A GENETIC APPROACH TO STUDY RHIZOBIAL NOD FACTOR AND MYCORRHIZAL FUNGI ACTIVATED SIGNALING

René Geurts

Promotor: dr. T. Bisseling, hoogleraar in de moleculaire biologie.

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A genetic approach to study rhizobial Nod factor and Mycorrhizal fungi activated signaling

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Niet de initiatie, maar de regulatie van infectiedraadgroei is het meest afhankelijk van de structuur van de door *Rhizobium* geproduceerde Nod-factor.
Ardourel *et al.* (1994) *Plant Cell* **6**: 1357-1374, *Dit Proefschrift*.

Het beter karakteriseren van *sym18* erwtenmutanten maakt het mogelijk aan te tonen of, naast *SYM2*^{A} bevattende erwtenlijnen, ook gecultiveerde erwten preferentieel door NodX geacetyleerde Nod-factoren herkennen.
LaRue *et al.* (1996) *Plant Soil* **180**: 191-195.

Het experiment beschreven door Temnykh *et al.* (1995) om vast te stellen of *SYM2*^{A} en *nod3-1* allelisch zijn, is ondeugdelijk door het niet gebruiken van flankerende merkers.
Temnykh *et al.* (1995) *Pisum Genet.* **27**: 26-28.

Het kloneren van fenotypisch gekarakteriseerde genen in plantensoorten met een relatief groot genoom kan worden vereenvoudigd door gebruik te maken van eventuele 'synteny' met een 'modelplant'.

In tegenstelling tot wat Barker *et al.* (1998) beweren, is niet alleen de tomaten RMC mutant, maar zijn ook de vlinderbloemige mutanten met een vergelijkbaar Myc$^-$-fenotype, waarschijnlijk verstoord in een mechanisme dat algemeen is voor mycorrhiza-gastheerplanten.
Barker *et al.* (1998) *Plant J.* **15**:791-797.

De eenzijdige investeringen door de Nederlandse overheid in conventionele infrastructuur in plaats van in een elektronische snelweg, laten zien dat politici niet alleen met hun wortels, maar helaas ook met hun visie zijn verankerd in de 20e eeuw.

'Nederland Brainport' moet meer inhouden dan het in elk klaslokaal plaatsen van een door het bedrijfsleven afgedankte computer.

De grote eenvormigheid van politieke partijen is het gevolg van het overmatig gebruik van advies- en reclamebureaus.

De factor geluk, die een niet te onderschatten rol speelt in de wetenschap, is niet te beïnvloeden.

Stellingen behorende bij het proefschrift:
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door ***René Geurts***,
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Contents

OUTLINE

Leguminous plants are able to interact with bacteria of the genera *Azorhizobium* , *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium*. This is a symbiotic interaction that results in the formation of a complete new organ, the root nodule. In these nodules the bacteria are hosted intracellularly and there they find the proper environment to reduce atmospheric nitrogen into ammonia, a source of nitrogen that can be used by the plant.

Root nodule formation involves growth responses in the epidermis as well as cortex of the root. This implies that the bacteria redirect the development of fully differentiated plant cells. The bacterial signals that set this in motion are the so-called nodulation (Nod) factors. Nod factors of the different *Rhizobium* species have a common basic structure; a β-1,4-linked N-acyl-D-glucosamine backbone of mostly 4 or 5 units, containing a fatty acid at the non-reducing terminal sugar. Furthermore, several species-specific decorations can be present at both terminal glucosamine residues and also the structure of the fatty acyl chain can vary. These substitutions play an important role in the host-specificity of the symbiosis.

Nod factors are active at low concentrations and their activity depends on their structure. This implicates that Nod factors are perceived by receptor(s). However, it is unclear how Nod factors are perceived, and how the signals are transduced. The aim of this thesis is to unravel Nod factor perception and transduction mechanisms by using a genetic approach. Furthermore, it is studied whether mycorrhizal fungi and rhizobia use similar mechanisms to establish an endosymbiotic relationship. This can provide insight in the phylogenetic origin of the legume mechanism controlling nodulation.

