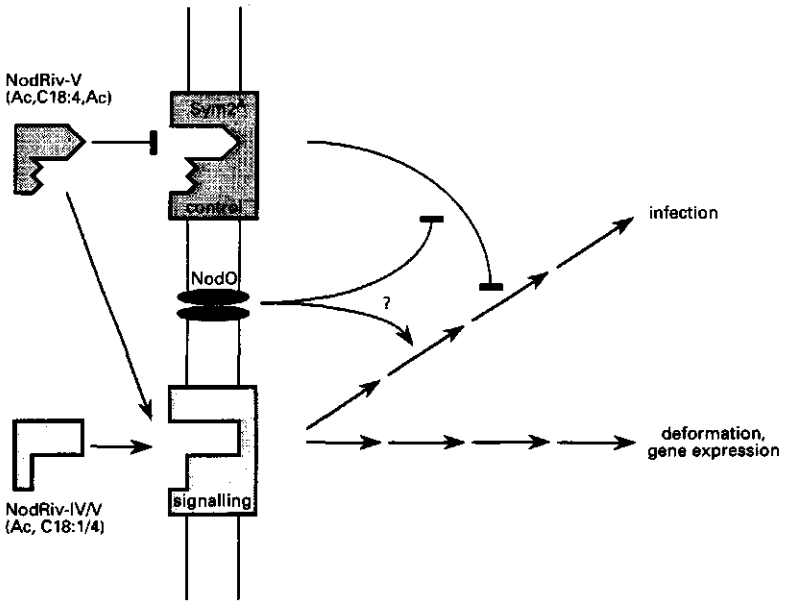

Figure 2. Model for the perception of Nod factors in the epidermis in *Sym2^A* peas. The signalling receptor, upon binding of Nod factor, induces root hair deformation and *ENOD12* gene expression as well as infection thread formation. Sym2^A is a 'control' receptor which in the unbound form results in a blocking of the infection. Upon binding, the specific NodX-modified Nod factor NodRlv-V (Ac, C18:4, Ac), Sym2^A is inactivated and subsequently the infection process induced by the signalling receptor can progress. NodO integrates into the plant plasmamembrane and either amplifies the signal transduction cascade leading to infection or partly inhibits the negative control by Sym2^A. In this way, NodO can complement for structure deficient Nod factors, allowing infection and nodulation by the Mutated *R.l.* bv. *viciae* strain secreting these deficient Nod factors. A similar model applies to *Sym2^C* peas where NodRlv-IV/V (Ac, C18:4) binds to the Sym2^C control receptor, thereby inactivating it, allowing infection to proceed.

continue to grow in the nodule primordia and the nodule. Infection thread growth in a cortical cell involves the same steps as the infection of a root hair. For every cell that is infected, a local hydrolysis of the cell wall is induced, where the plasmamembrane invaginates and a polar-growing infection thread is formed. The bacterial *nod* genes are expressed during the whole infection process (Sharma & Signer, 1990;

Schlaman *et al.*, 1991). These data suggest that after the second day Nod factor recognition still occurs and that a continuous activation of a receptor by Nod factor is required to allow the infection to progress. Therefore, it is very unlikely that Sym2 is the Nod factor receptor involved in inducing infection (uptake receptor, Ardourel *et al.*, 1994). It is plausible that another Nod factor receptor is involved and that this receptor, the signalling receptor, might induce both infection thread formation and deformation. When this hypothesis is correct and the signalling receptor is active in the epidermis, infection thread formation is induced in the epidermis of *sym2* peas by *R.l.* bv. *viciae* *nodX*$^-$ strains. Nevertheless, a strongly reduced number of infections is observed. Consequently, we postulate that Sym2 is part of a control mechanism that blocks infection, whereby Sym2 is a negative 'control' receptor binding the specific NodX-modified Nod factor. In the unbound form, Sym2 activates a blocking of the infection, whereas upon binding of NodX-modified Nod factor, Sym2 is inactivated and subsequently the infection process induced by the signalling receptor can progress. This model is depicted in Figure 2.

In peas lacking the *sym2* allele of Afghanistan (*sym2*^{A}) a Sym2-like receptor is probably present to control infection (here named Sym2^C, which is an allele of Sym2^A where C stands for cultivated peas). This Sym2^C control receptor would then be inactivated upon binding of Nod factor containing the unsaturated fatty acid (NodRlv-IV/V(Ac,C18:4)), enabling infection and subsequent nodulation by these strains.

Testing the model. In the proposed model (Fig. 2) Sym2^A acts as a negative control receptor and is not a receptor involved in the induction of infection (uptake receptor). Several lines of evidence support this hypothesis.

R.l. bv. *viciae* strain 248 (lacking *NodX*) nodulates *sym2*^{A} peas at 26 °C whereas it does not at 18 °C, the optimal growth temperature for peas. This indicates that Sym2^A function might be lost at high temperature. Alternatively, Sym2^A may bind Nod factors lacking the NodX modification at high temperature. Both explanations are consistent with the proposed role of Sym2^A as a control receptor. However, only the latter explanation is consistent with a role of Sym2^A as an uptake receptor.

Previously it was shown that the dominant character of *sym2*^{A} is strain-dependent. *sym2*^{A} is dominant for *R.l.* bv. *viciae* strain PRE (lacking *nodX*) and strain TOM*nodX*$^-$ whereas it is recessive for *R.l.* bv. *viciae* strain 248 (lacking *nodX*; Table 5; Lie, 1984; Kozik *et al.*, 1995; Geurts, Hadri & Heidstra, unpublished). However, *R.l.* bv. *viciae* strain 248*nodO*$^-$ (carrying a mutation in *nodO*) does not form nodules on heterozygous *sym2*^{A}*sym2*^{C} plants (Table 5; Geurts, Hadri & Heidstra, unpublished). Since NodO is not involved in the biosynthesis of Nod factors, but, it is more likely, interferes with Nod factor-activated signal transduction in the plant (see below), we conclude that *sym2*^{A} is a dominant gene. Therefore, this gene will be further designated *Sym2*^{A}. The dominant character of *Sym2*^{A} upon inoculation with *R.l.* bv. *viciae* strain PRE or strain TOM*nodX*$^-$ correlates well with the fact that these strains do not express a flavonoid-inducible NodO protein whereas *R.l.* bv. *viciae* strain 248 does (De Maagd *et al.*, 1989).

There are two general explanations for the dominant nature of *Sym2*^{A}:

(1) The formation of a poisoned complex leading to inactivation.

(2) Negative regulation of an induced response.

If Sym2^A functions as a multimer, the presence of both Sym2^A and Sym2^C in a heterozygous plant could disrupt the formation or activity of the multimer, resulting in a loss of function, as described for several dominant negative mutations (Herskowitz, 1987). In this case the same phenotype is expected for *R.l.* bv. *viciae* *nodO*$^-$ strains with or without *nodX*. However, whereas a *R.l.* bv. *viciae* *nodO*$^-$*nodX*$^-$ strain cannot nodulate heterozygous *Sym2*^{A}*Sym2*^{C} peas, a *R.l.* bv. *viciae* *nodO*$^-$ strain containing *nodX* can. Therefore, it seems unlikely that Sym2^A functions as a multimer.

The proposed role of Sym2^A as a control receptor is in accordance with the dominant character of Sym2^A. *R.l.* bv. *viciae* strains containing *nodX* produce in addition to the Nod factors of *R.l.* bv. *viciae* *nodX*$^-$ strains, also NodX-modified Nod factors. Therefore, Nod factors produced by *R.l.* bv. *viciae* *nodO*$^-$*nodX*$^+$ strains can bind to both Sym2^A and Sym2^C, thereby lifting the block on infection. However, the Nod factors produced by *R.l.* bv. *viciae* *nodO*$^-$*nodX*$^-$ strains bind to Sym2^C but not to the Sym2^A receptor which therefore remains active in heterozygous *Sym2*^{A}*Sym2*^{C} peas, blocking infection by these strains. Thus, the model explains why Sym2^A is dominant for *R.l.* bv. *viciae* *nodO*$^-$*nodX*$^-$ strains.

All our studies are consistent with the hypothesis that Sym2^A acts as a negative control receptor and they do not support its role as an uptake receptor. This model can also explain the phenotypes of the *R. meliloti* *nodFEL* mutants observed on alfalfa. The wild-type bacteria produces the fully decorated Nod factor that binds to the control receptor lifting the block on infection and nodulation occurs. In the case of the *nodFEL* mutuant, no infection is observed, indicating that the Nod factors produced by this mutant cannot bind to the control receptor and that the infection is blocked. The fact that the *nodFE* and *nodL* mutants are able to induce some infections suggests that binding of a partly modified Nod factor can cause some inactivation of the control receptor that in certain cases is sufficient to lift the block on

infection. Interestingly, in the interaction with the *nodFE* mutuant it was shown that, once the infection thread has passed the epidermis of the alfalfa root, infection proceeds normally (Ardourel *et al.*, 1994). This indicates that in alfalfa also the control receptor is only active in the epidermis.

NodO. Although $Sym2^A$ is dominant for $nodO^-nodX^-$ strains, *R.l.* bv. *viciae* $nodX^-$ strains expressing *nodO* (e.g. strain 248) can nodulate heterozygous $Sym2^ASym2^C$ plants. In case of homozygous $Sym2^A$ plants inoculated with *R.l.* bv. *viciae* strain 248 ($nodO^+nodX^-$) usually only up to five nodules are formed. However, after inoculation with a *R.l.* bv. *viciae* strain PRE ($nodO^-nodX^-$) no nodules are observed. So how does *nodO* compensate for the absence of NodX-modified Nod factors?

In experiments using a *R.l.* bv. *viciae* $nodE^-$ mutant it was shown that the presence of *nodO* enables this strain to nodulate pea and vetch, although producing only half the amount of nodules formed after inoculation with the wild-type during the same time period (Economou *et al.*, 1994). In the absence of *nodO* (*R.l.* bv. *viciae* $nodE^-nodO^-$ double mutuant) this strain is unable to nodulate pea and vetch, whereas its ability to induce root hair deformation is unaffected (Downie & Surin, 1990). The $nodE^-$ mutant produced Nod factors that only contain a C18:1 fatty acyl chain (Spaink *et al.*, 1991), indicating that here *nodO* compensates for the absence of NodE-modified Nod factors.

NodO is a secreted protein that is not involved in Nod factor biosynthesis (Sutton, Lea & Downie, 1994). When added to lipid bilayers, it forms channels that allow the movement of monovalent cations. Therefore it has been suggested that NodO integrates into the plant plasmamembrane and amplifies a Nod factor-induced signal transduction pathway (Economou *et al.*, 1994; Sutton *et al.*, 1994). We postulate that NodO does not compensate for a Nod factor structural deficiency by affecting Nod factor structure but either by amplifying the signal transduction cascade leading to infection or by partly inhibiting the negative control of the particular Sym2 receptor (Fig. 2).

Thus, *nodO* compensates the Nod factor structure deficiency of a *R.l.* bv. *viciae* $nodE^-$ as well as a $nodX^-$ mutant, enabling nodulation on $Sym2^C$ and $Sym2^A$ peas respectively, despite the fact that the structural deficiency in both mutants is different. Although, NodO more efficiently complements a *R.l.* bv. *viciae* $nodE^-$ than a $nodX^-$ mutation as indicated by the amount of nodules formed by either mutant; this might be a gradual effect. This indicates that $NodE^-$ and $NodX^-$ modified Nod factors bind to a similar receptor. In other words, the $Sym2^C$ control receptor binds NodRlv-IV/V(Ac,C18:4) containing the NodE-determined unsaturated fatty acid, whereas the $Sym2^A$ control receptor binds NodRlv-V(Ac,C18:4,Ac) containing the additional NodX- determined modification. The compensation by NodO for Nod factor structure deficiency also supports the hypothesis that the *Sym2* alleles encode a control receptor.

Sym2-like receptors in clover and soybean

If our hypothesis that Sym2 is a control receptor is correct it seems likely that other legumes will contain a similar receptor. Therefore, it is probable that a similar variation in Nod factor perception within a single species might also occur in other legumes. In fact, in clover and soybean, strain-specific nodulation phenotypes within the species are observed that might be caused by variations in a gene involved in Nod factor perception. These are *rwt-1* in clover and *Rj4* in soybean.

In clover the *rwt-1* gene is responsible for the inability of *R.l.*bv. *trifolii* strain TA1 to nodulate the subterranean clover cv. Woogenellup (Lewis-Henderson & Djordjevic, 1991*b*). Strain TA1 successfully nodulates various other subterranean clover cvs e.g. cv. Geraldton (Gibson, 1963), and *rwt-1*-containing subterranean clovers are readily nodulated by other *R.l.* bv. *trifolii* strains (e.g. strain ANU843, Gibson, 1968). The nodulation blocking by *rwt-1*is related to Nod factors, since for example, mutating the *nodM* gene of strain TA1 or introduction of the *nodT* gene from strain ANU843 into strain TA1 enables this strain to nodulate *rwt-1* clovers (Lewis-Henderson & Djordjevic, 1991*a*). The *nodM* gene encodes a protein with homology to glucosamine synthase, probably influencing the amount of Nod factor production (Marie, Barny & Downie, 1992). However, it cannot be ruled out that the ratio of the produced Nod factors is influenced by these genes.

The dominant locus *Rj4* prevents *B. japonicum* strain USDA61 and strains belonging to the USDA123 serogroup from nodulating, whereas other *B. japonicum* strains readily nodulate *Rj4* soybean (Vest & Caldwell, 1972; Sadowsky & Cregan, 1992). Like Sym2, *Rj4* probably interacts with Nod factors, since introduction of the *nolA* gene into USDA123 strains enable them to nodulate *Rj4* soybean (Sadowsky *et al.*, 1990, 1991). *nolA* regulates the expression of the *nod* genes, resulting in reduced production of Nod factors (Dockendorff *et al.*, 1994). Again, it cannot be excluded that it also leads to changes in the ratio of the produced Nod factors. Mutant USDA61 strains producing different ratios of Nod factors were capable of nodulating *Rj4* soybean (Stokkermans *et al.*, 1992).

Thus, it seems that, also in clover and soybean, Sym2-like receptors might control strain-specific nodulation, although it remains to be resolved whether rwt-1 and Rj4 monitor the amount of Nod factors or interact with a specific Nod factor.

Candidates for a signalling receptor

At present it cannot be concluded that one of the *sym* genes encodes a Nod factor signalling receptor. In most cases only one mutant has been isolated, so it is not known whether the mutation is a null-allele. Therefore, *sym* genes for which an inactive mutant has been isolated, as well as *sym* genes that confer a reduced activity, could still be receptor genes. To determine whether a gene is involved in the early stages of Nod factor perception or signal transduction it is essential to determine which signal transduction pathways are activated. For example, it has been shown that, like *Rhizobium*, *E. coli* strains producing cytokinin induce cortical cell division in alfalfa roots (Cooper & Long, 1994). Assuming that Nod factors activate a signal transduction pathway that results in an increased cytokinin level, it should be possible to induce cortical cell divisions with cytokinin in a Nod$^-$ mutant if the mutation is in a gene encoding a protein functioning in Nod factor perception or signal transduction upstream of the cytokinin-induced effect. Therefore, further studies to elucidate the signal transduction pathways involved in nodulation, and possibly the isolation of more alleles of *sym* genes, is required. Hence, at present none of the mutants is a clear candidate for encoding a signalling receptor.

CONCLUDING REMARKS

Genetic approaches can be used to isolate Nod factor receptors from legumes such as pea and soybean. Mapping studies positioned *Sym2* on chromosome 1 of pea between closely linked markers (Weeden, Kneen & LaRue, 1990; Kozik *et al.*, 1995). Unfortunately, the size of the pea genome cautions the use of YAC libraries and chromosome walking for cloning genes. PCR-based techniques comparing either two genomes (AFLP) or the mRNA pools of two plants (DDRT-PCR) circumvent this problem and presently differential display studies are being carried out using near isogenic lines differing for a small introgressed region including the *Sym2* gene.

A biochemical approach to the isolation of a Nod factor receptor is also feasible since large quantities of purified, as well as chemically synthesized Nod factors are available. A first report on Nod factor binding proteins has revealed a binding site that is present predominantly in the particulate fraction of alfalfa root extracts (Bono *et al.*, 1995). However, the affinity of this binding protein for the Nod factors is lower than the concentration at which Nod factors are active. Furthermore, it binds sulphated and non-sulphated factors with the same affinity whereas factors lacking the sulphate group are not active on alfalfa.

Alternatively, to study the mode of action of Nod factors and to isolate Nod factor receptors, it might be essential to explore the potential of non-legume systems. A few observations suggest that Nod factors are recognized by non-legumes, indicating the presence of a putative Nod factor receptor. For example, expression of rhizobial *nod* genes in tobacco affects the development of these plants (Schmidt *et al.*, 1993). Furthermore, a mutated carrot cell line that has lost the ability to form somatic embryos can be rescued by Nod factors (De Jong *et al.*, 1993), and most convincingly, applying Nod factors to tobacco protoplasts induces these cells to divide (Röhrig *et al.*, 1995). The latter response, for example, may be used as an assay to screen for mutants in Nod factor perception and signal transduction.

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CHAPTER 2

Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*

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Root Hair Deformation Activity of Nodulation Factors and Their Fate on *Vicia sativa*[1]

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We used a semiquantitative root hair deformation assay for *Vicia sativa* (vetch) to study the activity of *Rhizobium leguminosarum* bv *viciae* nodulation (Nod) factors. Five to 10 min of Nod factor-root interaction appears to be sufficient to induce root hair deformation. The first deformation is visible within 1 h, and after 3 h about 80% of the root hairs in a small susceptible zone of the root are deformed. This zone encompasses root hairs that have almost reached their maximal size. The Nod factor accumulates preferentially to epidermal cells of the young part of the root, but is not restricted to the susceptible zone. In the interaction with roots, the glucosamine backbone of Nod factors is shortened, presumably by chitinases. NodRlv-IV(C18:4,Ac) is more stable than NodRlv-V(C18:4,Ac). No correlation was found between Nod factor degradation and susceptibility. Degradation occurs both in the susceptible zone and in the mature zone. Moreover, degradation is not affected by NH_4NO_3 and is similar in vetch and in the nonhost alfalfa (*Medicago sativa*).

The *Rhizobium*-legume interaction starts with the exchange of signal molecules between both partners. Flavonoids secreted by the roots of the host plant trigger the expression of the nodulation (*nod*) genes of *Rhizobium*, resulting in the synthesis of specific lipooligosaccharides named Nod factors (Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993).

The structure of the major Nod factor produced by *Rhizobium meliloti* was the first to be elucidated (Lerouge et al., 1990). At present, the structure of Nod factors produced by several *Rhizobium* species has been determined (Dénarié and Cullimore, 1993), showing that all Nod factors are molecules consisting of a β-1,4-linked *N*-acetyl-D-glucosamine backbone varying in length between three and five sugar units. The nonreducing terminal sugar moiety is substituted on the C-2 position with a fatty acid group, the structure of which is variable. Depending on the *Rhizobium* species, additional substitutions on the terminal sugar residues are present (Spaink, 1992; Dénarié and Cullimore, 1993).

The Nod factors produced by *Rhizobium leguminosarum* bv *viciae* have an *O*-acetyl group at the C-6 position on the nonreducing terminal sugar residue; there is no substitution present on the reducing sugar. The acyl moiety is a C_{18} chain with either four or one double bond(s) (Spaink et al., 1991).

Nod factor nomenclature is based on the similarities of all published Nod factors (Spaink, 1992). In this nomenclature the species indication (e.g. Rlv for *R. leguminosarum* bv *viciae*) is followed by a roman numeral, referring to the number of glucosamine units, and a term in parentheses indicating the length of the acyl chain and degree of unsaturation and substitutions on nonreducing and reducing sugar residues [e.g. NodRlv-V(C18:4,Ac)].

After the discovery of the rhizobial Nod factors, the extent to which these molecules could induce steps of the nodulation process was studied (Fisher and Long, 1992; Spaink, 1992; Dénarié and Cullimore, 1993). In vetch, root hair deformation is induced by both C18:4- and C18:1-containing NodRlv factors, whereas cortical cell divisions and preinfection thread formation can be induced only by NodRlv factors containing a C18:4 acyl group (Spaink et al., 1991; Van Brussel et al., 1992). The Nod factor-induced nodule primordia in vetch become arrested at an early stage in development (Vijn et al., 1993), but in some plant species Nod factors are able to induce the development of complete nodule structures (Fisher and Long, 1992; Dénarié and Cullimore, 1993). Purified Nod factors are not able to promote the formation of genuine infection threads, but they induce the expression of the infection-related early nodulin genes in pea as well as in transgenic *Medicago varia* roots (Horvath et al., 1993; Pichon et al., 1993).

Thus, Nod factors play a key role in the induction of early steps of nodulation, and therefore it is important to elucidate the mechanisms by which these *Rhizobium* signal molecules induce these early steps. For this purpose, it is essential to use assays in which the activity of Nod factors can be perceived, preferably shortly after application of the factor. According to this criterion the best assays available are the depolarization of alfalfa root hair membranes, which occurs within 10 min (Ehrhardt et al., 1992), and the induction of ENOD12 gene expression in the root epidermis of alfalfa, which is induced in about 3 h (Pichon et al., 1993).

Root hair deformation has frequently been used to study the activity of Nod factors because it is a simple assay. In an assay developed for clover (*Trifolium repens* L.), root hair

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Abbreviations: CHAPS, (3-[3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; Nod, nodulation.

deformation was scored 3 h after inoculation with *R. leguminosarum* bv *trifolii* and these deformed root hairs were present in a zone located 2 to 5 mm from the tip (Bhuvaneswari and Solheim, 1985). However, in all but one (Firmin et al., 1993) of the studies to determine Nod factor activity, root hair deformation was examined several days after addition of Nod factor (Lerouge et al., 1990; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993).

We adapted the assay described by Bhuvaneswari and Solheim (1985) to study *Vicia sativa* root hair deformation and we show that the first deformation can be observed after only 1 h and that maximal deformation is established after 3 h. A period of Nod factor-root contact as short as 10 min is sufficient to induce root hair deformation. This root hair deformation assay can be used as a semiquantitative assay by which the activity of different Nod factors is compared. Furthermore, we compared the fate of radioactive Nod factors containing four or five GlcNAc residues and studied the effect of NH_4NO_3 on deformation and on the the fate of Nod factors.

MATERIALS AND METHODS

Plant Material

Vicia sativa subspecies *nigra* seeds were sterilized and germinated as described by Van Brussel et al. (1982). Germinated seeds were transferred to modified Fåhraeus slides (Bhuvaneswari and Solheim, 1985) in small trays containing Fåhraeus medium (Fåhraeus, 1957). Each slide contained 1 mL of medium and six plants. The plants were grown at 22°C with a 16-h light period for 2 d. After 1 d the slides were transferred to Petri dishes and put in a slanted position and the medium was replaced by fresh medium. After 2 d the roots had grown about 1 cm and they were used in a deformation assay. The same procedure was followed for the alfalfa seeds.

Root Hair Deformation Assay

Before applying Nod factors the roots of all plants were examined microscopically and Fåhraeus slides containing plants with deformed root hairs were discarded. The medium was replaced by medium containing Nod factor and the Fåhraeus slide was incubated in a Petri dish at 22°C. After 3 h the roots were microscopically examined. The amount of deformed root hairs in the susceptible zone was determined and deformation was rated 0, 1, or 2 corresponding to 0–20%, 20–60%, and >60% deformed root hairs. At least two Fåhraeus slides were used for each incubation and deformation was scored blindly by two persons.

The length of the root hairs in the susceptible zone was determined over a 24-h period using an ocular micrometer to ascertain if these hairs were still growing during the time in which they were susceptible to Nod factor. Root hair deformation was followed in time using a video camera. A Fåhraeus slide was placed under the microscope and the susceptible zone was located. After exchanging the medium with medium containing Nod factor, some root hairs in the susceptible zone were followed in time by continuous recording for 4 h.

Isolation and Labeling of Nod Factors

NodRlv factors were purified using the overproducing *R. leguminosarum* bv *viciae* strain LPR5045(.pIJ1089) ("wild-type") (Spaink et al., 1991), and the concentration of purified Nod factors was determined according to Spaink et al. (1991). NodRlv-V(C18:4,Ac) (1 mg) was incubated with 1 unit of exochitinase (*Streptomyces griseus*, Sigma) for 24 h under conditions described by the manufacturer. The resulting material was applied to a reversed-phase HPLC column (5 μm 4 × 250 mm, Pharmacia LKB) with acetonitrile:water (30:70) as a mobile phase (flow rate, 0.7 mL/min). NodRlv-III(C18:4,Ac) and NodRlv-II(C18:4,Ac) were purified by elution with an acetonitrile:water gradient (30:70 to 65:35 in 25 min and monitoring the eluate at 206 and 303 nm). The structure of the purified compounds was confirmed by MS analyses.

NodRlv factors were labeled in vivo by adding 0.5 mCi of [^{14}C]acetate (56 mCi mmol^{-1}) to a 200-mL naringinin-induced culture of the overproducing *R. leguminosarum* bv *viciae* (LPR5045.pIJ1089) strain as described by Spaink et al. (1991). By using labeled acetate it is likely that both the acyl moiety and the sugar backbone are labeled. The specific activity was about 26 mCi mmol^{-1} for each Nod factor.

The [^{3}H]NodRlv-V(C18:0,Ac) compound was made (at the Commissariat a l'Energy Atomique, Gif sur Yvette, France) by reducing the acyl group of NodRlv-V(C18:4,Ac) with Pd-C under a $^3H_2$ atmosphere by a procedure derived from the method described by Roche et al. (1991b). NodRlv-V(C18:4,Ac) was reduced in a solution of *n*-butanol:water (1:1) instead of using methanol, and the catalyst was extracted with DMSO after labeling instead of filtering the catalyst and collecting the methanol filtrate. The specific activity of this compound was about 200 Ci mmol^{-1}.

Nod Factor Extraction and TLC Analysis

Fåhraeus slides containing vetch plants were incubated with 6 × 10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv factor for 10, 60, or 180 min. At each time point the medium of three slides was collected and extracted with *n*-butanol. The roots of the plants were washed five times with water, the roots were collected and ground in liquid nitrogen in a small mortar, and the ground material was extracted with *n*-butanol. The *n*-butanol fractions were evaporated and redissolved in *n*-butanol. The complete root extract and one-third of the medium extract were analyzed by TLC. The same procedure was used for Fåhraeus slides containing alfalfa plants or Fåhraeus slides incubated with [^{3}H]NodRlv-V(C18:0,Ac).

To determine the degradation in the susceptible and the mature zone of the vetch roots, 100 plants were harvested 6 h after application of 5 × 10^{-9} M (10^6 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac), and segments of 2 to 3 mm of the susceptible zone and the zone containing mature root hairs were isolated using a dissecting microscope and *n*-butanol extracted as described above. The extracts were analyzed by TLC.

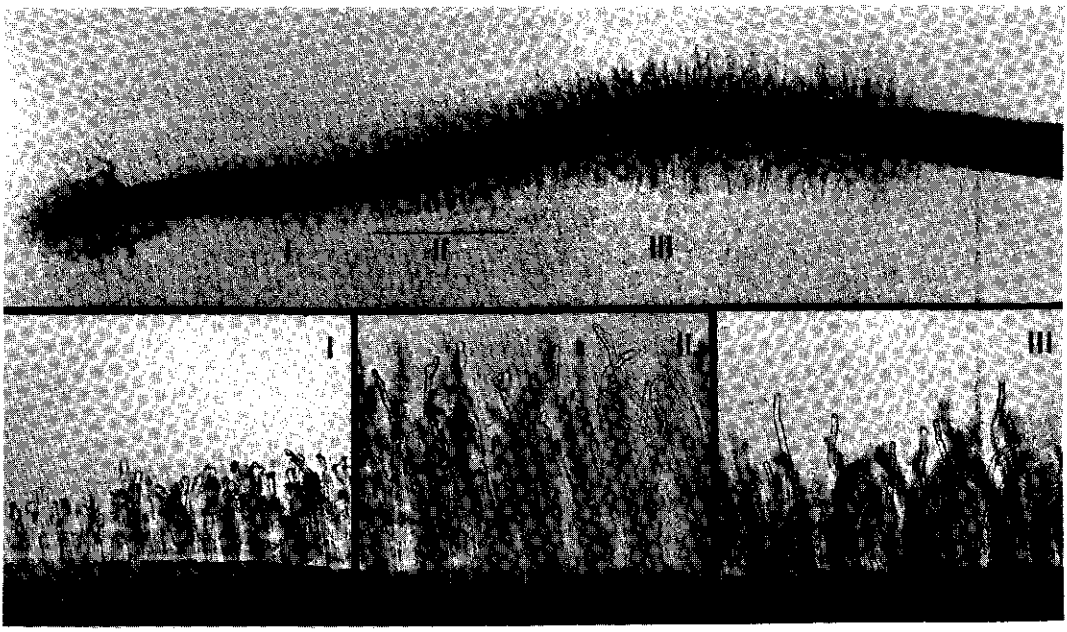

Figure 1. Root hair deformation on vetch roots. After applying NodRlv factor root hairs present in the susceptible zone (indicated by the line) deform after 3 h. The susceptible zone (II) of a vetch root encompasses young root hairs that have almost reached their mature state. This zone is about 2 mm long. Neither the young elongating root hairs (I) nor the older mature root hairs (III) are included in the susceptible zone.

The TLC system used for analysis consisted of Silica Gel 60 plates (Merck) with *n*-butanol:water:acetic acid (6:2:2) as mobile phase. A second system, used for the confirmation of co-migration with references, consisted of C_{18} reversed-phase TLC plates (Sigma) with acetonitrile:water (1:1) as a mobile phase. Both systems are described by Spaink et al. (1992).

The TLC plates were exposed for 2 d to a phosphor screen. The screen was scanned and the data were quantified using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). As an alternative, autoradiograms of TLC plates containing tritiated compounds were scanned using a computing densitometer (Molecular Dynamics).

Accumulation of [^{3}H]NodRlv-V(C18:0,Ac) on Vetch Roots

Fåhraeus slides containing vetch plants were incubated with 10^{-9} M (2×10^5 cpm mL^{-1}) or 10^{-10} M (2×10^4 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac) for 2 and 10 min. Each experiment was performed two times using 10 Fåhraeus slides. Subsequently, the roots were washed five times with Fåhraeus medium containing 0.01% CHAPS, and the remaining radioactivity was determined after solubilizing the roots overnight in 0.5 mL of Soluene-350 (Packard) using liquid scintillation. The average percentage remaining on the roots was determined and the corresponding SE was calculated using the equation:

$$\mathrm{SE}(\bar{x}) = \frac{\sigma_{n-1}}{\sqrt{n}}.$$

Sectioning of *V. sativa* Roots

Fåhraeus slides containing vetch plants were incubated for 3 h with 2×10^5 cpm (10^{-9} M) [^{3}H]NodRlv-V(C18:0,Ac), after which the roots were washed five times with water containing 0.01% CHAPS. The roots were collected and fresh longitudinal sections (70 μm) were made using a fibratome (Bio-Rad). The sections were dried overnight on polylysine-coated slides. The slides were coated with Amersham LM-1 nuclear emulsion diluted 1:1 with 600 mM NH_4Ac and exposed for 2 weeks at −20°C. They were developed in Kodak D19 developer for 3 min and fixed in Kodak Fix. Sections were stained with 0.025% toluidine blue O for 5 min and mounted with DPX (BDH).

RESULTS

Vicia Root Hair Deformation Assay

V. sativa plants were grown in Fåhraeus slides as described in "Materials and Methods." Each Fåhraeus slide contained 1

mL of Fåhraeus medium and six plants that were grown for 2 d in these slides. Before the medium was exchanged with medium containing Nod factor, the root hairs were examined microscopically and Fåhraeus slides containing plants with deformed root hairs were discarded.

After addition of 10^{-9} M NodRlv-V(C18:4,Ac), root hairs deformed within 3 h and the deformed root hairs occurred in a narrow zone of the root. This susceptible zone was about 2 mm long, and as illustrated in Figure 1, encompassed young root hairs that had almost reached their mature size, but did not include either young elongating root hairs or old mature root hairs. The length of the root hairs in the deformation zone at time 0 increased by 10 to 20% over 24 h to reach the same size as the old root hairs that did not grow during this time. Root hair deformation was followed in time with a video camera and a typical timelapse series is shown in Figure 2. At time 0, 10^{-9} M NodRlv-V(C18:4,Ac) was added. Within 30 min cytoplasmic streaming was increased (data not shown), and within 1 h the root hair tip started to swell; this swelling was more pronounced after 1.5 h. After 2 h polar growth was initiated at the swollen root hair tip and after 3 h about 80% of the root hairs in the susceptible zone had the typical deformed appearance shown in Figure 2.

To study whether root hair deformation can be used as a semi-quantitative assay, we applied a series of concentrations ranging from 10^{-7} to 10^{-13} M NodRlv-V(C18:4,Ac) to the Fåhraeus slides and examined deformation after 3 h. Root hair deformation was scored blindly by two persons and at least two Fåhraeus slides were examined for each concentration. Concentrations of 10^{-7} to 10^{-11} M NodRlv-V(C18:4,Ac) had similar effects on root hair deformation, since at these concentrations about 80% of the hairs in the susceptible zone deformed. At the higher concentrations of this factor (10^{-7}, 10^{-8} M), a small percentage (1–5%) of the hairs in the susceptible zone branched. Since branching was a rather rare event and occurred only at high concentrations of Nod factor, we did not use this effect to score Nod factor activity. When plants were treated with 10^{-12} M NodRlv-V(C18:4,Ac), the percentage of deformed root hairs decreased to about 40%. At 10^{-13} M less than 10% of the root hairs deformed, and this level of deformation could not be distinguished from the level in control plants. We rated root hair deformation as 0, 1, or 2 according to the average percentage of root hairs that deformed 3 h after addition of Nod factor. A rating of 0 was given when 0 to 20% of the root hairs in the susceptible zone deformed, whereas ratings 1 and 2 were defined as 20 to 60% and >60% deformed root hairs, respectively. The reliability of this assay lies in the fact that over 90% of the plants tested with a certain concentration of Nod factor respond with an almost identical degree of deformation. So, by applying dilution series of Nod factors, this deformation assay can be used as a semiquantitative assay.

Using this assay, we tested the ability of the naturally occurring NodRlv factors [NodRlv-V(C18:4,Ac), NodRlv-IV(C18:4,Ac), NodRlv-V(C18:1,Ac), NodRlv-IV(C18:1,Ac)] and the radiolabeled NodRlv-V(C18:0,Ac). Each Nod factor was applied to vetch roots in a concentration range that varied from 10^{-7} to 10^{-13} M. The results in Table I show that C18:4- and C18:1-containing factors were able to induce root hair deformation (rating 2) at concentrations as low as 10^{-11} M, whereas the minimal concentration of NodRlv-V(C18:0,Ac) resulting in this rating was 10^{-10} M (Table I). This shows that the unsaturation of the acyl group is not a major determinant in the ability of NodRlv factors to induce root hair deformation.

Time of Nod Factor-Root Contact Required for Induction of Root Hair Deformation

As described above, swelling of root hairs tips was observed within 1 h after incubation with Nod factor, suggesting that only a short period of Nod factor-root contact is required to induce root hair deformation. The minimal period of Nod factor-root contact that leads to root hair deformation was determined by incubating vetch plants for 1, 2, 5, and 10 min with the lowest concentration of NodRlv-V(C18:4,Ac), NodRlv-IV(C18:4,Ac), or NodRlv-V(C18:0,Ac) that could induce deformation in a 3-h incubation period (Table I, rating 2). After incubation the Fåhraeus slides were washed five times with Fåhraeus medium containing 0.01% CHAPS and the plants were further incubated in Nod factor-free medium. Root hair deformation was scored 3 h later. Table II shows

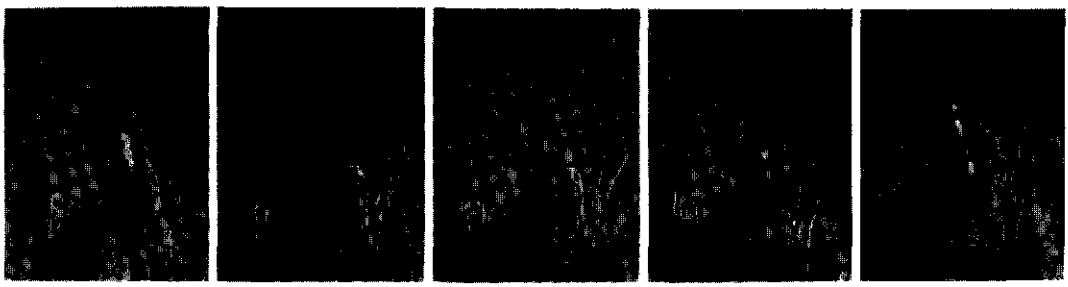

Figure 2. Root hair deformation followed in time. After applying 10^{-9} M NodRlv-V(C18:4,Ac) deformation was followed for 3 h using a video camera. After about 1 h the tips of the root hairs started to swell and this swelling was more pronounced after 1.5 h. After 2 h polar tip growth was initiated from the swollen root hair tips, and after 3 h the root hairs had a typical deformed appearance.

Table I. *The activity of Nod factors and Nod factor-derived compounds on root hair deformation*

Nod factors were applied to Fåhraeus slides containing vetch plants in a concentration range of 10^{-7} to 10^{-13} M. Deformation was examined as described in "Materials and Methods." Ratings 0, 1, and 2 correspond to 0 to 20, 20 to 60, and >60% deformed root hairs in the susceptible zone, respectively. Each experiment was performed at least five times using at least two Fåhraeus slides for each concentration of Nod factor.

Nod Factor	Concentration ($-\log_{10}$ M)						
	7	8	9	10	11	12	13
NodR1v-V(C18:4,Ac)	2	2	2	2	2	1	0
NodR1v-IV(C18:4,Ac)	2	2	2	2	2	1	0
NodR1v-V(C18:1,Ac)	2	2	2	2	2	1	0
NodR1v-IV(C18:1,Ac)	2	2	2	2	2	1	0
NodR1v-V(C18:0,Ac)	2	2	2	2	1	0	0
NodR1v-III(C18:4,Ac)	2	2	0	0	0	0	0
NodR1v-II(C18:4,Ac)	2	1	0	0	0	0	0

that the minimal incubation time with 10^{-11} M NodRlv-V(C18:4,Ac) and NodRlv-IV(C18:4,Ac) resulting in root hair deformation (rating 2) was 10 min, whereas a 5-min incubation period with 10^{-10} M NodRlv-V(C18:0,Ac) was sufficient to induce deformation (rating 2). Because more NodRlv-V(C18:0,Ac) was applied, a shorter time may be required for an amount of Nod factor able to induce complete root hair deformation to become associated with the root than for NodRlv-V(C18:4,Ac).

The amount of Nod factor remaining on the roots after washing was determined by incubating Fåhraeus slides for 2 or 10 min with either 10^{-9} M (2×10^5 cpm mL^{-1}) or 10^{-10} M (2×10^4 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac). Subsequently, the Fåhraeus slides were washed five times with Fåhraeus medium containing 0.01% CHAPS and the radioactivity bound to the roots was determined (see "Materials and Methods"). Table III shows that 1 to 2% of the originally applied radioactivity remained on the roots after washing.

Table II. *Minimal Nod factor incubation time for induction of root hair deformation*

Fåhraeus slides containing vetch plants were incubated with Nod factor for 1, 2, 5, or 10 min, after which the roots were washed with Fåhraeus medium containing 0.01% CHAPS and the plants were further incubated without Nod factor. As a control, Fåhraeus slides were incubated continuously in the presence Nod factor. Each experiment was performed at least three times using at least two Fåhraeus slides for each concentration of Nod factor.

Nod factor	Concentration	Incubation Time	Rating
	M	*min*	
NodR1v-V(C18:4,Ac)	10^{-11}	1	0
NodR1v-IV(C18:4,Ac)		2	0
		5	1
		10	2
		180	2
NodR1v-V(C18:0,Ac)	10^{-10}	1	0
		2	1
		5	2
		10	2
		180	2

Considering that only 1 to 2% of the applied Nod factor accumulated on the roots when a concentration of 10^{-10} M was applied and that a continuous incubation with a Nod factor concentration lower than 10^{-11} M did not lead to root hair deformation, it appears that the processes leading to deformation are initiated within 10 min.

Localization of Nod Factor Accumulation in Relation to Deformation

To determine whether Nod factors accumulate on a specific part of the roots, vetch plants were incubated with 10^{-9} M [^{3}H]NodRlv-V(C18:0,Ac) (2×10^5 cpm mL^{-1}) for 3 h. Fresh sections (70 μm) were made and exposed to a photographic emulsion. These studies showed that the label accumulated on the surface of the root; a relatively high amount was also detected on the root hairs (Fig. 3). Most of the radioactivity was found on the epidermal cells in the young part of the root, a region encompassing mature, susceptible, and actively growing root hairs. Less Nod factor accumulated on the old epidermal cells. Therefore, Nod factors preferentially accumulate on the young region of the root, but this accumulation is not restricted to the susceptible zone.

A detail of the susceptible zone containing deformed root

Table III. *Percentage of [^{3}H]NodR1v-V(C18:0,Ac) bound by vetch roots*

Ten Fåhraeus slides were incubated with 10^{-9} M (2×10^5 cpm mL^{-1}) or 10^{-10} M (2×10^4 cpm mL^{-1}) [^{3}H]NodR1v-V(C18:0,Ac) for 2 or 10 min, after which the roots were washed and the radioactivity remaining on the roots was determined. Calculation is as described in "Materials and Methods." Each experiment was performed two times using 10 Fåhraeus slides for each time point.

Concentration	Incubation Time	Percent Bound
M	*min*	
10^{-9}	2	1.1 ± 0.1
	10	1.7 ± 0.2
10^{-10}	2	0.9 ± 0.1
	10	1.8 ± 0.2

hairs shows that the Nod factor is mostly evenly distributed over the root hairs (Fig. 4). However, on some deformed root hairs label is present in a tonsure-like pattern around the tip of the root hair (indicated by arrowheads), whereas the newly formed tip itself does not contain any label. Plasmolyzed root hairs show that Nod factor is located in the cell membrane (indicated by arrows).

The Fate of Nod Factors during Root Hair Deformation

To determine the stability of Nod factors during the period in which root hair deformation takes place, we incubated vetch plants with 6×10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv-V(C18:4,Ac) or [^{14}C]NodRlv-IV(C18:4,Ac). After 10, 60, and 180 min the medium of three Fåhraeus slides was collected and the roots were washed five times with Fåhraeus medium. The medium and the roots were extracted with *n*-butanol. Both the water phase and the *n*-butanol phase were analyzed by TLC. Since we detected hardly any radiolabeled compounds in the water phase, these data are not shown. It is likely that GlcNAc would be present in the water phase. Therefore, we believe that the GlcNAc molecules that were cleaved off were quickly metabolized or immobilized. Alternatively, our TLC system may not be suitable for analyzing these sugars.

The results of a typical experiment with [^{14}C]NodRlv-V(C18:4,Ac) are shown in Figure 5. Three new compounds (B, C, and D) were produced in the medium and on the roots. By comparing the R_F values with those of references, using two different TLC systems, it was shown that the R_F values of spots B, C, and D corresponded with NodRlv factors with a shortened glucosamine backbone, a tetramer ([^{14}C]NodRlv-IV(C18:4,Ac)), a trimer ([^{14}C]NodRlv-III(C18:4,Ac)), and a dimer ([^{14}C]NodRlv-II(C18:4,Ac)), respectively. When NodRlv-IV(C18:4,Ac) was applied, C (trimer) and D (dimer) were formed. This suggests that Nod factors are degraded by chitinases.

The amount of radioactivity of the different compounds was determined with a PhosphorImager and expressed as a percentage of the total amount of radioactivity recovered from the medium and from the roots at each time point. We performed three experiments in which we compared the fate of the pentamer [NodRlv-V(C18:4,Ac)] and the tetramer [NodRlv-IV(C18:4,Ac)]. These experiments gave similar results; results from a representative experiment are shown in Figure 6.

In the medium 19% of the pentamer was degraded after 3 h and the major breakdown product was the tetramer (13%). The trimer and dimer were present in equal amounts (3%). In contrast, only 6% of the tetramer was degraded into trimer (2%) and dimer (4%). Thus, the pentamer was degraded more rapidly than the tetramer.

Nod factors accumulated on the roots during incubation and after 3 h about 15% of the originally applied radioactivity was associated with the roots and their Nod factors were rapidly degraded. Within 10 min one-half of the pentamer was already degraded. However, degradation of the tetramer during this time was much slower. When the pentamer was applied to the roots, its degradation product, the tetramer

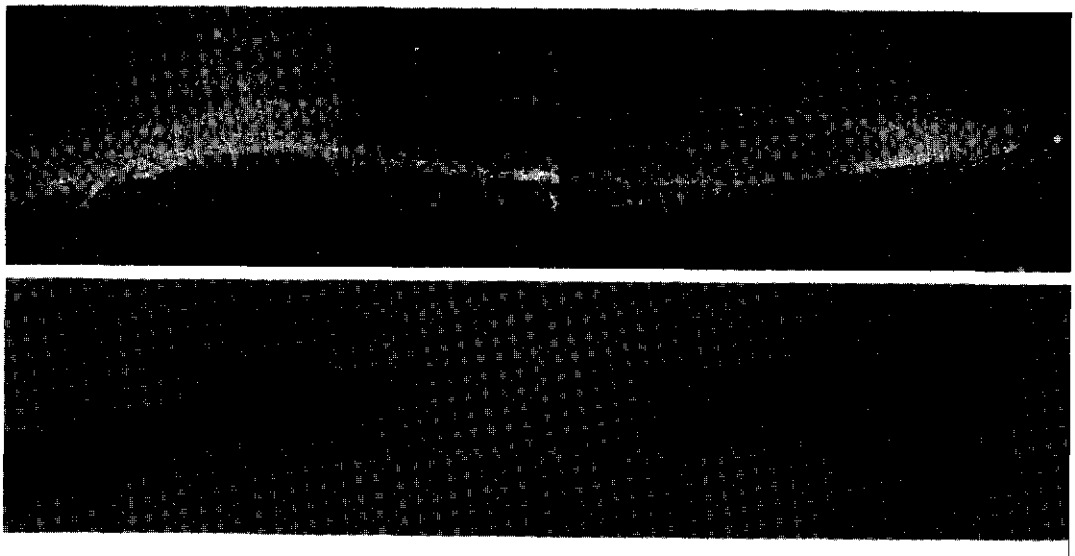

Figure 3. Localization of [^{3}H]NodRlv-V(C18:0,Ac) on vetch roots. Fresh longitudinal sections were made of roots incubated for 3 h in the presence of 10^{-9} M (2×10^5 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac) after washing the roots five times with Fåhraeus medium containing 0.01% CHAPS. The dark-field and bright-field pictures show that the label is present on the epidermal cells (including root hairs) of the root, but this accumulation is not restricted to the susceptible zone (indicated by the line).

Figure 4. Detail of Figure 3 using epipolarization microscopy. Some deformed root hairs contained label in a ring around the swollen root hair tip (indicated by arrowheads) but not on the polar outgrowth formed from this tip. Other root hairs that are plasmolyzed contain label in the cell membrane (indicated by arrows).

was always found at higher levels than the pentamer (except after 10 min). These observations confirm that the tetramer is more stable than the pentamer. The ratio of dimer to trimer was similar to that in the medium. When the pentamer was applied, a 1:1 ratio of dimer to trimer was found on the roots (approximately 3% of each after 3 h), whereas the dimer and trimer were found in a ratio of 2:1 (approximately 4% versus approximately 2% after 3 h) after incubation with the tetramer.

In addition to the chitinase degradation products, occasionally a product was formed that co-migrated with palmic acid. We assume that this was the released C18:4 fatty acid (data not shown).