Leguminous plants mutated in a gene encoding a key component of the Nod factor perception or transduction pathway will not respond to Nod factors. In *CHAPTER 2*, we describe a pea mutant, Sparkle-R25, which shows such phenotype. Sparkle-R25 is mutated in the *SYM8* gene. We demonstrate that rhizobial Nod factors are unable to trigger the early nodulin genes *PsENOD5* and *PsENOD12A* , whereas in wild type pea they do. This shows that SYM8 is required for the induction of both genes by Nod factors. Besides that Sparkle-R25 does not respond to Nod factors, it is also unable to establish a mycorrhizal symbiosis, showing that SYM8 is also involved in this endosymbiotic interaction. We demonstrate that mycorrhizal fungi are as well able to trigger the expression of both early nodulin genes in wild type peas, but are unable to do so in Sparkle-R25. This indicates that mycorrhizal signals activate a signal transduction cascade sharing SYM8 in common with a Nod factor induced signal transduction cascade.

The studies in *Chapter 3, 4, 5 & 6* are focused on the *SYM2* gene. *SYM2* is first identified in the pea ecotype Afghanistan ($SYM2^A$), where it inhibits nodulation by *Rhizobium* strains secreting only Nod factors without a specific substitution at the reducing terminal sugar residue. In *Chapter 4* we show that these specific substitutions can either be an acetyl or a fucosyl group. SYM2 is specifically involved in the infection process (*Chapter 3*). In order to study the mode of action of *SYM2* a suppressor mutant has been isolated by mutagenizing a $SYM2^A$ harboring pea line (*Chapter 5*). Bacteria that do not produce properly substituted Nod factors are blocked in their ability to trigger infection thread formation on $SYM2^A$ harboring peas, whereas other Nod factor controlled responses occur normally. Genetic analysis showed that SYM2 plays a role in controlling infection thread growth and its activity depends on Nod factor structure. This suggests that SYM2 is involved in a Nod factor recognition mechanism. In *Chapter 6* the first steps towards the cloning of the *SYM2* gene are described. By using differential RNA display several root hair cDNAs are identified which a genetically linked to the *SYM2* locus. One of these clones, encoding a putative receptor kinase, shows tight linkage to *SYM2*. This clone can serve as marker for further research to clone *SYM2*.

In the Discussion of this theses (*Chapter 7*) the common aspects between the rhizobial and the mycorrhizal symbiosis are described and it is discussed whether mycorrhizal fungi and rhizobia use similar mechanisms to establish an endosymbiotic relationship.

INTRODUCTION

René Geurts and Ton Bisseling

INTRODUCTION

In addition to 'classical' hormones like auxin and cytokinin, several other growth factors play a role in plant development. Examples of such signals are (small) peptides, brassinosteroids and oligosaccharidebased signals. Members belongingto the latter group are the lipo-chitin oligosaccharides(LCOs) based nodulation (Nod) factors of *Rhizobium* species, which trigger developmental processes at their leguminous host plants. Also, there are several indications that oligosaccharide based signals play a more general role in plant development. For example in embryogeneses of the brown algae *Fucus spiralis*, the cell wall has been shown to be a source of signals that determine cell fate (Berger *et al.*, 1994). Furthermore, by using a bio-assay, in which the developmental fate of epidermal cells of tobacco (*Nicotina tobaccum*) leaves can be monitored, it has been shown that certain products derived from hydrolysed plant cell walls can change the developmental fate of cells (Marfà *et al.*, 1991). However, only the Rhizobial Nod factors have been studied in more detail.