Root hair deformation was induced by NodRlv factor at concentrations as low as 10^{-11} M. The relatively high concentration of 6×10^{-7} M [^{14}C]NodRlv-V(C18:4,Ac) used in these studies might not be physiological. Therefore, we also examined degradation of [^{3}H]NodRlv-V(C18:0,Ac), which had a higher specific activity (200 Ci mmol^{-1}). Fåhraeus slides containing vetch plants were incubated with 10^{-9} M (2×10^{5} cpm mL^{-1}) NodRlv-V(C18:0,Ac), and degradation of this factor was studied as described above. Because a tritiated molecule was used, the radiograms were quantified with a computing densitometer. The rate of degradation of NodRlv-V(C18:0,Ac) was similar to that of NodRlv-V(C18:4,Ac), and the percentage of the pentamer that bound to the roots was comparable. The tetramer was also the major breakdown product. However, the 5:1 ratio of dimer to trimer found on the roots differed from the 1:1 ratio observed during NodRlv-V(C18:4,Ac) incubation.

To determine whether the trimer and the dimer are able to induce deformation, these compounds were applied to vetch plants in concentrations varying from 10^{-7} to 10^{-13} M. As described above, the pentamer and tetramer induced deformation at concentrations as low as 10^{-11} M. However, as shown in Table I, the minimal concentration of trimer and dimer that induced deformation (rating 2) was much higher, 10^{-8} and 10^{-7} M, respectively, indicating that a GlcN backbone of four or five sugar residues is optimal for induction of deformation. Therefore, it is unlikely that degradation, which leads to the formation of Nod factors at least 1000-fold less active than the originally applied Nod factors, is involved in the induction of root hair deformation.

Nod Factor Degradation in Relation to Biological Activity

To determine whether the degradation of Nod factors controls where and when Nod factors are active, we studied the correlation between the ability to respond to Nod factors by root hair deformation and the ability to degrade Nod factors. Since root hair deformation occurs only in the susceptible zone of the root, we studied whether Nod factors are degraded only in the zone with mature root hairs.

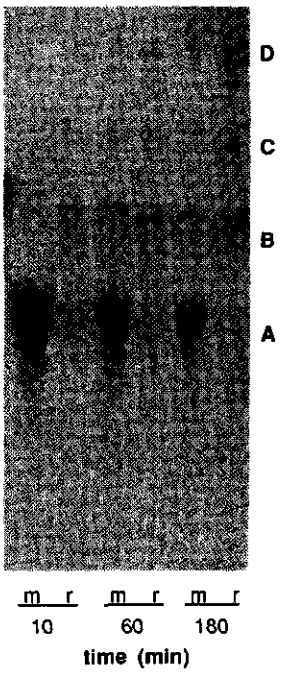

Figure 5. A typical TLC analysis of *n*-butanol-extracted medium (m) and roots (r). At 10, 60, and 180 min after applying 6×10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv-V(C18:4,Ac) to Fåhraeus slides, *n*-butanol extracts of the medium and roots of three Fåhraeus slides were made, and one-third and all of the extract was spotted on TLC, respectively. The TLC system used consisted of Silica Gel 60 plates with *n*-butanol:water:acetic acid (6:2:2) as a mobile phase. The positions of the reference compounds A ([^{14}C]NodRlv-V(C18:4,Ac)), B ([^{14}C]NodRlv-IV(C18:4,Ac)), C ([^{14}C]NodRlv-III(C18:4,Ac)), and D ([^{14}C]NodRlv-II(C18:4,Ac)) are indicated.

Sixty Fåhraeus slides containing vetch plants were incubated for 3 h with 5×10^{-9} M (10^6 cpm mL^{-1}) [^{3}H]NodRlv-V(C18:0,Ac), and after washing the slides the susceptible zone and the mature zone were collected. *n*-Butanol extraction, TLC analysis, and quantification were performed as described in "Materials and Methods." As is shown in Figure 7, Nod factors are degraded in a similar manner in the mature and susceptible zone, indicating that degradation of Nod factors does not control the susceptibility of regions of the root. Also shown is the ratio of 5:1 for the dimer and trimer as described above.

The nonhost alfalfa does not respond to Nod factors lacking a sulfate group on the reducing sugar (Lerouge et al., 1990). Therefore, we compared the degradation of NodRlv-V(C18:4,Ac) on vetch and alfalfa roots. Alfalfa plants were germinated and grown in Fåhraeus slides as described for vetch and incubated for 3 h with 6×10^{-7} M (15,000 cpm mL^{-1}) [^{14}C]NodRlv-V(C18:4,Ac). *n*-Butanol extraction, TLC analysis, and quantification were performed as described for vetch. As shown in Figure 8, a similar amount of Nod factor is degraded on vetch and alfalfa after a 3-h incubation period. Therefore, the inability of alfalfa to respond to NodRlv factors is not caused by an enhanced degradation of NodRlv factors on the root system of this nonhost plant.

The Effect of NH_4NO_3 on Root Hair Deformation and Nod Factor Degradation

Nitrate has been known for a long time to inhibit root hair deformation on legumes inoculated with *Rhizobium* (Thornton, 1936). Root hairs of vetch plants grown in the presence of 10 mM NH_4NO_3 are not able to deform after incubation with up to 10^{-7} M NodRlv-V(C18:4,Ac) (data not shown). To determine the time required for NH_4NO_3 to cause this block in deformation, vetch plants grown in Fåhraeus slides were preincubated with 10 mM NH_4NO_3 for 0 to 48 h. Root hair deformation was rated 3 h after applying 10^{-9} M NodRlv-V(C18:4,Ac). Table IV shows that only a preincubation period of 36 h or longer completely blocked root hair deformation.

Since the root keeps growing during this 36 h, we tested whether root hairs formed before NH_4NO_3 addition are affected in deformation ability. The root systems were examined at the time of NH_4NO_3 addition (time 0) and 36 h later when Nod factor was applied. The position of the susceptible zone of the roots was marked on the coverslip of the Fåhraeus slide at both time points. This experiment showed that the susceptible zones at 0 and 36 h do not overlap, since at 36 h the new susceptible zone was located in the region of the root that did not yet contain root hairs at time 0. This means that the old and the new susceptible zones were about 2 mm apart. So, a complete block of deformation was obtained only when a root hair was formed in the presence of NH_4NO_3.

To determine whether the inhibition of root hair deformation by NH_4NO_3 is reversible, we grew the vetch plants in the presence of 10 mM NH_4NO_3 for 48 h, after which the Fåhraeus slides were washed five times with Fåhraeus medium without NH_4NO_3. Subsequently, the plants were grown for 0 to 48 h in the absence of NH_4NO_3 before 10^{-9} M NodRlv-V(C18:4,Ac) was applied. As shown in Table V, root hair deformation can first be induced 36 h after transfer to an NH_4NO_3-free medium. This suggests that root hairs formed in the presence of NH_4NO_3 do not regain the ability to deform.

We tested whether the presence of NH_4NO_3 in the medium affects Nod factor degradation. Vetch plants were grown in Fåhraeus slides with Fåhraeus medium containing 10 mM NH_4NO_3 and incubated for 3 h with 6×10^{-7} M (15,000 cpm mL^{-1}) NodRlv-V(C18:4,Ac). Figure 8 shows that NodRlv-V(C18:4,Ac) is degraded in a similar way on roots of control vetch plants and on roots of plants grown in the presence of 10 mM NH_4NO_3. Hence, it is very unlikely that the inhibito

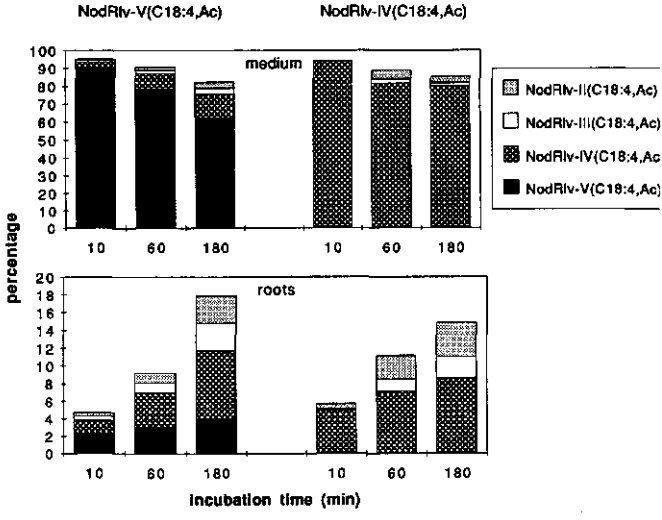

Figure 6. Degradation products present in the medium and on roots contained on Fåhraeus slides after incubation with [^{14}C]NodRlv-V(C18:4,Ac) and [^{14}C]NodRlv-IV(C18:4,Ac). On roots the pentamer was rapidly converted mainly into tetramer, which is always present at higher levels than the pentamer (except at 10 min). In both medium and roots the ratio of dimer to trimer present after incubation with the pentamer was 1:1, whereas after incubation with the tetramer the ratio was 2:1.

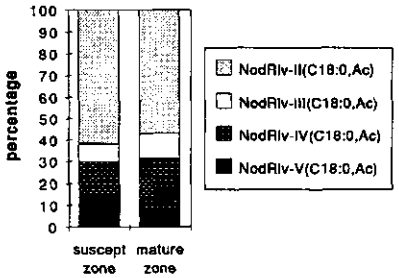

Figure 7. Comparison of degradation of [^{3}H]NodRlv-V(C18:0,Ac) in the susceptible zone and the mature zone. Fåhraeus slides were incubated with [^{3}H]NodRlv-V(C18:0,Ac) (2×10^{-9} M, 2×10^5 cpm mL^{-1}) for 3 h. The experiment was performed in duplicate and the amount of radioactivity was considered 100% for each zone. In both zones about equal degradation took place.

effect of NH_4NO_3 on root hair deformation is caused by an increased Nod factor degradation.

DISCUSSION

We show here that the root hair deformation assay described is fast and simple and can be used as a semiquantitative assay to determine the activity of Nod factors. Furthermore, root hair deformation can be followed microscopically. Nod factor-induced root hair deformation is a rapid response of the plant. The first deformation occurs within 1 h after Nod factor application, whereas an increased cytoplasmic streaming occurs within 30 min (data not shown). In most other studies, root hair deformation was first scored several days after Nod factor application (Lerouge et al., 1990; Spaink et al., 1991; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Mergaert et al., 1993). Therefore, it is not clear whether such studies provide an accurate comparison of the abilities of different Nod factors to induce deformation. We also used this deformation assay to study alfalfa root hair deformation and showed that NodRm factors induce clear deformation within 3 h (data not shown). Since clover root hair deformation is also induced within 3 h (Bhuvaneswari and Solheim, 1985), it is likely that for several small legumes this root hair deformation assay will prove to be a rapid and simple assay with which to study the activity of Nod factors.

As described, root hair deformation is induced by both C18:4- and C18:1-containing NodRlv factors, whereas cortical cell divisions and preinfection thread formation can be induced only by NodRlv factors containing a C18:4 acyl group (Spaink et al., 1991; Van Brussel et al., 1992). This shows that the deformation assay is less specific with regard to the structure of Nod factors. Consequently, the root hair deformation assay cannot be used to study the interaction between Nod factor and legume roots that requires such a high structure specificity.

Monitoring root hair deformation with a video camera showed that the existing tips of root hairs swell before polar growth is induced. A similar swelling of root hair tips can be induced by a treatment with hydrolytic enzymes (Cocking, 1985). Therefore, it is possible that Nod factors induce tip swelling by targeting hydrolytic enzymes to the tips of root hairs. The tip swelling is followed by new polar growth from the swollen root hair tip. During the formation of root hairs, the induction of tip growth is preceded by a local hydrolysis of the epidermal cell wall (Dazzo et al., 1987; Bakhuizen, 1988). Thus, the sequence of events during root hair formation and deformation are similar. Therefore, we postulate that the mechanism of root hair deformation is derived from the process controlling root hair development. This hypothesis is supported by the ability of Nod factors to stimulate root hair development (Roche et al., 1991a).

Root hair deformation is induced only in a small zone of the root containing root hairs that have almost stopped growing. However, in *Medicago* ENOD12 gene expression and root hair membrane depolarization are induced in a broader zone, including mature and young root hairs (Ehrhardt et al., 1992; Pichon et al., 1993). These results show that this entire zone is susceptible to Nod factor, whereas only in a limited part of this zone do root hairs deform. This again suggests that only at a specific stage of development are root hairs able to deform.

Nitrate has been shown to be an effective inhibitor of root hair deformation (Thornton, 1936). Here we show that root hairs that developed in the presence of NH_4NO_3 are unable

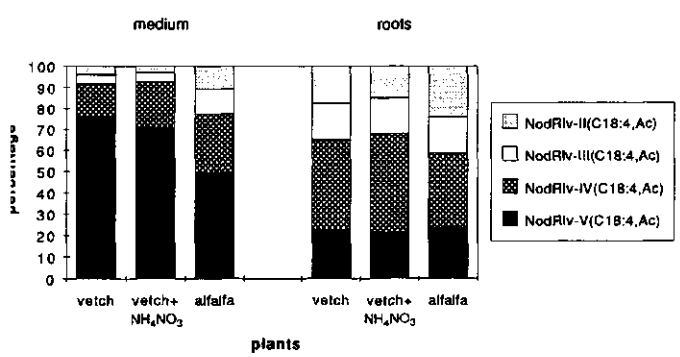

Figure 8. Comparison of degradation with vetch, vetch grown in the presence of 10 mM NH_4NO_3, and alfalfa. Fåhraeus slides were incubated with [^{14}C]NodRlv-V(C18:4,Ac) (6×10^{-7} M, 15,000 cpm mL^{-1}) for 3 h. Each experiment was performed at least two times and the results of these experiments were similar. The amount of radioactivity was considered 100% for the medium and the roots. The degradation pattern and rate in both medium and roots did not differ significantly from that observed for the control vetch plants.

Table IV. *Minimal NH_4NO_3 preincubation time required to inhibit root hair deformation*

Fåhraeus slides were preincubated with 10 mM NH_4NO_3 for 0 to 48 h. Deformation was examined 3 h after applying 10^{-9} M NodR1 V(C18:4,Ac). *Each experiment was performed at least five times using at least two Fåhraeus slides.*

Incubation time (h)	0	3	6	12	24	36	48
Rating	2	2	2	2	1	0	0

to deform, whereas NH_4NO_3 could not block deformation in root hairs that developed in the absence of NH_4NO_3. This suggests that NH_4NO_3 blocks the synthesis of a component(s) involved in the perception or transduction of Nod factors. Alternatively, NH_4NO_3 may alter root hair development so that root hairs can no longer deform.

Studies on the fate of Nod factors during incubation with vetch roots showed a rapid degradation of these factors, most likely by chitinases. This chitinase activity is associated mainly with the roots, since the relative amount of breakdown products on the roots is markedly higher than in the medium. Incubation with 10^{-9} M [^{3}H]NodRlv-V(C18:0,Ac) results in a 5:1 ratio of dimer to trimer. But because dimers are the terminal products formed after chitinase digestion of Nod factors, we assume that relatively more dimer is produced because the substrate concentration is markedly lower and the reaction is enzyme limited.

Because Nod factors are rapidly degraded, it can be questioned whether the rate of Nod factor degradation determines which part of the root system will respond to Nod factors. We showed that after 3 h the degradation of Nod factors is similar in the susceptible zone and the zone containing mature root hairs, and consequently, that degradation of Nod factors does not appear to be a major determinant of the susceptibility to Nod factors. This does not exclude the possibility of a differential degradation at an earlier time point, e.g. at 10 min. However, since the amount of degradation products is markedly lower at 10 min, it was not possible to perform this experiment. Furthermore, we showed that 3 h after application the degradation of NodRlv-V(C18:4,Ac) by the nonhost alfalfa is similar to the degradation by vetch. Moreover, we showed that NH_4NO_3, which completely blocks deformation, had no effect on Nod factor breakdown. Since our experiments did not show a correlation between Nod factor degradation and the potential to deform root hairs, we conclude that degradation cannot account for the differential response of root hair deformation. The fact that the degradation products can induce root hair deformation only at 1000-fold higher concentrations indicates that Nod factor degradation is not of importance in deformation.

A 10-min period of Nod factor-root contact appears to be sufficient to induce root hair deformation, but when the Nod factor-treated roots were extensively washed, about 2% of the originally applied Nod factor remained on the root sy tem. Therefore, it cannot stated with certainty that this sma quantity of Nod factor does not induce root hair deformatic at a later stage. However, since we used the minimal concer tration of Nod factor that can efficiently induce deformatic and since Nod factors are rapidly degraded on the ro surface, we think it is most likely that within 10 min No factor perception occurs and signal transduction has bee initiated. This conclusion is consistent with the observatic of Ehrhardt et al. (1992), who showed that root hair mem brane depolarization is induced within 10 min. In sever animal systems it has been shown that a short period ligand-receptor recognition is sufficient to set a signal-tran duction process in motion (Chao, 1992; Lefkowitz, 199 Therefore, the fact that Nod factors are active at concentr tions as low as 10^{-11} M and our observation that a 10-m period is sufficient to start root hair deformation suppo the hypothesis that a receptor is involved in Nod fact recognition.

If a receptor is involved, it is unknown which molecu would bind to such a putative receptor. Is this the unmodifi Nod factor or are Nod factor-derived molecules recognize Degradation of Nod factors occurs within 10 min; hence, v cannot be certain that NodRlv-III(C18:4,Ac) or NodRl II(C18:4,Ac) is not recognized by a receptor. However, sin the trimer and dimer have strongly reduced abilities to eli deformation, it is not very likely that these compounds pl a role in the induction of root hair deformation.

A comparison of the degradation rate of NodRl V(C18:4,Ac) and NodRlv-IV(C18:4,Ac) showed that the t tramer is the more stable of the two. On vetch roots t pentamer was degraded rapidly, and within 10 min af application it encompassed less than 50% of the NodR compounds present on the root; at all time points test (except at 10 min) the tetramer was present at higher qua tities than the pentamer. The latter observation supports t conclusion that the tetramer is more stable than the pentam

Our studies are in agreement with those of Schultze et (1993), who showed that NodRm-V(C16:2,S) is degrad faster than NodRm-IV(C16:2,S). Schultze et al. (199 showed that the NodRm-pentamer is 100-fold less active inducing *Medicago* root hair deformation than the NodR tetramer. This is in contrast to the equal potential of NodR

Table V. *Reversibility after NH_4NO_3 treatment*

Fåhraeus slides were preincubated for 48 h in the presence of 10 mM NH_4NO_3. Subsequently, the slides were washed and the plants w further incubated in the absence of NH_4NO_3 for 0 to 48 h, after which 10^{-9} M NodR1v-V(C18:4,Ac) was added and deformation v *examined 3 h later. Each experiment was performed at least five times using at least two Fåhraeus slides.*

Time of NH_4NO_3 absence (h)	0	3	6	12	24	36	48
Rating	0	0	0	0	1	2	2

pentamer and NodRlv-tetramer to induce root hair deformation in vetch (Spaink et al., 1991). We showed that NodRlv-pentamer is rapidly converted into the tetramer, which is equally active on vetch roots. However, removal of the terminal sugar of the sulfated NodRm-pentamer results in the nonsulfated tetramer, a compound inactive on alfalfa roots. Therefore, it is quite possible that vetch and alfalfa Nod factor receptors preferably recognize NodRlv-IV(C18:4,Ac) and NodRm-IV(C16:2,Ac,S), respectively.

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CHAPTER 3

Nod factor induced expression of leghemoglobin to study the mechanism of NH_4NO_3 inhibition on root hair deformation

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Molecular Plant-Microbe Interactions, in press

Nod factors secreted by *Rhizobium leguminosarum* bv. *viciae* induce root hair deformation, the formation of nodule primordia and the expression of early nodulin genes in *Vicia sativa* (vetch). Root hair deformation is induced within 3 h in a small susceptible zone (±2 mm) of the root. NH_4NO_3, known to be a potent blocker of nodule formation, inhibits root hair deformation, initial cortical cell divisions and infection thread formation. To test whether NH_4NO_3 affects the formation of a component of the Nod factor perception-transduction system, we studied Nod factor-induced gene expression. The differential display technique was used to search for marker genes which are induced within 1 to 3 h after Nod factor application. Surprisingly, one of the isolated cDNA clones was identified as a leghemoglobin gene (*VsLb1*), which is induced in vetch roots within 1 h after Nod factor application. By using the drug brefeldin A it was then shown that *VsLb1* activation does not require root hair deformation. The pVsLb1 clone was used as a marker to show that, in vetch plants grown in the presence of NH_4NO_3, Nod factor perception/transduction leading to gene expression is unaffected.

INTRODUCTION

The interaction between *Rhizobium* and its legume host plant starts with an exchange of signals and culminates in the formation of a root nodule. Flavonoids excreted by the plant induce the transcription of bacterial nodulation (*nod*) genes (Goethals et al. 1992). The proteins encoded by these *nod* genes are involved in the synthesis of specific lipo-chitin oligosaccharides, called Nod factors (Lerouge et al. 1990; Carlson et al. 1995), which can induce the initial responses in the *Rhizobium*-legume interaction such as root hair deformation, depolarisation of the root hair membrane potential, expression of some nodulin genes, and formation of nodule primordia (Lerouge et al. 1990; Spaink et al. 1991; Ehrhardt et al. 1992; Heidstra et al. 1994; Journet et al. 1994; Cook et al. 1995; Vijn et al. 1995).

Nod factors consist of a ß-1,4-linked N-acetyl-D-glucosamine backbone varying in length between three and six sugar units of which the non-reducing terminal sugar moiety is substituted on the C-2 position with a fatty acid of variable structure. Depending on the *Rhizobium* species, additional substitutions on the terminal sugar residues can be present (Carlson et al. 1995).

Recently, we developed a fast and simple semi-quantitative root hair deformation assay for vetch (*Vicia sativa*) that allows for the characterization of the root hair deformation process in more detail (Heidstra et al. 1994). It was found that root hair deformation is induced within 3 h after addition of Nod factors, in a small susceptible zone (±2 mm) of the root, encompassing young root hairs that have almost reached their mature size. One hour after the addition of Nod factors the root hair tip starts to swell; 1 h later, tip growth is initiated at the swelling; and 1 h after that (i.e. 3 h after the application of Nod factors), the root hair has the typical deformed appearance (Heidstra et al. 1994). Root hair deformation is induced by purified Nod factors at concentrations as low as 10^{-12}M (Heidstra et al. 1994).

NH_4NO_3 is known to be a potent inhibitor of nodule formation, an effect exerted soon after inoculation, inhibiting both initial cortical cell divisions and infection thread formation (Malik et al. 1987; Carroll and Mathews 1990). In addition, NH_4NO_3 is an effective inhibitor of root hair deformation (Thornton 1936; Heidstra et al. 1994). Root hairs developed in the presence of NH_4NO_3 do not deform when treated with Nod factors, whereas NH_4NO_3 is not able to block the deformation response if applied simultaneously with Nod factor (Heidstra et al. 1994). Therefore, NH_4NO_3 might preclude Nod factor perception and transduction.

To test the possible effect of NH_4NO_3 on the formation of a component of the Nod factor perception-transduction system, we studied Nod factor-induced gene expression. Genes that are rapidly activated after Nod factor addition are most suitable for that purpose . Previously, it was shown that in vetch Nod factors induce the expression of *ENOD5* and *ENOD12*, but these genes are activated after root hair deformation has been initiated (Vijn et al. 1995). Therefore, we searched for additional marker genes, the induction of which occurs shortly after Nod factor application and do not require deformation.

Although differential screening and subtractive hybridization methods have been succesfully used to identify differentially expressed nodulin genes (Scheres et al. 1990; Kouchi and Hata 1993; Cook et al. 1995), these methods often require a substantial amount of biological starting material, and are generally laborious. The differential display technique recently developed by Liang and Pardee (1992) does not have these drawbacks and has been succesfully applied to plants (Wilkinson et al. 1995; Goormachtig et al. 1996).

We have utilized a differential display analysis in an attempt to identify vetch genes induced within 3 h after Nod factor application. Here we report on one of these early Nod factor-induced genes for which a cDNA clone was obtained that was identified as leghemoglobin (*VsLb1*). The pVsLb1 clone was then used to show that Nod factor perception and transduction leading to gene expression are unaffected in vetch plants grown in the presence of NH_4NO_3.

RESULTS

Identification of Nod factor induced vetch genes.

We used differential display to identify genes that are induced within the time period of 1 to 3 h in

which root hair deformation occurs. Total RNA was isolated from the susceptible zone of 300 vetch roots either untreated or treated with 10^{-10} M NodRlv factors for 1 or 3 h. First-strand cDNAs were synthesized using 12 oligo-dT_{12}MN anchor primers. Subsequently, 23 upstream primers (10 mers) were used to amplify specific subsets of the first-strand cDNAs. By comparing the pattern of cDNA fragments of the different RNA samples obtained with each primer combination, cDNA fragments, present in the 1- and/or 3-h samples but absent from the 0-h sample were located. In this way, a total of 34 mRNAs were identified that apparently accumulated after Nod factor addition, 24 of which represented genes that were already induced after 1 h.

The 34 positive cDNAs were excised from the gel, reamplified, and cloned. Nine clones were randomly selected for further characterization. It was established that all nine cDNA clones represented genes that are expressed at a very low levels, because we were unable to detect the corresponding mRNAs by Northern (RNA) blot analysis. The cDNA sequence of eight of the nine clones showed no homology to sequences in any of the databases. These sequences contained an oligo-dT_{12}MN anchor primer sequence, and therefore the cDNAs most likely represent the 3' ends of mRNAs. It is known that in general the untranslated 3' ends of mRNAs are not conserved. These clones were not further used.

The sequence of the ninth cDNA clone, referred to as dd-3, showed that it did not represent the 3' untranslated region of a mRNA. Apparently by mispriming of the anchor primer, a 3' region of a coding sequence had been cloned, and that sequence surprisingly showed homology to the the 3' portion of the coding region of leghemoglobin genes from several plant species. Among the homologous sequences was the *VfaLbB* gene of *Vicia faba* to which it has a 64% homology at the nucleotide level (Kuhse and Pühler 1987; Fig. 1). Since the dd-3 cDNA clone is the first *Vicia sativa* leghemoglobin sequence, it was renamed pVsLb-1.

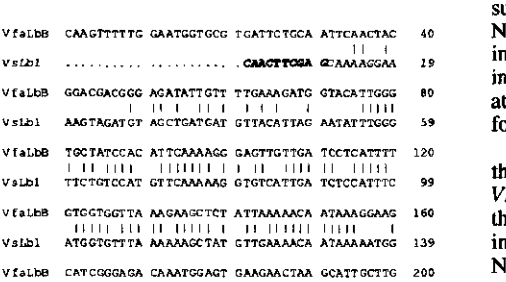

Figure1. Alignment of nucleotide sequences of the cDNA clones *VfaLbB* and *VsLb1*. The primer sequences used for the differential display are printed bold.

Early expression of leghemoglobin.

The time course of the induction of *VsLb1* during root hair deformation was determined by reverse transcriptase polymerase chain reaction (RT-PCR). At 0, 1, 3 and 8 h after Nod factor application, total RNA from 60 susceptible zones was isolated; and for each time point, the amount of *VsLb1* mRNA was quantified by RT-PCR using the primers indicated in Table 1. As shown in Figure 2, *VsLb1* expression is already detectable 1 h after application of NodRlv factor, and the amount of *VsLb1* mRNA then increased through 8 h after Nod factor addition. No *VsLb1* sequences were amplified when the RT reaction was omitted, indicating that the RNA preparations did not contain contaminating genomic DNA (data not shown).

To study whether de novo protein synthesis is required for the induction of *VsLb1* gene expression we used cyclohexamide (CHX). Preliminary experiments with a concentration series of CHX showed that 1 µM is the minimal concentration capable of completely inhibiting root hair deformation induced by 10^{-10} M NodRlv factor. When 1 µM CHX was applied together with 10^{-10} M NodRlv factor, no *VsLb1* mRNA was found in the RNA isolated 3 h after application, indicating that de novo protein synthesis is required for the activation of *VsLb1* (Fig. 2).

Since *VsLb1* is so rapidly expressed after addition of NodRlv factor, we wondered whether *VsLb1* is expressed abundantly in vetch root nodules or whether it is a specific gene expressed only early in the *Rhizobium*-legume interaction. Therefore,*VsLb1* sequences were amplified from nodule RNA by RT-PCR and the obtained cDNA fragment was cloned. The cDNA sequences of 10 randomly selected clones were 100% homologous to *VsLb1*, indicating that *VsLb1* is expressed in nodules as well as in the susceptible zone of roots shortly after application of NodRlv factor. These results suggest that *VsLb1* is induced at an early stage of the *Rhizobium*-plant interaction in the susceptible zone of the root and that at a later stage of the interaction, when the nodule is formed, the expression is strongly enhanced (Fig. 2).

We then compared the expression of *VsLb1* with that of the early nodulin genes *VsENOD5* and *VsENOD12*. Previously, it was shown by RT-PCR that vetch genes *VsENOD5* and *VsENOD12* were induced between 3 and 8 h after application of NodRlv factors (Vijn et al. 1995). However, in that study, primers based on the heterologous sequences from pea were used. We designed new primers complementary to the vetch sequences for quantitative RT-PCR experiments (Table 1). The time course of activation of *VsENOD5* and *VsENOD12* was determined with the same total RNA used for quantifying *VsLb1* mRNA accumulation at 0, 1, 3 and 8 h after NodRlv factor addition. In contrast to *VsLb1*,*VsENOD5* and *VsENOD12* appeared to be expressed at a low basal level in the

susceptible zone in the absence of NodRlv factor (Fig. 2). This low basal level was not due to contaminating DNA, since no *VsENOD5* and *VsENOD12* sequences were amplified if the RT reaction was omitted (data not shown). Three hours after NodRlv factor application the expression of *VsENOD5* and *VsENOD12* was enhanced 22-fold and 4-fold, respectively, and the level of those transcripts was slightly further increased after 8 h. The enhanced expression of both genes required de novo protein synthesis since the expression remained at a basal level when 1 µM CHX was applied together with 10^{-10} M NodRlv factor (Fig. 2).

Based on these results, we concluded that *VsLb1* is the most suitable marker gene available for studying the immediate effects of Nod factors, since *VsLb1* is expressed within 1 h after Nod factor addition, and there is no basal level of expression.

Uncoupling of root hair deformation and induction of gene expression.

Root hairs of vetch plants grown for 2 days in the presence of 20 mM of NH_4NO_3 do not deform (Heidstra et al. 1994). The rapid activation of *VsENOD5*, *VsENOD12* and especially *VsLb1* by NodRlv factors makes it possible to study the mode of action of NH_4NO_3 in inhibiting root hair deformation in more detail. For that purpose we first used the drug brefeldin A (BFA) to analyse whether induction of these these genes and root hair deformation are coupled. BFA inhibits exocytosis by altering the Golgi apparatus, an effect which is fully reversible (Misumi et al. 1986; Fujiwara et al. 1988; Satiat-Jeunemaitre and Hawes 1992; Driouich et al. 1993).

A concentration series showed that 2 µM BFA is the minimal concentration required for blocking root hair deformation with 10^{-10} M NodRlv factor without an observable effect on cytoplasmic streaming. If the BFA was then removed by washing the roots with Fårhaeus medium, and 10^{-10} M NodRlv factor was again applied, the root hairs still deformed within 3 h, showing that the effect of BFA is fully reversible. Subsequently, total RNA was isolated from the susceptible zones of the roots of 60 vetch plants treated for 3 h with 2 µM BFA and 10^{-10} M NodRlv factor, and *VsENOD5*, *VsENOD12* and *VsLb1* mRNA was amplified by RT-PCR as described. In these plants, expression of these genes after 3 h reaches a level similar to that in plants treated with NodRlv factor only (Fig. 2). Thus, the induction of expression of *VsENOD5*, *VsENOD12* and *VsLb1* does not depend on root hair deformation.

We then studied whether Nod factors can still induce the expression of *VsENOD5*, *VsENOD12* and *VsLb1* in the susceptible zone of vetch plants grown in the presence of 20 mM NH_4NO_3. For this purpose total RNA was isolated from the susceptible zone of the roots of 60 vetch plants grown in the presence of 20 mM NH_4NO_3 and treated for 3 h with 10^{-10} M NodRlv factor. Upon amplification of *VsLb1* mRNA by RT-PCR, it was found that the amount of *VsLb1*

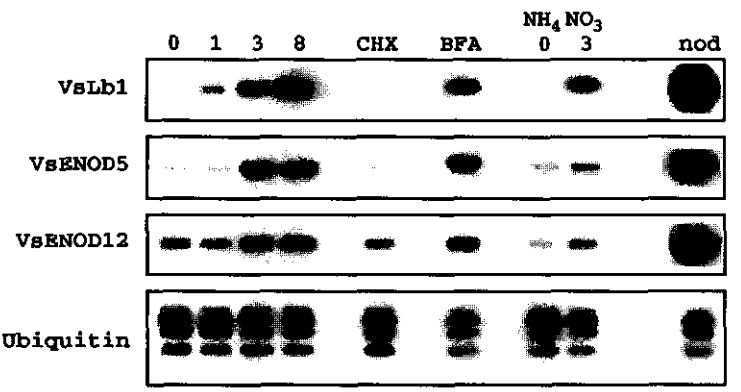

Figure 2. RT-PCR analysis of *VsLb1*, *VsENOD5* and *VsENOD12* expression. Vetch plants were either untreated or treated with 10^{-10} M NodRlv factor, and the susceptible zone was collected 1, 3 and 8 hours later. Total RNA was isolated for RT-PCR experiments. The effect of CHX and BFA on *VsLb1*, *VsENOD5* and *VsENOD12* expression was determined by treating vetch plants for 3 hours with 10^{-10} M NodRlv factor in the presence of 1 µM CHX or 2 µM BFA, respectively. Vetch plants grown in 20 mM NH_4NO_3 for 2 days were either untreated or treated with 10^{-10} M NodRlv factor, and the susceptible zone was collected 3 hours later. Total RNA was isolated and *VsLb1*, *VsENOD5* and *VsENOD12* mRNA was amplified by RT-PCR. Total RNA from 2 week old nodules (2w) was used for RT-PCR experiments to determine the expression in nodule. Due to the high expression in nodules the amplification of *VsLb1*, *VsENOD5* and *VsENOD12* reached a plateau well before the number of cycles that was used to quantify the expression in roots. Therefore the actual expression in nodules is higher than shown here. As an internal control *Ubi* RNA was amplified.

mRNA accumulated after 3 h was similar to thtat in plants grown in the absence of NH_4NO_3 (Fig. 2). However, only a twofold increase in expression of *VsENOD5* and *VsENOD12* was observed 3 h after NodRlv application when plants were grown in the presence of NH_4NO_3.

These results show that induction of *VsENOD5*, *VsENOD12* and *VsLb1* gene expression can be uncoupled from root hair deformation. Although in the presence of NH_4NO_3 *VsENOD5*, and *VsENOD12* gene expression is only marginally enhanced by NodRlv factors compared to plants grown in the absence of NH_4NO_3, *VsLb1* expression is induced to a similar level, showing that Nod factor perception and transduction still occur, even though deformation is not induced.

DISCUSSION

We have shown that the well-studied leghemoglobin gene, once thought to encode a characteristic late nodulin, now appears to be the earliest nodulin gene induced by Nod factors so far. Using *VsLb1* as an early marker for Nod factor-induced responses we showed that induction of gene expression is not coupled to root hair deformation. We used this marker to study the effect of NH_4NO_3 and found that Nod factor perception and transduction leading to gene expression are unaffected by NH_4NO_3.

Our studies show that the differential display technique is a useful method for identifying genes induced very early during the *Rhizobium*/Nod factor-plant interaction. A nearly complete screening using 12 dT_{12}MN anchor and 23 upstream primers resulted in the isolation of 34 putative positive cDNAs representing genes specifically induced by Nod factors within the time that root hair deformation occurs. One of these genes, the expression of which is induced in vetch roots within 1 h after Nod factor application appeared to be*VsLb1* . However, the induction of this gene was prevented in the presence of CHX and therefore requires de novo protein synthesis, which means that there must be genes induced earlier. In view of the low expression of *VsLb1* after 1 h, differential display may again be used to identify such still earlier expressed genes. The characterization of the remaining 33 cloned cDNAs will show whether such genes are already represented in this group.

VsLb1 is expressed at a high level in nodules in addition to being expressed in vetch roots upon Nod factor treatment. The function of *Lb* in nodules is to transport oxygen to the nitrogen-fixing bacteroids. The oxygen-binding characteristics ensure that there is an adequate supply of oxygen for bacterial respiration at the very low concentrations of free oxygen that prevail in infected cells. Damage to the oxygen-sensitive enzyme nitrogenase is avoided at these low oxygen concentrations (reviewed in Appleby, 1984). An explanation for *Lb* expression in the susceptible zone may be that deforming root hairs are rapidly respiring cells, as indicated by the increase in cytoplasmic streaming. This raises the question whether *Lb* formed in the susceptible zone also functions as an oxygen transporter, although the level of expression is low and the cell type, where it is expressed, is unknown.

Transgenic studies using the promoter of nonsymbiotic hemoglobin genes from *Parasponia* and *Casuarina* spp. show that these genes are expressed in the root meristem of tobacco and lotus plants, respectively (Bogusz et al. 1990; Jacobsen-Lyon et al. 1995). The function of hemoglobin in these tissues where oxygen supply is likely to be limiting was proposed to be that of an oxygen carrier for these rapidly respiring cells. Additional support for the oxygen-transport role comes from the study on a barley hemoglobin gene, which was induced under hypoxic conditions (Taylor et al. 1994). Vetch roots are grown in water, which is supposed to cause a poor supply of oxygen (water logging). Therefore, it is unlikely that Nod factors cause a rapidly hypoxic condition. Local expression of *Lb* during the initial *Rhizobium*-plant interaction may be involved in facilitating the flux of oxygen necessary for the mitochondial respiration in root hairs and cortical cells as has been reported for the nonsymbiotic *Lb* genes. So, besides a function as an oxygen carrier for the bacteroids in nodules, the *Lb* gene may also be employed in the early stages of the inteaction to support mitochondrial respiration in cells interacting with the bacteria.

In legume nodules the symbiotic *Lb* genes are expressed only in the infected cells where they are activated shortly after the release of the bacterium from the infection thread. However, high levels of the *Lb* mRNAs are found only in infected cells fully packed with the microsymbiont. The induction of *Lb* genes in nodules appears to require the presence of intracellular bacteria, since in uninfected nodules formed e.g. by mutant bacteria there is no *Lb* expression (Nap et al. 1989). This suggests that a signal released from the bacterium is required for the induction of *Lb* in the infected nodule cells. Since the *nod* genes are active in the area of the nodule where *Lb* is induced it is possible that Nod factors are also involved in triggering *Lb* expression in the nodule. However, the level of *Lb* expression in infected cells of the nodule is at least two orders of magnitude higher compared with that in the susceptible zone of roots a few hours after Nod factor addition. Therefore, additional factors may be involved in inducing *Lb* expression in the nodule.

VsLb1 now appears to be the earliest and most suitable marker gene of Nod factor-induced processes since it is induced within 1 h after Nod factor application, whereas *VsENOD5* and *VsENOD12* are only induced after 3 h. In addition, *VsENOD5* and *VsENOD12* are expressed at a low level in control plants, whereas *VsLb1* is not. This previously unobserved low expression in untreated roots is probably due to the specific primers complementary to the vetch *VsENOD5* and *VsENOD12* sequences

which makes the RT-PCR analysis more sensitive. The fact that these basal expression levels were observed in vetch and not in pea and alfalfa may be explained because we used whole root segments as starting material for RNA isolation and RT-PCR experiments. In contrast, in pea and alfalfa only gene expression in the epidermis was examined (Pichon et al. 1992; Horvath et al. 1993) thus avoiding the contribution of other tissues to the background level of *VsENOD5* and *VsENOD12* expression.

Root hair deformation is not required for the induction of *VsENOD5*, *VsENOD12* and *VsLb1*, as was shown in the experiment in which BFA was used to block deformation. Furthermore, in susceptible zones of plants grown in NH_4NO_3, in which root hairs are not able to deform, *VsLb1* is induced, showing that the effect of NH_4NO_3 is not due to the absence of Nod factor perception and transduction. The reason that *VsENOD5* and *VsENOD12* expression is only enhanced twofold by Nod factors in NH_4NO_3 grown plants suggests that additional host factors are required for their induction by NodRlv factors that are affected by the presence of NH_4NO_3. This is not unlikely, since in the expression of *VsENOD5* and *VsENOD12* is enhanced well after *VsLb1* is induced, which means that regulation of expression of these genes may be rather complex.

Not only does NH_4NO_3 effect on root hair deformation soon after inoculation, but it also inhibits both initial cortical cell divisions and infection thread formation (Malik et al. 1987; Carroll and Mathews 1990). If Nod factor perception and transduction are unaffected, then NH_4NO_3 should cause other changes affecting these different steps in nodule formation. Since the growth and morphology of plants in general is affected by NH_4NO_3 (Redinbaugh and Campbell 1991), it is likely to have a pleiotropic effect. Thus, although the way nodulation is affected remains unclear, Nod factor perception and transduction is unaffected by NH_4NO_3.

MATERIALS AND METHODS

Plant material and root hair deformation assay.

Vicia sativa spp. *nigra* seeds were sterilised and germinated as described by Van Brussel et al. (1982). Germinated seeds were transferred to modified Fåhraeus slides (Bhuvaneswari and Solheim 1985) in small trays containing Fåhraeus medium (Fåhraeus 1957) and treated as described by Heidstra et al. (1994). Nod factors were purified according to Spaink et al. (1991). The mixture of Nod factors as secreted by *Rhizobium leguminosarum* bv *viciae*, designated NodRlv factors, was used in all experiments described.

Each slide contains 1 ml of medium and 6 plants. Before applying NodRlv factors the roots of all plants were examined microscopically and slides containing plants with deformed root hairs were discarded. The medium was replaced by medium containing NodRlv factor and after 3 h the roots were microscopically examined. At least 2 slides were used for each treatment and deformation was scored blindly by 2 persons. In all experiments 10^{-10} M NodRlv factors were used for the induction of root hair deformation. When CHX (1 µM) and BFA (2 µM) were applied, that was done together with NodRlv factors. With NH_4NO_3, plants were grown for 2 days in Fåhraeus medium containing 20 mM NH_4NO_3.

RNA isolation.

For each RNA isolation, the susceptible zones of 60 vetch plants were collected in liquid nitrogen. The medium of the Fåhraeus slides was exchanged with medium containing NodRlv factors and susceptible zones of the plant roots were collected 1, 3 and 8 h later. To eliminate the possibility of dealing with genes induced by the exchange of medium, the susceptible zones of the 0-h timepoint were collected 3 h after the medium was exchanged with medium lacking Nod factors. The susceptible zones from roots treated with CHX and BFA were collected 3 h after application. All experiments were performed at least three times.

Total RNA was isolated (Pawlowski et al. 1994) and chromosomal DNA contamination was removed from the RNA samples according to Liang et al. (1993). The amount and quality of RNA was determined using spectrophotometry and agarose gel electrophoresis. Only RNA preparations having both A260/A280 and A260/A240 ≥ 2 were used for further studies.

Differential display of NodRlv factor induced genes in vetch.

Differential display, using total RNA isolated from susceptible zones of vetch roots 0, 1 and 3 h after Nod factor treatment, was performed essentially as described by Liang and Pardee (1992) with slight modifications. Reverse transcription was performed using 0.5 µg of total RNA in a 20 µl reaction volume with 300 U M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD). Duplicate cDNA samples, starting with the same amount of RNA, were prepared for the amplification by PCR in order to minimize errors in the PCR procedure which lead to spurious bands in one but not in the other lane. A ramp of

Table 1. Primers used in the RT-PCR assays.

Primers	Positions
1 5'-ATCCCATTGCCTTCTTAATC-3'	Compl. to nt 229-209 of the VsLb1 cDNA
2 5'-GGAAAAGTAGATGTAGCTGA-3'	Homolog. to nt 16-35 of the VsLb1 cDNA
3 5'-ACCGCACACACATATCTGT-3'	Compl. to nt 442-424 of the VsENOD5 cDNA
4 5'-TGATACTGTTGTCAATGAGG-3'	Homolog. to nt 145-164 of the VsENOD5 cDNA
5 5'-CTATGTTCTTCTGTAGCATG-3'	Compl. to nt 297-278 of the VsENOD12 cDNA
6 5'-TTCCTTGCTGCTCTTATCC-3'	Homolog. to nt 41-59 of the VsENOD12 cDNA
7 5'-ACCACCACG$^{G}/_{A}$AGACGGAG-3'	Compl. to the 3' end of a repeating ubiquitin unit
8 5'-ATGCAGAT$^{C}/_{T}$TTTGTGAAGAC-3'	Compl. to the 5' end of a repeating ubiquitin unit

3 s/°C was standard included in the PCR during the heating from the annealing to the extension temperature, resulting in better amplification when oligo-dT primers containing two pyrimidines as anchor were used. The PCR conditions were as follows: 94°C for 30 s, 40°C for 1 min, 72°C for 30 s for 40 cycles followed by 72°C for 5 min. Following the PCR, the reaction mixture was evaporated in a Speed Vac, redissolved in 5 µl formamide/water (1:1) dye solution, and the amplified cDNAs were separated on a 6% DNA sequencing gel. The PCR products were labeled with ^{32}P-dATP or preferably with ^{33}P-dATP, as the latter resulted in a much better resolution of the labeled fragments after separation on a DNA sequencing gel. The recovery and reamplification of the cDNA probes was according to Liang et al. (1993). The reamplified cDNA probes were cloned into the pGEM-T vector (Promega, Madison, WI). For at least 12 positives of each ligation it was determined whether only one or several different cDNAs were cloned originating from the same band on the sequencing gel.

The sequence of the inserts of the cDNA clones was determined with an automatic sequencing apparatus of Applied Biosystems, using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

RT-PCR.

The amounts of *VsLb1*, *VsENOD5*, *VsENOD12* mRNA were quantified using the RT-PCR method. Hybridization to the corresponding ^{32}P-labelled cloned inserts allowed the measurement of amplified products during the exponential phase of the PCR amplification. The amplification efficiency for each primer pair was determined by sampling a PCR reaction every 2 cycles between 8 to 28 cycles. This procedure was repeated for PCR reactions at different annealling temperatures and $MgCl_2$ concentrations to determine the optimal values. The PCR samples were analysed on agarose gel electrophoresis followed by Southern blotting (see below). Around the cycle number that was chosen to quantify gene expression, the amount of PCR product increased exponentially. Ubiquitin (*Ubi*) mRNA was used as an internal control (Horvath et al. 1993). The *Ubi* gene consists of five multiple units (Watts and Moore 1989) and the *Ubi* primers hybridize with the ends of each single unit.

Total RNA, 2.5 µg, was reverse transcribed in a total volume of 20 µl in 10mM Tris-Cl, pH8.8, 50 mM KCl, 5 mM MgCl2 containing 1 mM dNTPs, 20 U RNAguard, 1 µg oligo-dT_{12-18} (Pharmacia, Uppsala, Sweden) and 25 U AMV reverse transcriptase (Stratagene, La Jolla, CA). The reaction mixture was incubated for 10 min at room temperature and subsequently for 1 h at 42°C. After heating for 5 min at 95°C the the samples were diluted with water to 200 µl.

By amplifying an aliquot of the cDNA sample in separate PCR reactions the same batch of cDNA can be used in the analysis of multiple mRNAs. The cDNAs were amplified separately to avoid differences in amplification efficiencies between primer pairs. For the PCR reaction 5 µl of the cDNA sample was amplified in a total volume of 50 µl in 10 mM Tris-Cl, pH8.3, 50 mM KCl, 2.5 mM $MgCl_2$ containing 100 µM dNTPs, 100 ng 3' and 5' primer (Table 1) and 1 U *Taq* DNA polymerase. The *VsLb1* cDNA was amplified during 25 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s) while amplification of *VsENOD12* and *VsENOD5* was carried out during 23 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 30 s). The *Ubi* cDNA was amplified during 12 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s). Amplified DNA samples (10 µl) were run on a 1.5% agarose gel, and after alkaline blotting to Nytran-N$^+$ membrane (Schleicher and Schuell, Dassel, Germany) hybridized to ^{32}P-labeled inserts of the corresponding cDNA clones (Sambrook et al. 1989). Each experiment was performed in duplo.