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Azorhizobium* (here collectively called rhizobia) secrete LCOs, called Nod factors, which play a pivotal role in the symbiotic interaction of these bacteria and their leguminous host plants. A striking characteristic of this symbiosis is its host specific nature; a particular *Rhizobium* species can only nodulate a limited number of leguminous plant species. Nod factors play a major role in this host specificity. Furthermore, Nod factors trigger the processes leading to the development of a nodule. The first morphological changes induced by Nod factors occur at the epidermis where root hairs deform. Some of these root hairs form a shepherd's crook like curl by which the bacteria become entrapped and where an infection site is created. At these sites an inward-growing tubular structure, the infection thread, is formed by which the bacteria enter the plant. Concomitantly, Nod factors mitotically activate cortical cells, and these dividing cells will give rise to a nodule primordium. The infection threads grow toward these primordia. Subsequently, bacteria are released from the infection thread into the cytoplasm of the primordial cells and become surrounded by a plant-derived peribacteroid membrane. Then, the nodule primordium develops into a mature nodule, while the bacteria differentiate into their endosymbiotic form, the bacteroids. These bacteroids are able to reduce nitrogen into ammonia, which can subsequently be utilized by the plant. In the following paragraphs the biosynthesis of Rhizobial Nod factors and the processes they induce in legume roots are described in more detail.

NODULATION FACTORS

The rhizobial genes encoding the enzymes involved in the biosynthesis of Nod factors are silent when the bacteria do not grow in the vicinity of the root system of their host plants. There, rhizobia sense signals molecules secreted by the host. This are in general flavonoids. These molecules activate the transcriptional regulator(s) NodD. Activation of NodD leads to the transcription of the other bacterial nodulation (*nod*) genes, whose encoded proteins are responsible for the biosynthesis and secretion of Nod factors.

Nod factors made by the different *Rhizobium* species all have a similar basic structure; a ß-1,4-linked N-acyl-D-glucosamine backbone of mostly 4 to 5 units long, containing a fatty acid at the non-reducing terminal sugar. Both, genetic and biochemical studies have shown that the synthesis of this core structure is catalyzed by the NodA, NodB and NodC proteins. NodC is an N-acetylglucosaminyl-transferase and catalyzes the synthesis of the chitin oligomer and controls the length of this backbone (Geremia *et al.*, 1994; Spaink *et al.*, 1994; Kamst *et al.*, 1997). The terminal non-reducing glucosamine unit of this oligomer is deacetylated by NodB (John *et al.*, 1993), and subsequently substituted with an acyl chain by NodA (Atkinson *et al.*, 1994; Röhrig *et al.*, 1994).

Besides the length of the glucosamine backbone (Roche *et al.*, 1996), the biological activity of Nod factors is determined by certain substitutions at the terminal sugar residues as well as the nature of the acyl chain. Several other *nod* genes are responsible for these substitutions. As an example, the major Nod factors produced by *Sinorhizobium meliloti* and *Rhizobium leguminosarum* biovar *viciae* respectively, are described (For all Nod factors produced by these two species see table 1). The major factor produced by *R. meliloti* contains 4 glucosamine units, an acyl chain of 16 C-atoms in length with two unsaturated bounds, an acetyl group at the non-reducing and a sulfate group at the reducing terminal sugar residue (figure 1, Lerouge *et al.*, 1990; Schultze *et al.*, 1992). The sulfotransferase encoding gene *nodH* is responsible for the sulfation of the reducing end (Roche *et al.*, 1991a,b) whereas the NodL protein, which is an *O*-acetyltranferase, acetylates the non reducing end of the glucosamine backbone (Downie *et al.*, 1989, Spaink *et al.*, 1991, Bloemberg *et al.*, 1994).

Table 1. An overview of the different Nod factors produced by *S. meliloti* and *R. leguminosarum* bv *viciae*. The double bounds at position 2, 4 and 6 in the acyl chains are in *trans* conformation, whereas the double bounds at postion 9 or 11 or are in *cis* conformation. The decorations at the terminal glucosamine residues are present at postion 6. In between brackets the specifically required nod gene(s) is given.

Rhizobium specie	acyl chain	non-reducing sugar moiety	reducing sugar moiety	number of glucosamine units	reference
R. leguminosarum bv *vicae*	-C18:4 ($\Delta_{2,4,6,11}$) (*nodE*) -C18:1 (Δ_{11}) -C18:0 -C16:1 (Δ_9) -C16:0	O-acetyled (nodL)	-H and also O-acetylated pentamers if the strain harbors nodX	4,5	Spaink *et al.* (1991) Firmin *et al.* (1993) Bloemberg *et al.* (1994) Spaink *et al.* (1995)
S. meliloti	-C16:3 ($\Delta_{2,4,9}$) -C16:2 ($\Delta_{2,9}$) (both *nodE*) -C16:1 (Δ_9) -C18 to C26 (_-1)-hydroxylated (*nodD*$_3$, *syrM*)	H or O-acetylated (nodL)	O-sulphated (nodH, nodP,nodQ)	3,4,5	Lerouge *et al.* (1990) Roche *et al.* (1991a,b) Schultze *et al.* (1992) Demont *et al.* (1993)