The hybridization on the blots was quantified using the PhosphorImager and the accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

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CHAPTER 4

Ethylene provides positional information on cortical cell division but is not involved in Nod factor induced tip growth

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Submitted

Nod factors secreted by *Rhizobium leguminosarum* bv. *viciae* induce root hair deformation, involving a reinitiation of tip growth, and the formation of nodule primordia in *Vicia sativa* (vetch). Ethylene is a potent inhibitor of cortical cell division, an effect that can be counteracted by applying silver ions (Ag^+) or aminoethoxy-vinylglycine (AVG). In contrast to the inhibitory effect on cortical cell division, ethylene promotes the formation of root hairs (which involves tip growth) in the root epidermis of *Arabidopsis*. We investigate the possible paradox concerning the action of ethylene, putatively promoting Nod factor induced tip growth whilst, at the same time, inhibiting cortical cell division.

We show, by using the ethylene inhibitors AVG and Ag^+, that ethylene has no role in the reinitiation of root hair tip growth induced by Nod factors (root hair deformation) in vetch. However, induction as well as maintenance of tip growth in epidermal cells leading to root hair formation is controlled, at least in part, by ethylene. Furthermore, we show that ACC oxidase, catalizing the last step in ethylene biosynthesis, is expressed in the cell layers opposite the phloem in that part of the root where nodule primordia are induced upon inoculation with *Rhizobium*. Therefore, we test whether endogeneously produced ethylene provides positional information controlling the site where nodule primordia are formed by determining the position of nodules formed on pea roots grown in the presence of AVG or Ag^+.

INTRODUCTION

The interaction between *Rhizobium* and its legume host plant culminates in the formation of a nitrogen fixing root nodule. The rhizobial signals that play a pivotal role in the induction of the development of this organ are specific lipo-chitin oligosaccharides called Nod factors (Lerouge et al., 1990; Carlson et al., 1995). These molecules alter the growth of two cell types in the root as they induce root hair deformation by inducing tip growth in existing root hairs (see below) and, furthermore, activate cortical cells to resume mitosis resulting in nodule primordia that are predominantly formed opposite protoxylem poles (Bond, 1948; Libbenga and Harkes, 1973a; Lerouge et al., 1990; Spaink et al., 1991; Truchet et al., 1991). The phytohormone ethylene has opposite effects on tip growth and cell division in roots of dicotelydonous plants. On the one hand, ethylene promotes tip growth, i.e. root hair formation (Tanimoto et al., 1995), indicating that it may act as a second messenger in Nod factor-induced root hair deformation. On the other hand, ethylene blocks cortical cell division (Grobbelaar et al., 1971; Goodlass and Smith, 1979; Lee and LaRue, 1992). Here we study how *Rhizobium* overcomes this paradox and examine the role of ethylene in both processes.

Nod factor-induced root hair deformation can be followed when seedlings are grown in so-called Fåhraeus slides. Such studies showed that there is a correlation between the ability of root hairs to deform and their stage of development (Heidstra et al., 1994; N. de Ruijter, personal communication). In the development of root hairs three successive stages can be distinguished, represented by hairs located in adjacent zones on the root (Fig. 1). Zone I contains growing root hairs which do not deform upon Nod factor treatment. They have, just like other tip growing cells (review Sievers and Schnepf, 1981), a polarly organised cytoplasm. The apical region of these growing root hairs lacks big organelles, including vacuoles, hence it is called a clear zone (Fig. 1). Zone I root hairs show a reverse fountain type of cytoplasmic streaming that does not include the cytoplasm in the tip, and the direction of streaming is reversed below the clear zone. Other characteristics specific for the tip-growing root hairs of zone I are the occurrence of a spectrin-like antigen and a cytoplasmic Ca^{2+} concentration gradient in the tip (N. de Ruijter, personal communication). In zone II, the clear zone is no longer visible (Fig. 1), cytoplasmic streaming reaches into the tip but is still of the reverse fountain type, and the Ca^{2+} gradient and spectrin-like antigen are absent in the tip. The hairs of zone III are full grown a§nd characterized by the presence of a large vacuole, filling most of the hair, surrounded by a thin layer of cortical cytoplasm (Fig. 1). They show a rotation type of cytoplasmic streaming. Nod factors only induce deformation of root hairs in zone II, where about 80 % of the root hairs are able to deform. Upon Nod factor addition, a swelling of the tip is visible within 1 h, which is followed 1 h later by the formation of a new cylindrical outgrowth initiated at the swelling (Heidstra et al., 1994). This new outgrowth has the characteristics of a growing root hair since a clear zone is found at the tip (Fig. 1), cytoplasmic streaming is of the reverse fountain type, a cytosolic Ca^{2+} concentration gradient is built up, and the spectrin-like antigen accumulates. Hence, it has been concluded that root hair deformation involves the reinitiation of tip growth in zone II hairs (N. de Ruijter, personal communication). Here we show that, like in *Arabidopsis*, ethylene regulates the initiation of root hair formation in vetch, and we investigate whether ethylene has also a positive regulatory role in Nod factor induced tip growth.

Applied ethylene is a potent inhibitor of nodule formation, blocking the formation of nodule primordia, whereas the number of infections does not decrease. This inhibitory effect of ethylene can be counteracted by applying silver ions (Ag^+), which restores the ability to form nodules (Grobbelaar et al., 1971; Goodlass and Smith, 1979; Lee and LaRue, 1992). The silver ions are thought to interfere with the functioning of the ethylene receptor, thereby interfering with ethylene action (Burg and Burg, 1967; Beyer, 1976). Endogeneously produced ethylene also has a negative effect on nodulation. Upon treatment with amino-ethoxyvinylglycine

(AVG), an inhibitor of 1-aminocyclopropane-1-carboxylate (ACC) synthase, nodule formation on alfalfa and vetch was increased twofold (Peters and Crist-Estes, 1989; Zaat et al., 1989). The effect of ethylene as a negative regulator of nodule primordium formation is further demonstrated by a pea mutant (*sym5*) that has lost the ability to form nodule primordia. If *sym5* plants are treated with AVG, the roots regain the ability to form nodules. A similar effect is observed with Ag^+ ions (Fearn and LaRue, 1991; Guinel and LaRue, 1991). Thus, whereas Nod factors may employ ethylene to act as positive regulater in root hair deformation, at the same time ethylene is a negative regulator of primordium formation thereby creating a paradox concerning its action during the initial interaction between *Rhizobium* and its host. Here, we report on the role of ethylene during the induction both both processes.

The developing nodule primordia are predominantly found in areas of the root cortex opposite protoxylem poles (Bond, 1948; Libbenga and Harkes, 1973a). This implies that the plant provides positional information, e.g., by an interplay of positive- and negative-acting factors, controlling the site where the primordia will be formed. Recently, uridine was identified as a positive-acting factor of the stele from pea that, in combination with phytohormones, is capable of inducing cell divisions in the inner cortex of pea root explants (Smit et al., 1995). In this study, we determined where ethylene is most likely produced by locating the site where ACC oxidase mRNA accumulates, and we tested whether endogeneously produced ethylene provides positional information controlling the site where nodule primordia are formed.

RESULTS

Ethylene is a positive regulator of root hair formation in vetch

If vetch roots contained in Farhaeus slides are treated with either AVG or Ag^+, both in concentrations of 50, 10, 1 or 0.1 μM, the rate of root growth (± 0.2 mm/hr) is not affected. However, addition of 50, 10 or 1 μM Ag^+ had an immediate effect on the organisation of the cytoplasm in zone I root hairs resulting in the disappearance of the clear zone within 10 minutes (Fig. 1); root hair growth was completely stopped within 1 h. After adding 50 μM AVG, the clear zone in zone I hairs disappeared within 3 h (Fig. 1), and root hair growth was stopped within the next hour. After 8 h, the part of the root formed during the 1 μM Ag^+ treatment contained no root hairs (Table 1). Also the newly formed part of the root examined 4 h after root hair growth was blocked with 50 μM AVG contained no root hairs (Table 1). The observation that the area containing the youngest root hairs remained cone shaped confirmed that root hair growth was stopped upon treatment with Ag^+ or AVG (data not shown). The minimal concentrations of 1 uM Ag^+ and 50 uM AVG that completely blocked root hair growth within the 8 h time period of the experiment were used in further experiments.

Table 1. Ethylene triggers vetch root hair formation

Treatment	Root hairs / 50 μm
control	75
50 μM AVG	0
1 mM ACC + 50 μM AVG[1]	79
1 μM $^2Ag^+$	0

For each treatment, 3 roots were sectioned, and the root hairs present on 4 serial sections of 50 μm of the newly formed part of the root 8 h after treatment were counted and the average amount of root hairs per 50 μm was determined.

[1]The roots were preincubated for 3 h in medium containing 1 mM ACC.

[2]The medium was replaced every hour with fresh medium containing 1 μM Ag^+

To check whether the effect of AVG on root hair formation is caused by blocking ethylene biosynthesis, we preincubated vetch roots in medium with 1 mM ACC for 3 h before exchanging the medium with one containing 50 μM AVG and 1 mM ACC. If the effect caused by AVG was due to a block of ACC synthase, the addition of ACC should preserve root hair development as it enables the production of ethylene by ACC oxidase. Indeed, on the part of the root formed in the presence of the mixture of AVG and ACC, root hairs developed as on untreated roots (Table 1). Furthermore, the clear zone remained present in zone I hairs. These results show that root hair formation in vetch, i.e. induction of tip growth as well as continuation of tip growth, is regulated by ethylene.

Nod factor-induced root hair tip growth is ethylene independent

Since Nod factors induce tip growth leading to root hair deformation in zone II hairs, we investigated whether ethylene is part of the Nod factor-activated signal transduction pathway. Upon treatment of vetch roots for 0, 3 or 6 h with either 1, 10, 100 μM or 1 mM ACC or similar concentrations of the ethylene-releasing compound ethephon, neither compound induced root hair deformation in any of the applied concentrations. When 0, 3 or 6 h later the medium was replaced with medium containing 10^{-10} M Nod factors in addition to the different concentrations of ACC or ethephon, root hair deformation was induced in about 80 % of the root hairs in zone II, showing that these root hairs had remained fully competent to deform (Table 2). None of the treatments had any observable effect on the root hairs of zone I and III. These results show that ethylene alone is not sufficient to induce root hair deformation neither does it impede Nod factor-induced root hair deformation.

Subsequently, we tested whether induction of root hair deformation requires ethylene. Since root hair growth could be blocked with 50 μM AVG, this

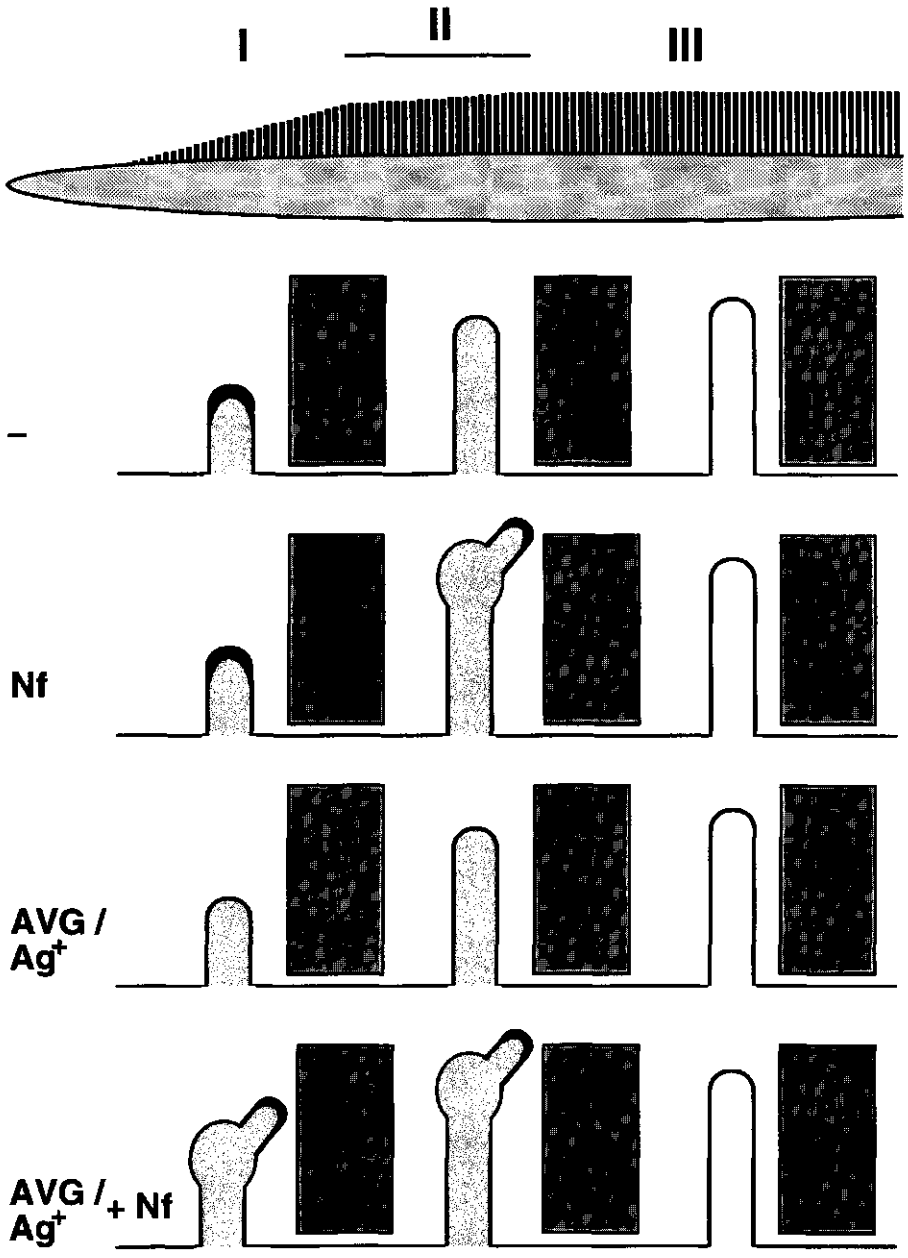

Fig. 1. Root hair deformation is correlated with developmental stage. In the development of root hairs three successive stages I, II and III are distinguished. Zone I root hairs are growing and have a clear zone at the tip (black) and cytoplasmic streaming is of the reverse fountain type (gray). In zone II the clear zone is no longer visible but cytoplasmic streaming is still of the reverse fountain type. After applying Nod factors, only root hairs present in zone II deform. Zone III contains mature root hairs lacking a clear zone and with rotation type cytoplasmic streaming (white). Addition of Ag^+ or AVG results in a disappearing of the clear zone. Subsequent addition of Nod factors leads to deformation of zone II and the altered zone I root hairs. Examples of root hairs are shown next to its schematic representative. All micrographs were made using Nomarski optics.

Table 2. Effect of ethylene and ethylene inhibtors on root hair deformation.

Compound	Concentration (μM)	Incubation time (h)	NodRlv factor[1] (M)	Deformation[2] (%) zone II	zone I
ACC	1, 10, 100, 1000	0, 1, 3, 6	-	0	0
			10^{-10}	>70	0
ethephon	1, 10, 100, 1000	0, 1, 3, 6	-	0	0
			10^{-10}	>70	0
AVG	50	0, 1	10^{-10}	>70	0
		3, 6	10^{-10}	>70	<5
Ag^+	1	0, 0.25, 1	10^{-10}	>70	40-60

The average percentage of deformed root hairs in a particular zone was determined. All experiments were performed at least 5 times.

[1]Mixture of Nod factors secreted by *Rhizobium leguminosarum* bv *viciae*.

[2]Deformation was scored after the indicated incubation time, or 3 h after application of NodRlv factor.

provided an internal control that ethylene-induced tip growth is blocked before adding Nod factors. The roots were pre-incubated for 0, 1, 3 or 6 h with 50 μM AVG before the medium was exchanged with medium containing 10^{-10} M Nod factors in addition to 50 μM AVG. Even after pretreatment of the roots with AVG for 3 or 6 h, which brings about a complete inhibition of root hair formation and growth of zone I hairs (see above) Nod factors induced root hair deformation in about 80 and 50 % of the zone II root hairs, respectively (Table 2). While zone I root hairs remained usually unaltered, occasionally some zone I root hairs (< 5 %) also deformed. These results show that ethylene is not required for Nod factor-induced root hair tip growth.

Ag^+ treatment renders zone I root hairs susceptible to Nod factors

The involvement of ethylene in root hair deformation was further tested by using Ag^+ as an inhibitor of ethylene action. The roots were pre-incubated for 0, 15 minutes or 1 h with 1 μM Ag^+ before the medium was exchanged with medium containing 10^{-10} M Nod factors in addition to 1 μM Ag^+. Addition of 1 uM Ag^+ resulted in the disappearance of the clear zone within 10 minutes and the zone I root hairs stopped growing within 1 h as found earlier (see above). However, even when the growth of zone I root hairs was blocked at the time Nod factors were added, root hair deformation was induced in about 80 % of the root hairs in zone II, just as in the experiments using AVG. Surprisingly, about 50 % of the zone I hairs were also deformed (Fig. 1, Table 2), whereas in control plants not treated with Ag^+, zone I root hairs did not deform upon Nod factor addition. These results indicate that the ability to deform is correlated with the cyto-architecture of the root hair. In addition, these results confirm that ethylene has no role in Nod factor-induced root hair deformation.

Ethylene controls the position where nodule primordia can be formed

Although ethylene is not involved in root hair deformation and there is no paradox in the function of ethylene during the initial stages of the interaction between *Rhizobium* and its host plant, the fact remains that endogeneous ethylene reduces the formation of nodule primordia (Peters and Crist-Estes, 1989; Zaat et al., 1989). As a first step in studying the effect of ethylene on cortical cell division in roots we localized the sites of ACC oxidase mRNA accumulation to determine where endogeneous ethylene may be synthesized.

The region of the root were root hairs just start to emerge is known to be the zone where primordia can be induced by *Rhizobium* and Nod factors. In situ hybridisation on 100-μm-thick cross sections of this zone using DIG labeled ACC oxidase antisense RNA, showed that ACC oxidase mRNA accumulates in the cell layers opposite the phloem poles (Fig. 2). Interestingly, nodule primordia are usually formed opposite protoxylem poles. Since it appears likely that the sites where ACC oxidase mRNA accumulates are also the sites of ethylene production, the ethylene produced in the cell layers opposite the phloem poles may suppress, in this area, cell division leading to nodule primordia.

To test this hypothesis, inoculated pea roots were grown in the presence of Ag^+ or AVG for 17 days. After sectioning the main root, the position of the formed nodules was determined. In the roots of plants grown in the absence of the ethylene inhibitors less than 1 % of the nodules is found opposite the phloem poles (Table 3). However, when plants were grown in the presence of the inhibitors, about 10 % of the nodules were located opposite a phloem pole (Table 3, Fig. 2). The amount of nodules formed

Table 3. Effect of Ag^+ and AVG on the position of the nodule on pea roots

Treatment[1]	Nodules	
	Total	Opposite phloem
-	169	1
100 μM Ag^+	247	23
50 μM AVG	182	15

Eight main roots of 17-day-old plants were sectioned in each treatment, and the total number and position of the nodules was determined.

[1]All plants were inoculated with *Rhizobium leguminosarum* bv *viciae* strain 248 at day 3 and alternatingly 300 ml water or Fåhraeus medium containing 50 μM $(Ag)_2SO_4$ or 50 μM AVG was applied 3, 6, 10 and 14 days after planting.

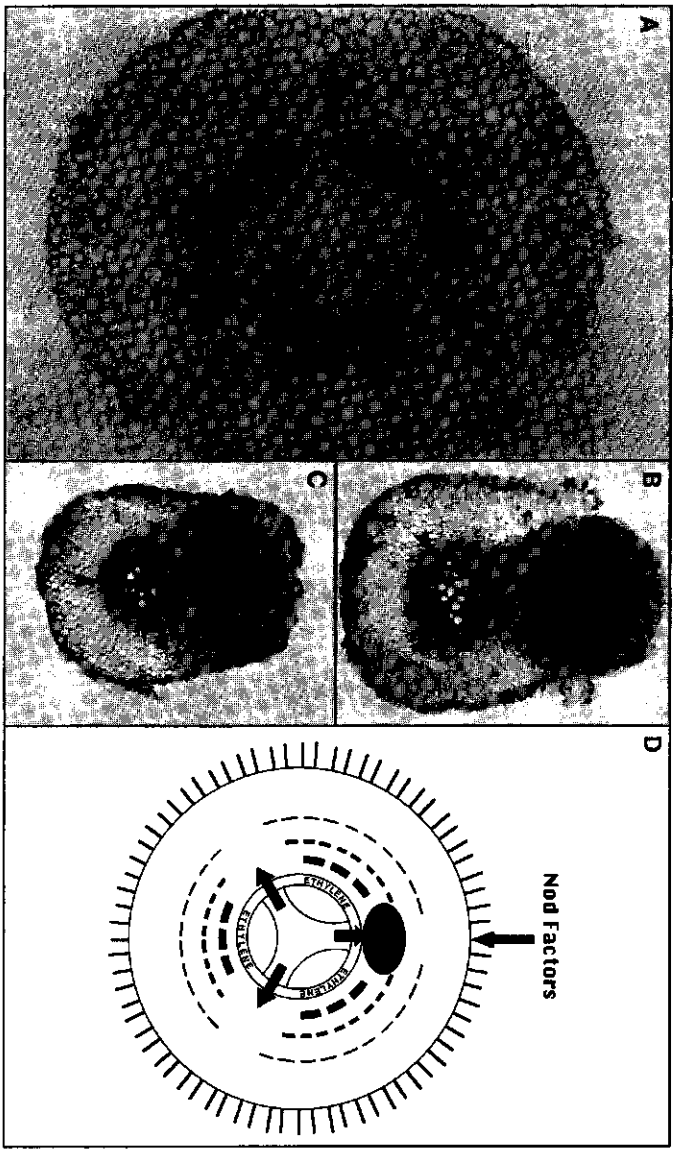

Fig. 2. The role of ethylene in positioning nodule formation. (A) In situ hybridisation of a cross section of a pea root at the region where nodule primordia can be formed with DIG labeled ACC oxidase antisense RNA, showing that ACC oxidase mRNA accumulates in the cell layers opposite the phloem poles. The position of the protoxylem is indicated with an arrowhead. Hybridisation using a sense probe showed no signal. (B,C) Examples of nodules formed opposite the xylem or phloem poles. More than 99 % of the nodules on pea roots in the absence of ethylene blockers (Ag^+ or AVG) are formed opposite the xylem poles (arrowhead) whereas in the presence of these inhibitors, ~10 % of the nodules are located opposite a phloem pole. Characteristic of nodules formed opposite the xylem poles (B) is the bifurcating vascular system coming from this xylem pole whereas with nodules formed opposite the phloem poles (C) the vascular system originates from the two xylem poles adjacent to the nodule. (D) Schematic representation of a gradient model involving positive- and negative-acting factors determining the position where nodule primordia can be formed. The developing nodule primordia are predominantly found opposite the protoxylem poles, implying that positional information is provided by the plant. In the root, a gradient system involving positive (e.g. uridine) and negative acting factors (e.g. ethylene) controls the position where cell division occurs upon stimulation by rhizobial Nod factors.

opposite the region between the phloem and xylem poles was similar in all experiments (data not shown). These experiments show that the local production of ethylene by the plant plays a role in positioning nodule primordium formation.

DISCUSSION

By using the ethylene inhibitors AVG and Ag^+, we showed that ethylene has no role in the reinitiation of root hair tip growth induced by Nod factors, i.e. root hair deformation, whereas tip growth in epidermal cells leading to root hair formation is promoted by ethylene. In addition, we show that ethylene is a negative-acting factor controlling the position where nodule primordia are formed.