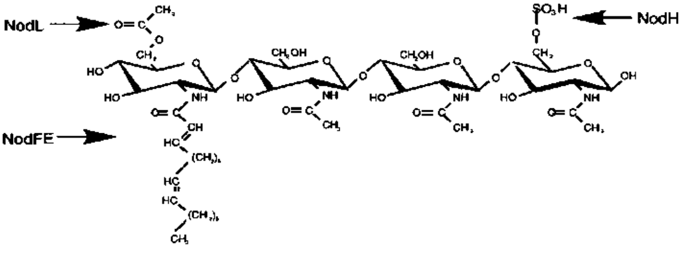
For the specific structure of the acyl chain two genes are responsible; *nodE* and *nodF*, encoding a β-ketoacyl transferase and an acyl carrier protein respectively (Demont *et al.*, 1993). In contrast to *S. meliloti*, *R. leguminosarum* bv *viciae* produces a mixture of factors that contains several major compounds. Depending on whether the bacterium contains the *nodX* gene either 4 or 6 major Nod factors are formed. The length of the glucosamine backbone is 4 or 5 units carrying an acyl chain of 18 C-atoms either with 1 or 4 unsaturated bounds, for which again *nodE* and *nodF* are responsible. These four Nod factors are *O*-acetylated at the non-reducing terminal sugar residue, which is nodL dependent (Spaink *et al.*, 1991). When the bacterium contains the *nodX* gene, encoding an *O*-acetyltransferase, the reducing terminal sugar residue of pentameric Nod factors are partially acetylated, whereas in the absence of this gene no substitution is present (Firmin *et al.*, 1993).

NOD FACTOR INDUCED EPIDERMAL RESPONSES

Nod factors trigger several responses in the root epidermis of their host plant, ranging from very fast physiological responses that occur within minutes, to morphological changes in the root hairs that become apparent after hours or days. In addition, symbiosis specific plant genes are induced. By analyzing where the various responses are induced, it has been determined which root epidermal cells are susceptible to Nod factors. The most clear data have been obtained with transgenic *Medicago* plants carrying the promoter of the Nod factor inducible *ENOD12* gene in front of the ß-glucuronidase (*GUS*) reporter gene. *ENOD12* encodes a proline rich protein with unknown function, which expression is induced in the root epidermal cells within hours after application of Nod factors (Scheres *et al.*, 1990a,b; Horvath *et al.*, 1993; Journet *et al.*, 1994). Later, when cortical cells start to divide, the gene is also expressed in these dividing cells (Pichon *et al.*, 1992). Studies with transgenic *Medicago ENOD12-GUS* plants have shown that the susceptible region of the epidermis starts just above the root tip, where root hairs have not yet emerged and extends to the area that contains mature root hairs (Pichon *et al.*, 1992; Journet *et al.*, 1994). In this susceptible region expression of *ENOD12* in reaction on the presence of Nod factor is independent of the presence of a root hair. Both, trichoblasts as well as atrichoblasts in the susceptible zone express *ENOD12* at similar levels. However, only cells that are in direct contact with Nod factor respond, indicating that *ENOD12* induction is a cell autonomous response (Journet *et al.*, 1994).