In the model for the development of root hairs in *Arabidopsis* presented by Tanimoto et al. (1995), it was proposed that ethylene, after being synthesized in the central region of the root, diffuses radially through the apoplast between the walls of the cortical cells towards the epidermis. With in situ hybridization experiments, we showed that ACC oxidase is expressed in the cell layers opposite the phloem in that part of the root where root hairs are being formed. This is most likely the site where ethylene is formed since ACC oxidase catalyzes the last step in ethylene biosynthesis, converting ACC into ethylene. Our data support the proposed model since ethylene produced in the inner layers of the root can subsequently diffuse towards the epidermis where it may induce root hair development. Although ethylene is specifically synthesized opposite the phloem, diffusion will ensure that it also reaches epidermal cells opposite the xylem poles.

Our results in vetch, showing that ethylene is the signal triggering root hair development, confirm the conclusions reached in studies using *Arabidopsis* (Tanimoto et al., 1995). In addition to blocking the induction of root hair formation in vetch, root hair growth was also blocked by AVG and Ag^+, showing that induction as well as maintenance of tip growth in the epidermis in vetch is dependent on ethylene.

Growing vetch plants in Farhaeus slides makes it possible to follow root hair deformation continuously and the zones I, II and III are easily recognized. Therefore, it is possible to predict where root hair deformation will be induced and quantify the number of root hairs that respond. This provides an ideal assay for studies on Nod factor action using blockers such as Ag^+ and AVG. In the case of AVG, the root hairs of zone I and zone II on the same root were monitored when ethylene-induced growth of zone I root hairs was stopped after 3 h. These zone I hairs represented the ideal internal control to show that even when root hair growth was stopped, subsequent application of Nod factors resulted in deformation of zone II root hairs. Although the region of the root where root hairs are emerging is the site where primordia and infection thread formation are induced by rhizobia, there is no reason to assume that the mechanism of root hair deformation induced by rhizobia is different from the root hair deformation induced in zone II by Nod factors in our assay. Therefore, we conclude that ethylene is not involved in root hair deformation and that there is no paradox concerning its action during the early *Rhizobium*-host plant interaction.

Whereas addition of Ag^+ had an immediate effect on the organisation of the cytoplasm in zone I root hairs, where the clear zone disappeared within 10 minutes, the same result was observed only after 3 h following treatment with AVG. Surprisingly, the altered zone I root hairs deformed upon Nod factor treatment, indicating that the ability to deform is correlated with the cyto-architecture of the root hair. Why the Ag^+ treated zone I hairs deform more efficiently than those treated with AVG is not clear. The slow response of zone I root hairs to AVG compared to Ag^+ may be the reason why only occasionally some of these root hairs deformed. The effect of Ag^+ is fast since probably it blocks the ethylene receptors present in the epidermal cells, whereas AVG has to reach the site where ACC synthase is located, which, in *Arabidopsis* roots was reported to be expressed in the root tip and in the stele (Rodrigues-Pousada et al., 1993). In addition, ACC and ethylene formed before ACC synthesis is blocked can still act until it has diffused out of the system. Alternatively, Ag^+ may, besides blocking ethylene action, affect the growth of root hairs in a way not related to ethylene inhibition. In any case, the fact that upon Ag^+ treatment zone I root hairs are able to deform confirms that ethylene is not involved in root hair deformation.

The developing nodule primordia are predominantly formed opposite the protoxylem poles in the root, implying some sort of positional information provided by the plant. This led Libbenga et al. (1973b) to postulate the presence of a transverse gradient system of endogeneous cell division factors which control the induction of primordium formation in the root cortex. Uridine represents a positive-acting factor since it occurs in the stele of pea roots and, when added to root cortex explants grown in the presence of auxins and cytokinin, induced cell division throughout the cortex (Libbenga et al., 1973b; Smit et al., 1995). These observations indicate that all pea cortical cells have the ability to divide in the presence of the proper cell division signals. However, in a bioassay with complete root explants containing the stele, cell divisions were first observed opposite protoxylem ridges, corresponding to the location where in vivo nodule formation is initiated. This suggests that also inhibiting factor(s) are released from the phloem areas of stele. Interestingly, ACC oxidase is expressed in the cell layers opposite the phloem, in the part of the root where nodule primordia can be formed. This expression pattern together with the inhibitory effect of ethylene on cell division suggested that ethylene can locally suppress the formation of nodule primordia. Indeed, by inhibiting ethylene synthesis or action a significant amount (~10 %) of the nodules is formed opposite the phloem. That the amount of the nodules opposite

xylem and phloem is not equal indicates that ethylene action is not completely blocked and/or that ethylene is not the only factor determining the positioning of nodule primordia. On the one hand, positive-acting factors like uridine, if specifically released from the protoxylem poles, induce cell division in a local manner, whereas on the other hand, negative-acting factors like ethylene, produced opposite the phloem poles, inhibit cell division locally. Both positive- and negative-acting factors could make up gradient systems that determine the position where cell division occurs (Fig. 2).

MATERIALS AND METHODS

Plant material and root hair deformation assay

Vicia sativa spp. *nigra* seeds were sterilised and germinated as described by Van Brussel et al. (1982). Germinated seeds were transferred to modified Fåhraeus slides (Bhuvaneswari and Solheim, 1985) in small trays containing Fåhraeus medium (Fåhraeus, 1957) and treated as described by Heidstra et al. (1994). Nod factors were purified according to Spaink et al. (1991). The mixture of Nod factors as secreted by *Rhizobium leguminosarum* bv *viciae*, designated NodRlv factors, was used at a concentration of 10^{-10}M in all experiments described. At least 2 Fåhraeus slides, containing 6 plants each, were used for each treatment and deformation was scored in a blind test by 2 persons, 3 h after addition of NodRlv factors. The average percentage of deformed root hairs in a particular zone was determined. All experiments listed below were performed at least 5 times.

NodRlv factors, AVG, ACC, ethephon or Ag^+ were applied to the roots of vetch plants by replacing the medium with medium containing one or a combination of the above compounds. If the roots were incubated in the presence of Ag^+ ions, the medium was replaced every hour with fresh medium containing Ag^+ ions because silver salts were readily formed due to the presence of chloride and phosphate in the medium, thereby lowering the free Ag^+ concentration. Stock solutions of AVG, ACC, ethephon and $(Ag)_2SO_4$ (Sigma) were made up in water, filter sterilized, and stored at -20°C.

In experiments with AVG or Ag^+ ions, root hair growth was determined using an ocular micrometer. Root growth and formation of root hairs was checked during 8 h at 1-h intervals by marking the position of the root tip and the emerging root hairs on the coverslip of the Fahraeus slide. After 8 h, the amount of root hairs on the newly formed part of the root was determined by counting the root hairs found on 50 µm cross sections of the root that were made using a fibratome (BioRad).

Pea seeds (*Pisum sativum* cv. Rondo) were grown in gravel as described (Bisseling et al., 1978) and *Rhizobium leguminosarum* bv *viciae* strain 248 was used for inoculation 3 days after planting. When pea plants were grown in the presence of Ag^+ or AVG, 300 ml water or Fåhraeus medium (alternating) containing 50 µM $(Ag)_2SO_4$ or 50 µM AVG was applied 3, 6, 10 and 14 days after planting. After 17 days, the main roots were collected, hand sectioned, and the number and position of the nodules was determined using light microscopy. The position of the nodules was scored as opposite xylem, opposite phloem or opposite the region in between xylem and phloem.

In situ hybridization

Segments of 6 day old pea roots (100 µm) were fixed in PBS containing 4 % paraformaldehyde, 0.25 % glutaraldehyde, 0.08 M EGTA, 10 % DMSO, and 0.1 % Tween 20, for 3 h at room temperature. In situ hybridization was performed essentially as described by Tautz and Pfeifle (1989), with modifications. The heptane washes were eliminated. Tissue was kept in ethanol, after fixation, at -20°C for 2 days. Before the proteinase K treatment, tissue was incubated for 30 min in 1:1 ethanol/xylene solution. This treatment was followed by a post fixation step in PBS containing 0.1 % Tween 20 and 5 % formaldehyde. After the proteinase K treatment the same post fixation step was applied. Prehybridization and hybridization took place at 42°C. For the post hybridization washes an RNase A treatment for 15 to 30 min was included (40 µg/ml RNase A in 500 mM NaCl, 10 mM Tris.Cl pH 7.5, 1 mM EDTA). Before use, the anti-digoxygenin antibodies coupled to alkaline phosphatase (Boehringer Mannheim) were pre absorbed in an acetone extract of fixed roots (overnight at 4°C). The final concentration of the antibodies used was 1:2000. Incubation with the antibody took place at 4°C overnight. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate, Boehringer Mannheim) and nitroblue tetrazolium (NTB, Boehringer Mannheim) was carried out for 30 min to several h. The cDNA clone PE8 encoding pea ACC oxidase (Peck et al., 1993) was used to make a DIG labeled antisense and sense probes.

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CHAPTER 5

Sym2 of *Pisum sativum* is involved in a Nod factor perception mechanism that controls the infection process in the epidermis

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In pea (*Pisum sativum*), tens of nodulation mutants are known, several of which are affected in early steps of the symbiotic interaction with *Rhizobium*. Here we describe the role of the *sym2* gene in 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* biovar *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 are not affected, we can conclude that more than one Nod factor perception mechanism is active. Furthermore, we show that $sym2^A$ mediated control of infection thread growth is affected by the bacterial nodulation gene *nodO*.

INTRODUCTION

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 wherein the bacteria fix nitrogen to ammonia, which can subsequently be utilised by the plant.

The symbiotic interaction of *Rhizobium* 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 lipo-chitin oligosaccharides, named Nod factors (Lerouge et al., 1990, Spaink et al., 1991). Nod factors consist of a tetra- or pentameric N-acetyl glucosamine backbone with a fatty acyl chain at the non-reducing 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 (reviewed in Carlson et al., 1994).

The role of Nod factor structure in host specificity is examplified as follows: alfalfa (*Medicago sativa*) belongs to the cross-inoculation group that can be nodulated by *Rhizobium meliloti*, which produces Nod factors with a sulphate group at the reducing sugar (Lerouge et al., 1990). In contrast, pea (*Pisum sativum*) is nodulated by *Rhizobium leguminosarum* biovar *viciae* (*R.l. viciae*) that produces Nod factors lacking a substitution at that position (Spaink et al., 1991). When the host specificity genes *nodH*, *nodP* and *nodQ* responsible for the sulphation of the Nod factors in *R. meliloti* are introduced into *R.l. viciae*, these bacteria can now induce non infected nodule like structures on alfalfa, but concomitantly lose the 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, like depolarisation 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), an 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 that 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 recognised by host receptors (reviewed in: Geurts and Franssen; 1996).

At present, our understanding of the mechanism of Nod factor perception is rather poor and only based on experiments with bacterial mutants and purified Nod factors. *Rhizobium* induced responses in the epidermis of alfalfa demand different structural features of Nod factors. Infection thread formation requires a Nod factor with C16:2 acyl group and a substitution of an acetyl group at the non reducing terminal sugar, whereas the specifc structure of the acyl chain and the acetyl substitution is not important for root hair deformation. Therefore it was proposed that more than one Nod factor perception mechanism are active in the epidermis (Ardourel et al., 1994). Like root hair deformation, the induction of *ENOD12* in alfalfa 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 furthermore illustrated by the fact that, in the interaction of *R.l. 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 a production of Nod factors that are mainly acylated with vaccenic acid (C18:1), while normally Nod factors with a highly unsaturated C18:4 acyl chain are also 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). It is furthermore 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 will now be important to have host mutants that are disturbed in such a mechanism. The characterisation 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 early steps of the symbiotic interaction with *Rhizobium*. For *sym2* an allele has been identified in the wild variety Afghanistan, $sym2^A$,

which only allows nodulation by specific *R.l. viciae* strains (Lie, 1984). A single bacterial nodulation gene, *nodX*, was shown to confer the ability to nodulate plants harbouring this $sym2^A$ allele from 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).

The allele in cultivated peas, homologous to $sym2^A$, will be named $sym2^C$. Which of the two *sym2* alleles is dominant in heterozygous plants is, suprisingly, determined by the *R.l. viciae* strain used as inoculum. For example, the *R.l. viciae* $nodX^-$ strains 248 and PF_2 form nodules on heterozygotic $sym2^Asym2^C$ plants, whereas a similar $nodX^-$ strain PRE does not (Lie, 1984; Kozik et al., 1995). Strikingly, *R.l. viciae* strains 248 and PF_2 produce significantly higher amounts of Nod factors than 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 introduction of the transcriptional activator *nodD* of *R.l. 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 $sym2^Asym2^C$ plants (Kozik et al., 1995).

Here we report on the role of $sym2^A$ in Nod factor perception. Our experiments show that *R.l. viciae* strains lacking *nodX* are specifically arrested in the infection process in their interaction with $sym2^A$ harbouring peas. Furthermore, we show that $sym2^A$ mediated control of infection thread growth is affected by *nodO*. By analysing the efficiency of Nod factor degradation it appears to be unlikely that $sym2^A$plays a role in Nod factor degradation.

RESULTS

$Sym2^A$ does not strongly enhance Nod factor degradation

In theory it is plausible that *R.l. viciae* strains harbouring *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 harbour an acetyl group at their reducing end are less stable on $sym2^A$ harbouring peas when compared to $sym2^C$ harbouring 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 Rondo-$sym2^Csym2^C$ and Rondo-$sym2^Asym2^A$. Two, 5 day old, seedlings were incubated for 1, 3, 8 and 24 h

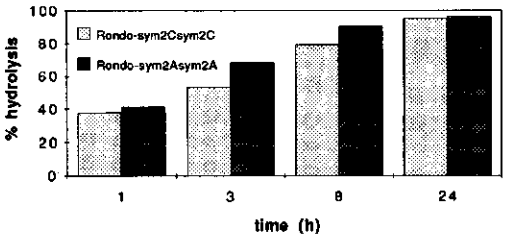

Figure 1. Degradation of [^{14}C]NodRlv-V(Ac,C18:4) by roots of Rondo-$sym2^Csym2^C$ and Rondo-$sym2^Asym2^A$. Two 5 day old pea seedlings were incubated in 4 ml medium with 25,000 cpm (6×10^{-7} M) labeled Nod factor for 1, 3, 8 and 24 h. The amount of radioactive pentameric Nod factor recovered from medium and root extracts was determined and compared to the amount initially added. The degradation rate of the Nod factor did not significantly differ in the presence of either of the roots.

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 analysed 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 was not significantly different between Rondo-$sym2^Csym2^C$ and Rondo-$sym2^Asym2^A$ (Fig. 1). Furthermore, the tetra-, tri- and di-meric Nod factor derivates were formed in similar amounts (data not shown). Thus, the precence of the $sym2^A$ allele does not significantly enhance degradation of Nod factors lacking the NodX-mediated acetyl group, indicating that $Sym2^A$ is unlikely to be involved in Nod factor degradation.

NodO can (partially) compensate for the absence of NodX-mediated Nod factors

Since the *R.l. viciae* strain used as inoculum determines which *sym2* allele is dominant, it is possible that those strains which can nodulate heterozygous Rondo-$sym2^Asym2^C$ plants may have extra genes compared with those strains that cannot. To address this, we analysed the nodulation behaviour of a pSym-cured derivative of *R.l. viciae* strain 248 containing large cloned *nod* gene regions from the Sym plasmid pRL1J1 (Fig. 2). The cured strain carrying pIJ1089 retained the characteristics of *R.l. viciae* strain 248 in that it nodulated heterozygous Rondo-$sym2^Asym2^C$ and homozygous Rondo-$sym2^Csym2^C$ plants (Table 1). Although the equivalent strain carrying pMP225 did nodulate Rondo-$sym2^Csym2^C$ plants, it could not nodulate heterozygous Rondo-$sym2^Asym2^C$ 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 $sym2^Asym2^C$ plants.

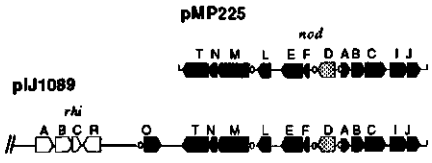

Figure 2. Map of the *nod-rhi* gene region of the *R.l. viciae* Sym-plasmid pRL1JI cloned in pMP225 (Spaink et al., 1987) and pIJ1089 (Downie et al., 1983). The *nod* genes are indicated as black arrows with the open circle indicating a *nod* box promoter. The constitutively expressed *nodD* is shown in gray and the *rhi* genes in white. pIJ1089 harbours in addition to the *nod* genes present in pMP225 a region containing *nodO, rhiABC, rhiR* and *nifH* (not shown).

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 analysed nodulation of *R.l. viciae* strain 248 derivatives carrying mutations in *nodO*, *rhiA* or *rhiR*. 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 while nodulation of the $sym2^C sym2^C$ genotype is not affected (Table 1). This demonstrates that *nodO* is essential for nodulation of heterozygous Rondo-$sym2^A sym2^C$ plants by *R.l. viciae* strain 248.

A role for *nodO* can also be seen in the nodulation of homozygous Rondo-$sym2^A sym2^A$ plants. *R.l. viciae* strain 248, carrying *nodO*, can nodulate the homozygous $sym2^A$ plants at a low level (up to 5 nodules), but when *nodO* is absent this nodulation is completely blocked. 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.l. viciae* strain 248 carrying *nodX* (Table 1). 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.l. 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 to be extremely difficult to analyse root hair deformation in a quantitative manner in pea, whereas *ENOD12* expression could be quantified by RT-PCR studies. Four-day old Rondo-$sym2^A sym2^A$ seedlings were inoculated with *R.l. viciae* strains 248, 248*nodO*$^-$ and 248*nodX*. The level of *ENOD12* mRNA in the root hairs was determined by RT-PCR after 24 hs. As shown in Figure 3 all strains trigger *ENOD12* expression to a similar level.

We studied infection thread formation and the induction of cortical cell divisions, using a spot-inoculation assay. By introducing a constitutively expressed ß-galactosidase (*lacZ*) gene 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 in the same segment the cortical cell divisions can be examined. Every experiment included at least 30 spot-inoculated roots, which were harvested after 10 days. Spot-inoculation of Rondo-$sym2^A sym2^A$ plants with *R.l. viciae* strain 248*nodX* resulted in more than 90% of the cases in the formation of a nodule within 10 days, which implied that both infection thread and nodule primordium were formed. If Rondo-$sym2^A sym2^A$ is inoculated with *R.l. viciae* strain 248 or 248*nodO*$^-$, the formation of a nodule primordium in the inner cortex was induced by both in about 70-90% of the cases (Table 2), but the cells never appeared to be infected. Figure 4 shows a cross section of a Rondo-$sym2^A sym2^A$ root segment, spot-inoculated with *R.l. viciae* strain 248*nodO*$^-$. A nodule primordium is formed in the inner cortex but there is neither differentiation into nodule tissues nor formation of a nodule meristem at the apex of the primordium. The primordia formed by *R.l. 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 is found in plants inoculated with *R.l. viciae* strain 248*nodO*$^-$.

Table 1. Nodulation behaviour of *R.l. viciae* strains on the cultivar Rondo- $sym2^C sym2^C$, the near isogenic line Rondo-$sym2^A sym2^A$ and the heterozygote Rondo-$sym2^A sym2^C$.

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

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

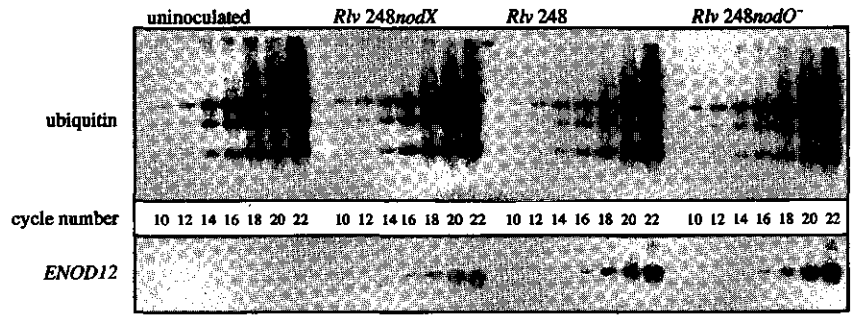

Figure 3. Induction of *ENOD12* expression in root hairs of Rondo-*sym2*^{A}*sym2*^{A}. *ENOD12* expression was analysed by RT-PCR using total RNA isolated from root hairs collected 24 hours after inoculation with *R.l. viciae* strain 248, 248*nodO*$^{-}$ or 248*nodX*. As a control *Ubiquitin* mRNA was amplified. Under the conditions used the amplifciation of *Ubiquitin* mRNA is exponential between 12 and 16 cycles, while *ENOD12* amplification is exponential up to 22 cycles.

Infection thread formation was only rarley found (less than 10% of the cases, Fig. 5), and these infection threads could only be detected in the epidermis and never grew into the inner cortical cell layers (Table 2).

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.l. viciae* strains lacking *nodX* formed a notably reduced number of infection threads while formed infection threads got 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). In 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 acitvity 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.l. viciae* strains 248 and 248*nodO*$^{-}$ on Rondo-*sym2*^{A}*sym2*^{A} was markedly increased when compared to the nodulation efficiency of both strains at the non-permissive temperature (18°C; Table 3).

We determined when *sym2*^{A} is active in the nodule formation process by growing plants for different periods at the permissive temperature and then transferring them to the non-permissive temperature. Rondo-*sym2*^{A}*sym2*^{A} roots were spot-inoculated with the *R.l. viciae* strains 248, 248*nodO*$^{-}$ and 248*nodX* and cultured for 1, 2 or 3 days at the permissive temperature (26°C) and, subsequently, the plants were cultured at the non-permissive temperature (18°C). The formation of nodules was scored 10 days post spot-inoculation. Every experiment included at least 20 spot-inoculated roots. A period of 3 days at 26°C post inoculation turned out to be sufficient to allow nodulation by *R.l. viciae* strain 248 with a similar efficiency as strain 248*nodX* (60-80%). *R.l. 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 4).

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

These observations demonstrate that after 2-3 days *sym2*^{A} has little or no control over infection thread development induced by *R.l. viciae nodX*$^{-}$

Table 2. Infection thread and primordium formation in the cortex of spot-inoculated Rondo-*sym2*^{A}*sym2*^{A}

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%)

Infection threads and primordia were scored 10 days after spot-inoculation

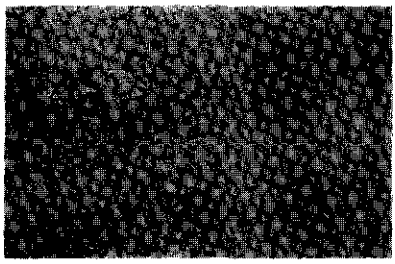

Figure 4. Nodule primordium formation on Rondo-*sym2^{A}sym2^A* by *R.l. viciae* strain 248*nodO*$^-$. A cross section (7 µm) is shown of a Rondo-*sym2^{A}sym2^A* root segment, 10 days after spot-inoculation with *R.l. viciae* strain 248*nodO*$^-$. A nodule primordium is formed in the inner cortex but the cells are not infected since there is no infection thread formed.

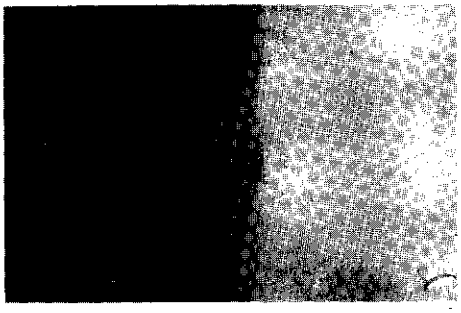

Figure 5. Infection thread formation in the epidermis of Rondo-*sym2^{A}sym2^A* by *R.l. viciae* strain 248*lacZnodO*$^-$. Root segments were collected 10 days after spot-inoculation with *R.l. viciae* 248*lacZnodO*$^-$ and infections were scored by staining the roots to detect b-galactosidase activity. Infection threads were rarely found in the epidermis and where never detected in the root cortex.

strains, 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 led us to conclude that *sym2^A* is only active in the first cell layer.

DISCUSSION

Here we show that Nod factors produced by *R.l. viciae* strains lacking *nodX* are perceived in the incompatible interaction with *sym2^A* harbouring peas, where they induce Nod factor specific responses. *R.l. viciae* strains lacking or harbouring *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 and growth of infection threads in the epidermis. When rhizobia produce the NodX substituted Nod factors infection thread formation takes place efficiently in the epidermis of *sym2^A* harbouring plants.

Sym2^A does not influence degradation of not NodX-mediated Nod factors

Substitutions at the reducing terminal sugar of Nod factors can reduce the efficiency by which they are degraded by root chitinases (Staehlin et al., 1994). Since NodX is responsible for acetylation of the reducing terminal sugar moiety of pentameric Nod factors, it was suggested that *sym2^A* might encode a chitinolytic enzyme that preferentially degrades Nod factors lacking this decoration (Firmin et al., 1993). Several lines of evidence now show that this is unlikely. Our results show that *R.l. viciae* Nod factors without a NodX-mediated modification are degraded with similar efficiency by *sym2^A* and *sym2^C* harbouring peas. Furthermore, 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. However, increasing Nod factor quantity does not overcome a deficiency in Nod factor structure (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, since nodulation by *R.l. viciae* strain 248 (harbouring *nodO*) is as effective as by strain 248*nodX* on heterozygous *sym2^{A}sym2^C* plants.

Taken together, these results suggest it is unlikely that Sym2^A establishes Nod factor structure dependent nodulation by general increase of degradation of Nod factors without a NodX-mediated modification. Instead it is more probable that Sym2^A functions in some way in the perception mechanism of the host.

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.l. viciae* strains in the interaction with heterozygous *sym2^{A}sym2^C* plants, whereas only a partial compensation is achieved in the interaction

Table 3. Temperature sensitive nodulation phenotype of Rondo-*sym2^{A}sym2^A*

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

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

Table 4. Temperature shift experiments with spot-inoculated Rondo-*sym2^{A}sym2^A*

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%)

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

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.l. viciae* strain 248*nodE*$^-$ (Economou et al., 1994). *R.l. viciae* strain 248*nodE*$^-$*nodO*$^-$ is seriously hampered in nodule formation on pea, while *R.l. 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. Hence it is well probable that in *R.l. viciae nodE*$^-$ mutants NodO also stimulates the infection process (Sutton et al., 1994).

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) and thereby it could amplify a step of the Nod factor induced signal transduction, which is needed for infection thread growth.

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.l. viciae* strains 248 or 248*nodO*$^-$, infection thread formation in the epidermis only occurs incidentally (less than 10%). Moreover, if infection occurs, the infection threads stop growing in the epidermis (Fig. 5). Furthermore, using the temperature sensitve nature of the *sym2^A* phenotype, we showed that this gene is only active 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 infection on alfalfa, while 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 on plant genes as well as *Rhizobium* mutants show that infection is controled in a more stringent way in the epidermis than in other layers of the root or root nodule.

The studies on *sym2^A* and *R. meliloti* mutants show that epidermal responses can be uncoupled. The data obtained with *R. meliloti* mutants led to the hypothesis that there are at least two Nod factor receptors in the epidermis; a signalling receptor and an entry receptor. The proposed entry receptor recognises only Nod factors with appropriate decoration and induces the formation of an infection site and initial ingestion of bacteria, while infection thread growth and root hair deformation is controlled by the signalling receptor which is less selective in Nod factor structure (Ardourel et. al., 1994).

Since *sym2^A* controls infection and requires specific Nod factors, the question arises whether *sym2^A* encodes the postulated entry receptor. Our studies show several kind of evidence that this is not the case. The suggestion that Sym2^A acts as an entry receptor would imply that *R.l. viciae* strains with or without *nodX* can nodulate heterozygous *sym2^{A}sym2^C* plants since both entry receptors are present. This is not the case, since *R.l. viciae* strain PRE and 248*nodO*$^-$ are unable to do so. Furthermore, the temperature sensitivity of the *sym2^A* phenotype could then only be explained if at higher temperatures (26°C) a decrease of stringency to Nod factor structure of the entry receptor occurs. The strongest argument that Sym2^A does not represent the proposed entry receptor is that in a spot-inoculation the *R.l. viciae* strains 248 and 248*nodO*$^-$ incidentally form an infection thread in the epidermis of Rondo-*sym2^{A}sym2^A* plants, but its growth is stopped. In contrast, the few infection threads induced by the *R. meliloti nodFE*$^-$ or *nodL*$^-$ mutants do not stop, but continue growth. This has led to the hypothesis that the entry receptor is only involved in infection site formation. Therefore it is unlikely that Sym2^A represents the proposed entry receptor and it seems more probable that *sym2^A* is involved in a mechanism controlling maintenance of infection

thread growth. In cases we do not observe infection thread formation in the incompatible interaction it can still mean that infection thread formation has been inhibited before a visible structure is present.

Since *sym2*^{A} demonstrates a relationship to a specific Nod factor structure it can be postulated that *sym2*^{A} encodes a Nod factor receptor that by interaction with the NodX-modifed Nod factor regulates maintenance of infection-thread growth in the epidermis. In the unbound form Sym2^A would repress infection thread growth, while upon binding of the proper Nod factor this receptor is inactivated (Fig. 6). Furthermore, our data suggest that there is a certain probability that infection threads can escape this repression mechanism and that this probability is increased by *nodO*. To explain the results on heterozygous Rondo-*sym2*^{A}*sym2*^{C} plants we postulate that *sym2*^{C} encodes a protein with similar function as Sym2^A but, whereas Sym2^A requires NodX modified Nod factors to be inactivated, Sym2^C requires a Nod factor with a highly unsaturated (C18:4) acyl chain. *R.l. viciae* strains that harbour *nodX* produce a mixture of both the NodX-substituted factors and unsubstituted Nod factors (Firmin et al., 1993; Kozik et al., 1995), so Sym2^A and Sym2^C would be inactivated leading to efficient nodulation. *R.l. viciae* strains that lack *nodX* produce only Nod factors that are able to inactivate *sym2*^{C}, whereas the block on infection due to Sym2^A remains, leading to inefficient nodulation. However, only half of the Sym2 proteins is the Sym2^A form which may result in a less active suppression mechanism (248*nodO*$^-$ makes up to 5 nodules on Rondo-*sym2*^{A}*sym2*^{C}, but 0 nodules on Rondo-*sym2*^{A}*sym2*^{A}). The presence of *nodO* in *R.l. viciae* strains lacking *nodX* then leads to an increase in the number of infections to an extent that nodulation is indistinguishable from nodulation by strains harbouring *nodX*.

Taken together, our studies strongly suggest that *sym2*^{A} is involved in a Nod factor recognition mechanism of the host plant controlling the infection process specifically in the epidermis. Whether *sym2*^{A} indeed encodes a Nod factor receptor remains to be proven by cloning this gene.

MATERIALS AND METHODS

Plant material and bacterial strains

For all experiments we used the near isogenic pea lines Rondo-*sym2*^{C}*sym2*^{C} (the cultivar Rondo) and Rondo-*sym2*^{A}*sym2*^{A} which was designated by Kozik et al. (1995) as line A.5.4.3. The backcross line contains approximately 10 cM of Afghanistan DNA around the *sym2* locus. Nodulation assays were performed in modified Leonard jars as described in Lie et al (1988).

All *Rhizobium* strains used are derivatives of *R.l. viciae* strain 248 (Josey et al., 1979). The bacterial strains and plasmids used in this study are listed Table 5. The plasmids pMP225, pIJ1089, pXLGD4, pMW1071 and pMW2102 were transferred to *R.l. viciae* strains using triparental mating with pRK2013 as a helper plasmid (Ditta et al., 1980). The *R.l. viciae* strains 248*nodO*$^-$, 248*rhiA*$^-$ and 248*rhiR*$^-$ were made by crossing in dervatives of pRL1JI carrying a Tn5 in *nodO*, *rhiA* or *rhiR* (Economou et al., 1990, Cubo et al., 1992) as described by Beringer et al. (1978). Selection of transconjugants was done on B$^-$ medium with the appropriate antibiotics (Spaink et al., 1989).

Spot inoculation

Sterilized pea seeds (15 min. commercial bleach, 15 min. 7% H_2O_2) were germinated at 18°C for 5 days on 1.5% agar plates. The seedlings were transferred to square petri dishes containing Fåhraeus medium (Fåhraeus, 1957)

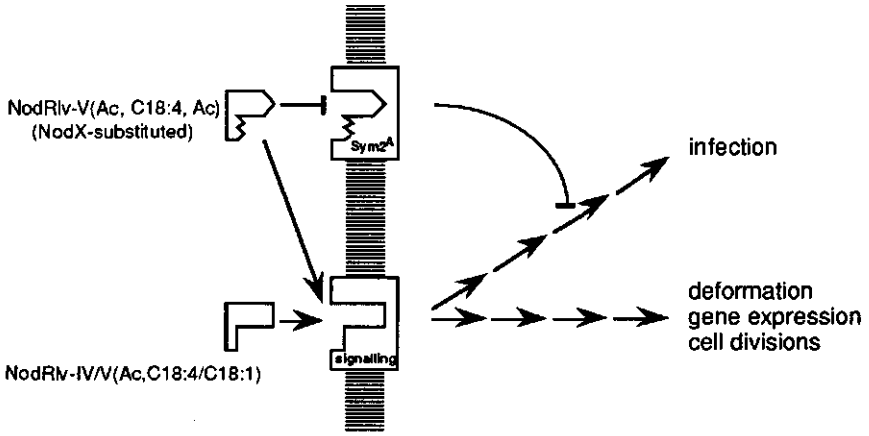

Figure 6. Model for the perception of Nod factors in the epidermis of *sym2*^{A} harbouring peas. One receptor, here called signalling receptor, triggers upon binding of a Nod factor, root hair deformation, *ENOD12* gene expression as well as infection thread formation. Sym2^A is involved in a block of infection thread growth, which can be lifted upon binding of the NodX-mediated Nod factor.

plus 1.5% agar. The plates have a hole in the rim, allowing the stem of the plant to grow out while the roots grow 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 5-7 days. For the temperature shift experiments the plants are grown at 26°C. The lateral roots were spot-inoculated at the just emerging root hairs with 0.2 µl bacterial culture ($O.D._{600}$ = 0.5). The position was marked in the agar using sterile ink.

For the quantification of infection threads *lacZ* containing *R.l. viciae* strains were used. ß-Galactosidase activity was assayed as described in Boivin et al. (1990) using X-gal as substrate. Blue staining of bacteria was visible within 24 h using 10x magnification. Cell divisions can be quantified after bleaching the root segments for 15 min. in commercial bleach.

Plastic embedding and sectioning

The spot-inoculated root segment was fixed for 1-2 h in 0.5% glutardialdehyde + 4% paraformaldehyde in 0.1 M sodiumphosphate buffer pH 6.85, washed 4 times 15 min. with phosphate buffer and 2 times 15 min. with water and dehydrated by ethanol series. Plastic infiltration was done according to the protocol of Kulzer Histo-Technik 8100.

RT-PCR to quantify ENOD12 expression

Pea plants were cultured as described by Bisseling et al. (1978). Four day old seedlings were inoculated (3 ml bacterial culture ($O.D._{600}$ = 0.5) for each plant). Root hairs were harvested from 5 day old seedlings (Gloudemans et al., 1989). Total RNA was isolated according to Pawlowski et al. (1994) followed by a DNaseI (Promega) treatment. cDNA was made from 2.5 mg total RNA in a volume of 20 ml of 10 mM Tris/Cl pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 mg oligo $dT_{12\text{-}18}$ (Pharmacia), 17 U RNA guard (Pharmacia) and 20 U AMV reverse transcriptase (Stratagene) for 10 min. at room temperature followed by 1 h at 42°C and 5 min. 95°C. The PCR reactions are performed with 1 ml of the cDNA solution in 10 mM Tris/Cl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ 100 mm dNTPs, 50 ng primer each and 1 U Taq polymerase (Bhoeringer) in a total volume of 50 ml. *ENOD12* as well as ubiquitin were amplified using the PCR program 30 s 94°C, 30 s 58°C, 30 s 72°C by using the primers psENOD12-for: 5'-TCACTAGTGTTGTTCCTTGC-3', psENOD12A-rev: 5'-CCATAAGATGGTTTGTCACG-3' to amplify only *psENOD12A*, and UBIQ-for: 5'-ATGCAGAT$^{C}/_{T}$TTTGTGAAGAC-3', UBIQ-rev: 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 to a nylon membrane (Hybond-N^{+}, Amersham) hybridized to ^{32}P-labelled *psENOD12* 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.l. viciae* strain 248^{c}.pIJ1089 as described in Heidstra et al. (1994). The specific activity of the Nod factor was about 10 mCi $mmol^{-1}$. Two 5 day old pea seedlings were incubated in 4 ml medium with 25,000 cpm (6 x 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), as described in Heidstra et al. (1994). The TLC plates were exposed to a phosphor screen and amount of radioactivity was quantified using the PhosphorImager (Molecular Dynamics).

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Table 5. *Rhizobium leguminosarum* bv *viciae* strains and plasmids used in this study.

Rhizobium strains	Relevant characteristics	Reference
248	*R.l. 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*$_{94}$::Tn5	This study
248*rhiA*$^{-}$	1391/pRL1JI*rhiA*$_{4}$::Tn5	This study
248*rhiR*$^{-}$	1391/pRL1JI*rhiR*$_{1}$::Tn5	This study
248*nodO*$^{-}$*nodX*	1391/pRL1JI*nodO*$_{94}$::Tn5 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*$_{94}$::Tn5 carrying pXLGD4	This study
plasmids		
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.l. viciae* strain TOM cloned in pMP2733	This study
pMP225	*nodABCIJDFELMNT* of pRL1JI	Spaink et al. (1987)
pIJ1089	*nodABCIJDFELMNTO rhiABCR nifH* of pRl1JI	Downie et al.(1983)
pXLGD4	*hemA*:*lacZ* fusion in pGD499	Leong et al.(1985)

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CHAPTER 6

Concluding remarks

Nod factors play a crucial role in the initiation of nodule formation. The research described in this thesis concerns the question how Nod factors start the first reactions leading to the nodule formation. For answering this question it is essential to have an assay system in which the effects of Nod factors can be followed. For this purpose we used a root hair deformation assay adapted for *Vicia sativa*. In this chapter we will discuss how our studies have contributed to understanding the mechanism by which Nod factors elicit different plant responses. With respect to the perception of Nod factors by the host plant we examined the possibility that the *sym2* gene of Afghanistan pea encodes a Nod factor receptor.

Root hair deformation as a tool to study the action of Nod factors

When the rhizobial Nod factors were discovered, fast assays to study the activity and mode of action of the Nod factors in roots were missing. We applied vetch grown in so-called Fårhaeus slides in a root hair deformation assay originally developed for clover (Bhuvanaswari & Solheim, 1985), to study the activity of Nod factors excreted by *Rhizobium leguminosarum* bv *viciae*. The advantages of this assay are its simplicity, reproducibility and rapid response. The first visible deformation occurs within 1h after Nod factor application. Furthermore, the deformation process can be continuously followed under the microscope.

Monitoring root hair deformation of vetch plants growing in Fårhaeus slides with a video camera showed that within 30 minutes after Nod factor application cytoplasmic streaming is increased and, within 1 h, the existing tips of root hairs start to swell before new growth of root hairs is induced. There turned out to be a correlation between the ability of root hairs to deform and a specific developmental stage. Only the root hairs of a small zone (zone II) of the root are susceptible and able to deform upon Nod factor application and about 80% of all root hairs in zone II are deformed after 3 h (Chapter 2). Recently, the root hair deformation process has been characterized in more detail at the cytological level and these studies have confirmed that there is a correlation between deformation ability and the cyto-architecture of the root hair at a specific developmental stage (N. de Ruijter, personal communication).

This correlation between a certain developmental stage, or the cyto-architecture, and the ability to deform was also found in experiments with the ethylene inhibitors aminoethoxyvinylglycine (AVG) and silver ions (Ag^+). Addition of Ag^+ ions especially had an immediate effect on the organisation of the cytoplasm in zone I root hairs resulting in the disppearance of the clear zone within 10 minutes, whereas this did not happen until 3 h after addition of AVG. Although zone I root hairs normally do not deform upon Nod factor treatment, the Ag^+ and AVG treated zone I root hairs surprisingly deformed upon Nod factor treatment indicating that these hairs had been shifted into a developmental stage resembling that of zone II hairs (Chapter 4).

Meanwhile, also other plant responses such as root hair membrane potential depolarization and calcium spiking in root hairs are used to study Nod factor activity (Ehrhardt et al., 1992, 1996; Felle et al., 1995; Kurkdjian, 1995). These assays show that the time period that Nod factors require to induce a response is short, in the order of minutes, whereas root hair deformation first becomes apparent within 1 h. Therefore, the early timing of depolarization and calcium spiking places them closer to the primary signal perception event, which makes them a powerfull tool in assays for early host responses combined with host genetic studies. The best candidates for perception and transduction mutants would be those that do not show depolarization and/or calcium spiking upon Nod factor application. However, these systems require a complex experimental setup and, therefore, our rapid and simple deformation assay remains a useful tool to study the mode of action of Nod factors. For example, our assay is very usefull to determine the correlation between the time course of root hair deformation and the induction of gene expression by Nod factors, as well as for testing the effect of all kinds of inhibitors to gain insight in how Nod factors are perceived and transduced. In addition, the root hair deformation assay can be used as a sytem to study the initiation and mechanism of root hair tip growth.

Isolation of early Nod factor induced genes

Root hair deformation occurs within 3 h and is dependent on transcription of genes and the synthesis of new proteins, indicating that gene expression occurs very fast after Nod factor application (Vijn et al., 1996). However, the earliest markers for vetch sofar, *VsENOD5* and *VsENOD12*, are only induced after 8 h. Therefore, we searched for earlier induced genes using differential display.

Differential RNA display appaers a useful technique for isolating early Nod factor induced genes. Using small amounts of biological starting material it is still possible to identify low abundant messengers. We identified 34 cDNAs putatively corresponding to genes that were induced within the 3 h time period in which root hair deformation occurs. None of the cDNAs from a subset of nine clones used as a probe on Northern blots gave a signal, indicating that the corresponding genes are low expressed. Sequencing each of the nine clones led to the conclusion that one cDNA corresponded to a gene encoding leghemoglobin. Subsequently, RT-PCR analysis proved that this gene, which was named *VsLb1*, is induced within 1 h after application of Nod factor, which makes this leghemoglobin gene the earliest marker of Nod factor activity found sofar (Chapter 3). The early expression of a leghemoglobin gene suggests leghemoglobin has an oxygen transporter function in the early stages of the Rhizobium-legume interaction and might presumably

support mitochondrial respiration in cells interacting with the bacteria. Upon comparing the expression of *VsLb1* with that of *VsENOD5* and *VsENOD12* we found that the latter two genes are expressed at a basal level in vetch roots, and that their expression was enhanced within 3 h upon Nod factor application (Chapter 3).

Although the expression of the *VsLb1* gene is induced within 1 h after Nod factor application, its expression was prevented in the presence of cycloheximide (Chapter 3). This means that leghemoglobin induction requires de novo protein synthesis and therefore, there should be genes whose expression is induced even earlier than that of the leghemoglobin gene. To identify such genes it may be meaningfull to repeat the differential display with RNA isolated from material that has been treated with Nod factor for a period shorter than 1 h.

Uncoupling of root hair deformation and gene induction

Nitrate has been shown to be an effective inhibitor of root hair deformation, infection thread formation and cortical cell division (Thornton, 1936; Malik, Calvert & Bauer, 1987; Caroll & Mathews, 1990). With our root hair deformation assay we have confirmed that root hairs are unable to deform upon Nod factor treatment in the presence of 20 mM NH_4NO_3. However, we found that only the root hairs that were produced when the root was grown in the presence of NH_4NO_3 were unable to deform, indicating that there is a correlation between the development of root hairs and their ability to deform (Chapter 2).

The rapid activation of *VsENOD5*, *VSENOD12* and especially *VsLb1* by Nod factors makes it possible to study NH_4NO_3 inhibition of root hair deformation in more detail. In the presence of the drug brefeldin A, that interferes with vesicle budding and the function of the Gogi apparatus, root hair deformation is prevented but the induction of these genes by Nod factors is unaffected showing that root hair deformation can be uncoupled from the induction of gene expression. Furthermore, in susceptible zones of vetch plants grown in the presence of NH_4NO_3 the *VsLb1* gene was still induced upon addition of Nod factors (Chapter 3). This makes it unlikely that NH_4NO_3 affects the formation of a component of the Nod factor perception/ transduction system. Expression of *VsENOD5* and *VsENOD12* is enhanced only twofold by Nod factors in NH_4NO_3 grown plants suggesting that NH_4NO_3 affects additional host factors that are required for their induction (Chapter 3). This explanation indicates that regulation of *VsENOD5* and *VsENOD12* expression is complex, which is not unlikely since their expression is enhanced well after *VsLb1* is induced. Taken together these data show that although the way NH_4NO_3 blocks nodulation remains unclear, Nod factor perception and transduction are unaffected.

The role of ethylene during the initial stages of nodule formation

Nod factors induce root hair deformation by inducing tip growth in existing root hairs and activate cortical cells to resume mitosis resulting in the formation of nodule primordia. Interestingly, ethylene induces tip growth in epidermal cells of *Arabidopsis* resulting in the formation of root hairs (Tanimoto et al., 1995). Both formation and deformation of root hairs involves tip growth which makes ethylene a possible candidate to function as a second messenger in eliciting the effects brought about by Nod factors. However, ethylene inhibits cortical cell division. So, when ethylene would have a role in transducing the Nod factor signal then a paradox exists concerning the action of ethylene. By using the ethylene inhibitors AVG and Ag^+ we showed that there is no such paradox, since ethylene has no role in the reinitiation of root hair tip growth induced by Nod factors (Chapter 4).

By in situ hybridization experiments we showed that ACC oxidase, that catalizes the last step in ethylene biosynthesis, is expressed in the cell layers opposite the phloem poles, in that part of the root where root hairs are being formed (Chapter 4). This observation is in agreement with the model of Tanimoto et al. (1995) which proposes that ethylene is produced in the central regions of the root and difuses radially, through the apoplast between the walls of the cortical cells, towards the epidermis where it can induce root hair development.

Interestingly, nodule primordia always arise opposite the proto-xylem poles which implies some sort of positional information provided by the plant. Libbenga et al. (1973) postulated the occurrence of a transverse gradient system of endogeneous cell division factors which controls the induction of primordium formation in the root cortex, with uridine representing a positive-acting factor (Smit et al., 1995). In a bioassay with pea root cortex explants it was demonstrated that cell divisions were induced throughout the cortex in explants without stele, or opposite the xylem poles in explants with stele. These observations indicated that in pea all cortex cells are able to dedifferentiate into dividing cells, given the proper cell division signals, and that this process may be influenced by positive- as well as by negative-acting factors. Considering that ethylene inhibits primordium formation and is produced opposite the phloem in the stele, ethylene could be a negative-acting factor in the dedifferentiation of cortical cells. This hypothesis was supported by the observation that about 10% of the nodules formed in pea roots grown in the presence of the ethylene inhibitors Ag+ and AVG were located opposite a phloem pole compared to less than 1% in control roots (Chapter 4). Hence, it is likely that positive- (e.g. uridine) and negative- (e.g. ethylene) acting factors control the position where cortical cell division induced by rhizobia, leading to primordium formation, occurs in the root.

Nod factor perception

In Chapter 1 of this thesis we have discussed the complexity of Nod factor perception and transduction. Nod factors rapidly induce responses in the epidermis, cortex and pericycle and studying these responses, like membrane potential depolarization, root hair deformation, gene expression, infection and induction of cell division, can improve our understanding of the mechanism of Nod factor perception.

The very low concentrations of Nod factors required to induce the epidermal responses suggest that a receptor will be involved in Nod factor perception. Subsequent analyses of the structural requirements of Nod factors for the different responses revealed that more than one receptor seems to be active in the epidermis. For induction of root hair deformation and *ENOD12* gene expression, the structure and degree of unsaturation of the fatty acyl group and the presence of the O-acetyl group at the non-reducing sugar residue is not very important (Roche et al., 1991; Spaink et al., 1991; Ardourel et al., 1994; Journet et al., 1994; Heidstra et al., 1994). On the other hand, efficient induction of root hair membrane depolarization and infection thread formation requires a Nod factor with both the unsaturated fatty acyl group and the O-acetyl group at the non-reducing sugar (Ardourel et al., 1994; Felle et al., 1995). Based on their studies with *Rhizobium meliloti nod* mutants Ardourel et al. (1994) proposed that there are at least two Nod factor receptors in the epidermis; a 'signalling' receptor and an 'entry' receptor. The proposed entry receptor recognises only Nod factors with appropriate decoration and induces the formation of an infection site and initial ingestion of bacteria, while infection thread growth and root hair deformation is controlled by the signalling receptor which is less selective in Nod factor structure.