Morphological responses

Nod factor-secreting bacteria as well as purified Nod factors induce morphological changes in root hairs. By using bioassays some insight in the mechanism by which Nod factors alter the growth pattern of root hairs has been obtained. Such studies have most extensively been done with vetch (*Vicia sativa*). Vetch root hairs that deform after Nod factor application have almost terminated growth, whereas the younger active growing hairs, as well as the mature hairs do not respond (Heidstra *et al.*, 1994). Growth termination is accompanied by the disappearance of the cytoplasmic dense region as well as the gradient of free calcium at the tip of the hair, which both are typical for tip growing cells (De Ruijter *et al.*, 1998). Upon application of Nod factor, the tips of these susceptible root hairs swell. The swelling of the root hair tip is accompanied by the formation of a calcium gradient at the plasmamembrane and it requires protein synthesis (Vijn *et al.*, 1995; De Ruijter *et al.*, 1998). Therefore, swelling of the root hair is the result of growth. However, since polarity is lacking it is isotropic instead of polar growth. After polarity is established the isotropic growth turns into tip growth. A tip growing tube emerges, which shows a strong resemblance with normal root hair growth.



Response	sulfate	acetate	unsaturated bounds acyl chain	references
membrane depolarization	+	-	-	Felle *et al.* (1995) Kurkdjian (1995)
cytoplasmic alkalanization	-	-	-	Felle *et al.* (1996)
Root hair deformation	+	-	-	Roche *et al.* (1991a,b)
ENOD12 induction	+	-	-	Journet *et al.* (1994)
Infection thread formation	+	+	+	Ardourel *et al.* (1994)

Figure 1. Structural requirements to Nod factor structure of *S. meliloti* to trigger responses on alfalfa. Also the responsible Nod proteins responsible for the specific structural decorations are given.

Nod factors induce new growth of root hairs leading to a deformed phenotype of the hair. However, generally only when bacteria are present, shepherd's crook like curling occurs in some root hairs, showing that additional information coming from the bacteria is required to guide the growth of the hair. *Rhizobium* requires the micro environment of a curled root hair to establish an infection site. In the curl of the hair the plant cell wall becomes locally degraded, followed by uptake of the bacteria via invagination of the root hair plasma-membrane. Vesicles in the root hair cell are directed to the tip of the invagination leading to the formation of an inward tip growing tubular structure, the infection thread. Despite the fact that Nod factors are insufficient to trigger root hair curling, they play a crucial role in the infection process, since infections are only initiated when the bacteria secrete specific Nod factors (Ardourel *et al.*, 1994).

Physiological responses

Several physiological changes are induced within a short time period after Nod factor application. Most of these studies have been performed on alfalfa (*Medicago sativa*) root hairs. Therefore the physiological responses induced in this host plant are summarized. Application of Nod factor causes immediately a decrease of the calcium concentration in the environment between the root hairs, which is probably due to an influx of calcium into the hairs (Felle *et al.*, 1998). Shortly after the induction of this calcium flux, an opposite directed flux of chloride ions occurs, which is accompanied by a depolarization of the root hair membrane (Ehrhardt *et al.*, 1992, Felle *et. al.*, 1995; Kurkdjian, 1995; Felle *et al.*, 1998). This depolarization is thought to be a consequence of the rapid release of chloride ions by the root hairs. Since for chloride ions there is a steep outwardly directed electrochemical gradient, they rapidly leave the root hair cytoplasm which could cause a temporary short-circuiting of the proton pumps leading to depolarization of the membrane (Felle *et al.*, 1998). Depolarization of the root hair membrane is Nod factor concentration dependent and occurs transiently. The depolarization is probably stopped due to charge balancing potassium fluxes whereas repolarization could occur by the activity of proton pumps (Felle *et al.*, 1995; 1998). The observed ion fluxes can be mimicked by calcium ionophores and are inhibited by calcium channel antagonists. This indicates that Nod factors activate a calcium channel leading to an influx of calcium into the root hair resulting in a depolarization of the root hair membrane (Felle *et al.*, 1998).

After Nod factor application also an alkalinization of 0.2-0.3 pH units of the root hair cytoplasm as well as of the environment between the root hairs occur (Felle *et al.*, 1996; 1998). These alkalinizations are significantly slower than the calcium and cloride ion fluxes. In contrast to the alkalinization of the root hair environment, the alkalinization of the root hair cytoplasm persists as long Nod factors are present. Since both, the root hair cytoplasm, as well as the direct environment around the root hair alkalinizes, these processes seem to be contradictory. The underlying mechanisms leading to both processes are not fully understood

(Felle *et al.*, 1996; 1998).