To try to identify and characterize the receptor one can choose to take a biochemical approach and do binding studies with labeled Nod factors. We have chosen the genetic approach and analysed whether among the available symbiosis mutants in legumes there might be one with a mutation in a gene encoding a putative Nod factor receptor. We focussed our studies on the *sym2* gene of Afghanistan pea since, if this gene is present, more stringent structural demands are put on the Nod factors produced by *Rhizobium* for succesful nodulation (Chapter 5).

The $sym2^A$ allele of Afghanistan pea is responsible for the inability of European *Rhizobium* strains (248, PRE) to nodulate Afghanistan pea whereas *Rhizobium* strains from the Middle East like strain TOM can nodulate $sym2^A$ peas as efficiently as peas lacking $sym2^A$ (Lie, 1984, Kozik et al., 1995) The extended host range of strain TOM is due to *nodX* which encodes an acetyl transferase responsible for adding an O-acetyl group at the C-6 position on the reducing sugar residue of pentameric Nod factors (Firmin et al., 1993). This suggests that $sym2^A$ is specifically involved in recognizing these NodX modified Nod factors.

Sym2 controls infection thread growth

A detailed characterization of the phenotype of $sym2^A$ peas showed that cortical cell divisions and activation of *ENOD12* gene expression are both induced by *Rhizobium* strains with and without *nodX*. However, the structural demands on Nod factors are more stringent for infection since infection thread formation is only induced incidentally by strains lacking *nodX*. In the few cases that infection was observed, the infection thread was aborted in the epidermis. Furthermore, from the temperature sensitive nature of the $sym2^A$ phenotype followed that this gene is only active during the first days of the interaction. At the permissive temperature (26°C) the nodulation effieciency of *Rhizobium* strains lacking *nodX* is markedly increased compared to nodulation at the non-permissive temperature (18°C). A period of 3 days at 26°C after inoculation equal efficient nodulation by *Rhizobium* strains with and without *nodX*. At this time the infection threads formed during the interaction with these *Rhizobium* strains were not grown beyond the root hairs (Chapter 5). Taken together the data suggest that $sym2^A$ is active in the epidermis in controling the infection process but, that it is unable to block nodulation once the infection thread has reached the cortical cells.

An interesting role was found for the bacterial NodO protein in the incompatible interaction between $sym2^A$ peas and *Rhizobium* strain 248 lacking *nodX*, since the presence of *nodO* in this strain seems to stimulates the infection process. For example, strain 248$nodO^-$ was able to nodulate $sym2^A$ peas at 26°C, but the number of succesfull infections was lower compared to the number observed in the interaction with the wild type strain 248 or strain 248*nodX*. Furthermore, on heterozygous $sym2^A sym2^C$ plants the presence of *nodO* fully compensates for Nod factor structure deficiency of strain 248, leading to the same nodulation effeciency as strain 248*nodX*, and a partial compensation is achieved with homozygous $sym2^A$ peas (Chapter 5). Previously it was shown that NodO similarly compensates for Nod factor structure deficiency, in this case for the absence of the highly unsaturated fatty acyl group, of strain 248$nodE^-$ for nodulation on pea and vetch (Economou et al., 1994). How NodO compensates for these Nod factor structure deficiency is unclear. Since NodO is a secreted protein that can integrate into membranes forming ion channels it is possible that it may do so in the host plasmamembrane where it enhances, under the control of a plant protein, the Nod factor induced signal needed for infection thread growth.

Our studies on *sym2* 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 (Chapter 5). Similar results have been obtained by using *R. meliloti nod* mutants (Ardourel et al., 1994). Hence, studies on plant genes as well as *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. Given that *sym2*^{A} controls infection and requires specific Nod factors, the question arises whether *sym2*^{A} encodes the postulated uptake receptor in the model of Ardourel et al. (1994). There is evidence that suggests this is not the case. First, if *sym2*^{A} is an uptake receptor this would imply that heterozygous *sym2*^{A}*sym2*^{C} peas can be nodulated by *Rhizobium* strains lacking *nodX* (strain PRE, 248*nodO*$^-$), which is not the case. Second, upon spot-inoculation of *Rhizobium* strains 248 and 248*nodO*$^-$ infection threads do incidentally form in the roots of homozygous *sym2*^{A} peas, but their growth is stopped in the epidermis. In contrast, the few infection threads induced by the *Rhizobium meliloti nodFE*$^-$ or *nodL*$^-$ mutants do not stop, but continue growth, which has led to the hypothesis that the entry receptor is only involved in infection site formation. Therefore, it is unlikely that Sym2^A represents the proposed entry receptor and it is more probable that *sym2*^{A} is involved in a mechanism controlling the infection thread growth.

A model can be proposed where *sym2*^{A} encodes a Nod factor receptor, because of its relation to a specific Nod factor structure, which regulates infection thread growth in the epidermis. In the unbound form Sym2^A would repress infection thread growth, while upon binding of the NodX modified Nod factor the block is lifted and infection can proceed. A certain amount of infection threads can escape this repression mechanism which is increased by the presence of *nodO* in the inoculated *Rhizobium* strain. The occurence of a similar receptor in cultivated peas can be proposed, i.e. Sym2^C, to explain the nodulation behavior of heterozygous *sym2*^{A}*sym2*^{C} plants. For inactivating the controler function of the Sym2^C receptor Nod factor with a highly unsaturated C18:4 fatty acyl chain would then be required. Unlike *Rhizobium* strains with *nodX* that produce both the NodX acetylated and non-acetylated factors enabling the inactivation of Sym2^A and Sym2^C, strains without *nodX* can only inactivate Sym2^C, but not Sym2^A, whereby the inhibition of infection evenso leads to inefficient nodulation. The occurence of NodO during the infection of these heterozygous plants might be more effective if only half of the Sym2 receptors is Sym2^A resulting in more infections to such an extent that equal nodulation efficiency is observed for strains with or without *nodX*. This fits with the experimental data (Chapter 5). Finally, the presence of a receptor controling infection implicates the presence of a second receptor responsible for the induction of infection. Similar to the model of Ardourel et al. (1994) this receptor may also be involved in induction of root hair deformation and gene expression in the epidermis. Although this model may explain all observed phenotypes of *sym2*^{A} peas, the cloning of the *sym2*^{A} gene will give the final answer whether it encodes a Nod factor receptor.

Samenvatting

Rhizobium bacteriën zijn in staat de wortels van vlinderbloemigen binnen te dringen en daar de vorming van een nieuw orgaan, de wortelknol, te induceren. Deze symbiontische interactie begint met wederzijdse herkenning waarna de bacteriën aan de wortelharen hechten. De wortelharen deformeren/krullen waarbij de bacteriën worden ingesloten en de bacteriën dringen de wortel binnen door middel van een infectie draad die door de wortelhaar naar binnen groeit richting de cortex. Tegelijkertijd worden cellen in de cortex geactiveerd en tot delen aangezet wat leidt tot de vorming van het knolprimordium. De infectiedraad groeit richting dit primordium en daar aangekomen infecteren de bacteriën de gastheercellen. Het primordium groeit vervolgens uit tot een knol en de bacteriën differentieren tot endosymbiontische bacteroïden. In de wortelknol worden de bacteroïden gehuisvest in omstandigheden die ideaal zijn voor het omzetten van atmosferische stikstof in ammonia, dat de plant vervolgens gebruikt voor zijn groei. Bij alle stappen van de knolvorming zijn specifieke planteneiwitten betrokken waarvan de informatie is opgeslagen in stukjes van het DNA die noduline genen worden genoemd.

De interactie tussen rhizobia en hun gastheer planten begint met het uitwisselen van signaalmoleculen. Door de plant worden flavonoïden uitgescheiden die de transcriptie van bacteriele nodulatie genen (nod genen) induceren. De eiwitten, die worden gecodeerd door deze genen, zijn betrokken bij de synthese van specifieke lipo-oligosacchariden of Nod factoren, de signaalmoleculen die worden gemaakt en uitgescheiden door *Rhizobium* bacteriën. De Nod factoren zijn essentieel voor het induceren van de eerste stappen van de interactie tussen rhizobia en hun gastheer, te weten; wortelhaardeformatie, genexpressie, infectie en knolprimordium formatie.

Niet iedere *Rhizobium* bacterie is in staat een symbiose aan te gaan met elke vlinderbloemige plant. Er bestaat een strikte gastheerspecificiteit wat betekent dat *Rhizobium leguminosarum* biovarieteit *viciae* bacteriën alleen knolvorming induceren op planten van de genera *Pisum* (erwt), *Vicia* (wikke), *Lathyrus* (siererwt) en *Lens*. Terwijl andere *Rhizobium* varieteiten, zoals *Rhizobium leguminosarum* bv *trifolii* alleen met *Trifolium* (klaver) knollen maken en *Rhizobium meliloti* alleen met *Melilotus* (alfalfa). Deze gastheerspecificiteit wordt mede bepaald door de structuur van de Nod factoren zoals die gemaakt worden door rhizobia.

Een aantal reacties van de wortel - zoals wortelhaardeformatie, genexpressie en inductie van knolprimodia - wordt ook geïnduceerd door gezuiverde Nod factoren alleen, waarbij de aanwezigheid van *Rhizobium* bacteriën niet nodig is. Hoe Nod factoren de verschillende wortel reacties induceren en hoe de perceptie van Nod factoren in z'n werk gaat is echter niet duidelijk. Experimenten om deze vragen te beantwoorden en meer inzicht te krijgen in de processen zelf zijn de basis van dit proefschrift.

Wortelhaardeformatie als systeem om de werking van Nod factoren te bestuderen

Het begin van de knolvorming kan alleen goed bestudeerd worden met planten waarbij onderzocht kan worden met welke moleculaire reacties de morfologische reacties geïnduceerd door Nod factoren gepaard gaan. Wij hebben gekozen om bij *Vicia sativa* (wikke) plantjes te kijken naar wortelhaardeformatie die kan worden geïnduceerd door Nod factoren van *Rhizobium leguminosarum* bv *viciae*. Na kieming van de *Vicia* plantjes worden de wortels verder gegroeid tussen twee glaasjes (zgn. Fåhraeus glaasjes) met een beetje medium. Door het medium te vervangen met medium waarin Nod factoren zitten wordt wortelhaardeformatie geïnduceerd. Wortelhaardeformatie wordt gekarakteriseerd door het zwellen en opnieuw uitgroeien van de wortelhaartip. Het voordeel van de Fahraeus glaasjes is dat ze onder de microscoop bekeken kunnen worden zodat het deformatie proces op de voet gevolgd kan worden en we hebben het hele proces dan ook op video vastgelegd. Dat filmen was geen probleem want wortelhaardeformatie wordt al zichtbaar na een uur en binnen 3 uur na toevoeging van Nod factoren zijn bijna alle wortelharen in de gevoelige zone gedeformeerd (Hoofdstuk 2).

Zoals al aangegeven zijn niet alle haren in staat tot deformatie, alleen de haren die bijna zijn gestopt met groeien zijn gevoelig voor Nod factoren, en niet de groeiende of de volwassen haren. Daar deze deformatie test zeer reproduceerbaar is kan nauwkeurig voorspeld worden welke haren zullen deformeren. Naast het feit dat de test simpel en snel is, is wortelhaardeformatie bovendien zeer gevoelig wat blijkt uit het feit dat Nod factoren al in picomolaire concentraties deformatie kunnen induceren. Zelfs de aanwezigheid van Nod factoren voor maar 5 minuten is al genoeg om deformatie op gang te brengen (Hoofdstuk 2). Aangezien Nod factoren bovendien snel worden afgebroken in aanwezigheid van de plant en deze afbraakprodukten niet of nauwelijks actief zijn is het waarschijnlijk dat de Nod factoren binden aan eiwitten of receptoren die het signaal doorgeven en op die manier wortelhaardeformatie en andere wortelreacties induceren.

Genexpressie tijdens wortelhaardeformatie

De wortelhaardeformatie geeft al aan dat Nod factoren snel werken, daarom is de deformatie test is ook gebruikt als basis voor het zoeken naar genen die in een vroeg stadium, binnen een uur, tot expressie worden gebracht door Nod factoren. Hiervoor is de differential RNA display methode gebruikt. Deze methode berust op het zichtbaar maken van de aanwezige boodschapper RNAs (messengerRNA), de tussenstap van DNA op weg naar het maken van eiwitten. Omdat deze mRNAs in heel kleine hoeveelheden voorkomen worden van de mRNAs van onbehandelde en met Nod factor behandelde worteltjes weer DNA (complementaryDNA) gemaakt, waarna deze cDNA poelen worden geamplificeerd met PCR (polymerase ketting

reactie). Met behulp van PCR wordt zoveel DNA gemaakt als nodig is waarna het op grootte van elkaar wordt gescheiden op een gel. Door het patroon van beide cDNA poelen te vergelijken kunnen de aanwezige verschillen, genen die geïnduceerd zijn door Nod factoren, vervolgens uit de gel gehaald worden.

Op deze manier is een leghemoglobine (Lb) cDNA geisoleerd, een gen waarvan gedacht was dat het alleen in de knol tot expressie kwam en waarvan nu blijkt dat het al binnen een uur in de wortel wordt geïnduceerd door Nod factoren (Hoofdstuk 3). De functie van het Lb eiwit in de knol is, vergelijkbaar met hemoglobine in bloed, het transporteren van zuurstof in dit geval naar de bacteroïden die druk doende zijn met het binden van stikstof. Mogelijk heeft het eiwit dat zo vroeg al in de wortel aanwezig is eenzelfde functie maar dan om de actieve wortelcellen, waarin allerlei processen zijn geïnduceerd, van zuurstof te voorzien.

Ondanks dat het *Lb* gen binnen een uur wordt geïnduceerd is voor de inductie nog steeds de novo eiwitsynthese nodig wat betekent dat er nog eerder genen worden geïnduceerd door Nod factoren (Hoofdstuk 3). Daarom zou dezelfde differential display methode kunnen worden gebruikt om te proberen deze genen op te pikken door al 10 minuten of een half uur na toevoeging van Nod factoren het RNA te isoleren en te analyseren.

In aanwezigheid van nitraat in de bodem hebben vlinderbloemigen geen behoefte aan een symbiose en treedt er dan ook geen wortelhaardeformatie en knolvorming op. Juist omdat het *Lb* gen zo snel wordt geïnduceerd kan het worden gebruikt om te kijken of planten in aanwezigheid van nitraat nog wel ontvankelijk zijn voor Nod factoren. Het blijkt dat nitraat weliswaar de knolvorming remt, maar ondanks dat er geen deformatie optreedt wordt het *Lb* gen toch geïnduceerd door Nod factoren (Hoofdstuk 3). De manier waarop nitraat knolvorming remt blijft dus een raadsel maar wel is duidelijk dat het de signaal transductie van Nod factoren niet aantast.

De rol van ethyleen in de knolvorming

Het is al gezegd dat Nod factoren wortelhaardeformatie induceren door het opnieuw initieren van groei van de wortelhaartip van gevoelige wortelharen. Dit mechanisme van groei inductie doet sterk denken aan het ontstaan van wortelharen in de epidermis van de wortel waarbij tipgroei wordt geïnduceerd door het plantenhormoon ethyleen. Ethyleen zou aldus aangewend kunnen worden door Nod factoren om tipgroei te induceren. Een ander effect van Nod factoren is dat ze cellen in cortex van de wortel aanzetten tot deling waardoor een knolprimordium wordt gevormd. Echter, ethyleen is een effectieve remmer van de vorming van knolprimordia. Zelfs de hoeveelheid ethyleen die door de plant zelf wordt geproduceerd remt knolvorming zodanig dat er in aanwezigheid van ethyleenremmers twee maal zoveel knollen gemaakt kunnen worden. Dit betekent dat wanneer ethyleen een onderdeel zou zijn in de signaal transductie van Nod factoren, dat er een paradox bestaat wat betreft de functie van ethyleen tijdens de knolvorming.

Deze paradox is er niet want door gebruik van ethyleenremmers blijkt dat hoewel het vormen en de groei van wortelharen afhankelijk is van ethyleen, wortelhaardeformatie dit niet is (Hoofdstuk 4). Desalnietemin is het interessant om te onderzoeken hoe en waar ethyleen de knolvorming remt. Daarom is de plaats waar ethyleen vermoedelijk gemaakt wordt in de wortel bepaald door het lokaliseren van ACC oxidase mRNA (het enzym voor laatste stap in de ethyleen biosynthese). Het blijkt dat dit mRNA zich ophoopt in de cellagen tegenover het floeem in de wortel (Hoofdstuk 4). Nu is het zo dat knolprimordia gewoonlijk gevormd worden tegenover de xyleem polen. Deze informatie gecombineerd met de remmende werking van ethyleen op de vorming van knolprimordia geeft aan dat ethyleen wel eens betrokken zou kunnen zijn bij het bepalen van de plaats waar deze primordia gevormd worden. Al eerder was aangetoond dat uridine (of stelefactor), geisoleerd uit de stele van de wortel, overal celdeling kan induceren in wortelplakjes (explanten) zonder stele, maar wanneer de stele aanwezig is worden alleen primordia gevormd tegenover de xyleem polen. Door erwten in aanwezigheid van ethyleenremmers te groeien om zodoende de positie informatie die ethyleen geeft (ten dele) uit te schakelen blijkt dat meer knollen tegenover de floeem polen worden gevormd (Hoofdstuk 4). Dit betekent dat er positieve (bijv. stelefactor) en negatieve (bijv. ethyleen) factoren zijn die informatie geven en bepalen op welke plaatsen in de wortel een knol wordt gevormd.

Op zoek naar een Nod factor receptor

Omdat Nod factoren actief zijn in zeer lage concentraties is al aangegeven dat er waarschijlijk receptoren betrokken zijn bij het doorgeven van de Nod factor signalen. Uit, onder andere, experimenten met alflalfa en *Rhizobium meliloti* mutanten bleek dat voor infectie stringentere eisen aan de Nod factor structuur werden gesteld dan voor wortelhaardeformatie of genexpressie. Mede hierom werd gepostuleerd dat er meerdere Nod factor receptoren in de epidermis aanwezig zijn. Ook in de erwt is deze korrelatie gevonden. Een methode om zo'n receptor te pakken te krijgen is door bindingsproeven met gemerkte Nod factoren uit te voeren en zo de receptor op te zuiveren. Het alternatief is om van de plant mutanten te analyseren waarbij deze mutanten preferentieel verstoord moeten zijn tijdens de vroege interactie met de bacterie. Het liefst moet het fenotype van deze mutant (wel of geen knollen) dan ook nog gekorreleerd zijn aan een specifieke Nod factor struktuur, want dat is een aanwijzing dat de plant gestoord is in de perceptie van deze Nod factoren.

Bij de erwt is er een wilde soort afkomstig uit Afghanistan die zo'n fenotype bezit en het hiervoor verantwoordelijke gen is *sym2*. Erwten met het $sym2^{Afghanistan}$ gen kunnen alleen knollen maken wanneer de bijbehorende *Rhizobium* bacterie het

nodX gen bezit. Dit gen is er verantwoordelijk voor dat de bacterie specifieke Nod factoren kan maken met een extra acetaat groep op een specifieke plaats aan het reducerende uiteinde van de Nod factor. Met andere woorden, de aanwezigheid van *sym2*^{A} is gekorreleerd met een Nod factor struktuur nodig voor knolvorming wat kan betekenen dat het *sym2*^{A} eiwit betrokken is bij de perceptie van Nod factoren. Een alternatief is de mogelijkheid dat dit eiwit een chitinase is dat er voor zorgt dat Nod factoren die zonder extra acetaat groep snel worden afgebroken zodat de bacterie geen interactie aan kan gaan met *sym2*^{A} planten. Dit blijkt echter niet waarschijnlijk te zijn omdat deze Nod factoren door erwten met of zonder *sym2*^{A} even snel worden afgebroken (Hoofdstuk 5).

Om iets te kunnen zeggen over de functie van het *sym2*^{A} eiwit moet het fenotype van erwten met dit gen nauwkeurig worden bepaald. Dat Nod factor perceptie complex is blijkt namelijk al uit het feit dat een ander bacterieel nod gen, *nodO*, het ontbreken van een specifieke Nod factor struktuur kan compenseren waardoor bijvoorbeeld een bacterie zonder *nodX* toch knollen kan maken op *sym2*^{A} erwten. Op heterozygote erwten, die maar een kopie van het *sym2*^{A} gen bevatten, compenseert de aanwezigheid van *nodO* zelfs zodanig dat evenveel knollen worden gemaakt als met bacteriën die wel *nodX* bevatten (Hoofdstuk 5).

Nu blijkt dat voornamelijk de infectie van *Rhizobium* bacteriën zonder *nodX* verstoord is op *sym2*^{A} erwten, terwijl expressie van het noduline gen *ENOD12* in de epidermis even effectief wordt geïnduceerd door bacteriën met of zonder *nodX*. Er vinden minder infecties plaats en als ze er zijn stoppen ze in de epidermis. Bovendien blijkt dat als deze planten drie dagen bij hoge temperatuur worden gegroeid dat er wel knollen gemaakt kunnen worden door bacteriën zonder *nodX*. Op dit moment waren de infectiedraden de wortelharen nog niet gepasseerd (Hoofdstuk 5). Deze gegevens wijzen erop dat het Sym2^A eiwit actief is in de epidermis waar het de infectie controleert, maar dat het de knolvorming niet kan stoppen zodra de infectiedraad de corticale cellen heeft bereikt. Bovendien blijkt dat, net als in alfalfa, ook in *sym2*^{A} bevattende erwten stringentere eisen aan de Nod factor struktuur worden gesteld voor infectie dan voor genexpressie. Geheel naar verwachting blijkt dat de aanwezigheid van het *nodO* gen in bacteriën zonder *nodX* de infectie te stimuleren en op die manier te compenseren voor het ontbreken van een specifieke Nod factor struktuur (Hoofdstuk 5).

Een model kan gepostuleerd worden waarbij sym2^A codeert voor een Nod factor receptor die de infectie in de epidermis controleert en blokkeert. Alleen als NodX gemodificeerde Nod factoren binden aan de Sym2^A receptor wordt de blokkade opgeheven en kan infectie en knolvorming plaatsvinden. Een aantal infecties ontsnapt aan deze controle als het gaat om *nodO* bevattende bacteriën. Tenslotte kan uit de aanwezigheid van een receptor die infectie controleert worden afgeleidt dat er ook een receptor moet zijn die infectie induceert. Alhoewel het fenotype van *sym2*^{A} erwten met dit model kan worden verklaard zal de klonering van het *sym2*^{A} gen uitwijzen of het werkelijk codeert voor een Nod factor receptor.

Curriculum vitae

Op 31 augustus 1966 ben ik in Zwaagwesteinde (Fr) geboren. Na vier jaar gemeentelijke MAVO te Kollum, behaalde ik in 1985 the eindexamen Atheneum aan de Rijksscholengemeenschap te Leeuwarden. Datzelfde jaar ben ik begonnen met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. De ingenieursstudie werd in november 1990 afgesloten met als hoofdvakken moleculaire biologie (prof. dr. A. van Kammen), proceskunde (prof. dr. J. Tramper) en een stage voor de vakgroep Molculaire Biologie uitgevoerd in het Department of Biological Sciences van de University of Warwick te Coventry, UK (dr. D.A. Hodgson). Vanaf december 1990 was ik als assistent in opleiding en vervolgens als onderzoeksmedewerker verbonden aan de Landbouwuniversiteit Wageningen. Het in dit proefschrift beschreven onderzoek is uitgevoerd onder leiding van dr. T. Bisseling en prof. dr. A. van Kammen bij vakgroep Moleculaire Biologie en gefinancierd door een Pionier subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) toegekend aan dr. T. Bisseling. Sinds 1 januari 1997 ben ik werkzaam als onderzoeksmedewerker (post-doc) bij de vakgroep Moleculaire Celbiologie van de Universiteit Utrecht.

Nawoord

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