Minutes after the application of Nod factors, regular oscillations of the cytoplasmic calcium can be detected. The process takes place in a distinct spatial pattern. The calcium oscillations are initiated around the nucleus and propagate radially (Ehrhardt *et al.*, 1996). Unknown is the relation of the calcium spiking and the postulated influx of calcium ions which occurs just a few seconds after Nod factor application.

NOD FACTOR PERCEPTION MECHANISMS

The above described epidermal responses are Nod factor structure dependent and are induced at very low concentrations (10^{-8}-10^{-12} M). Therefore it is plausible that Nod factors are perceived by plant receptors. By using *Rhizobium* mutants it was shown that the requirements to Nod factor structure varies with the type of response that is induced (Ardourel *et al.*, 1994). The formation of infection threads shows a higher demand to Nod factor structure than all other responses (figure 1). This has led to the hypothesis that more than one Nod factor perception mechanism is present in the epidermis of the root (Ardourel *et al.*, 1994). Support for this hypothesis was found by studying alkalinization of the root hair cytoplasm. On alfalfa, this response can even be induced by Nod factors lacking the sulfate decoration at the reducing terminal glucosamine residue (figure 1; Felle *et al.*, 1996). When plants have been treated with Nod factor, subsequent application of the same Nod factor does not induce a further increase of the pH. However, if plants treated with a sulfated Nod factor, are subsequently treated with unsulfated factor, or visa versa, further alkalinization is accomplished. This suggests that sulfated and unsulfated Nod factors are perceived by different receptors (Felle *et al.*, 1996).

NOD FACTOR INDUCED RESPONSES IN THE INNER CELL LAYERS OF THE ROOT

Nodule primordium formation

To form a root nodule primordium, fully differentiated root cortical cells have to be developmentally reprogrammed, which starts with mitotic activation of these cells. This is induced by Nod factors and leads to the formation of nodule primordia (Spaink *et al.*, 1991;

Truchet *et al.*, 1991). Upon infection by rhizobia these primordia subsequently differentiate in nodules. In some host plants, purified Nod factors are sufficient to induce this organogenesis, whereas in others the differentiation of primordia into nodules seem to require infection by the bacterium. Which cortical cells will form a nodule primordium is determined by the host plant. Primordia are mainly formed opposite the proto-xylem poles. Furthermore, the host species determines whether inner or outer cortical cells are involved in primordium formation. It is unclear whether Nod factors are translocated to the cortical cells that divide, or whether a secondairy signal is generated that triggers the division of the cortical cells. Despite this, it has become clear that endogenous plant growth factors provide positional information by which nodules are only formed at specific places. Several studies have shown that ethylene is a potent inhibitor of cortical cell division (Goodlass & Smith, 1979; Peters & Crist-Estes, 1989; Lee & LaRue, 1992). In the susceptible zone of pea root, the gene encoding 1-aminocyclopropane-1-carboxylate (ACC) oxidase, that catalyzes the last step in the ethylene biosynthesis, is predominantly expressed in the cells of the pericycle opposite the phloem poles (Heidstra *et al.*, 1997). This strongly suggests that ethylene is produced in these cells. Both, biochemical and genetic data show that this local production of ethylene provides positional information controlling where primordia are formed. A biochemical approach in pea showed that the positional information in the root cortex is lost when ethylene perception or biosynthesis by the plant is disturbed by silver ions or amino-ethoxyvinylglycine (AVG) (Heidstra *et al.*, 1997). The *Medicago truncatula* mutant Sickle is insensitive to ethylene and makes significantly more nodules than the wild type (Penmetsa & Cook, 1997). Also it is disturbed in the positioning of the nodules. Whereas normally cell divisions occur specifically opposite the protoxyleme poles, in Sickle they occur completely random (D.R. Cook, pers. com.).

In addition to a negative regulation by ethylene, also plant compounds regulating cell division in a positive way play a role in nodulation. Uridine, a compound present in the stele of the root, stimulates cell divisions in the inner cortex of pea root explants (Smit *et al.*, 1995). This compound is probably released from the vascular bundle and was previously described as stele factor (Libbenga *et al.*, 1973). However, whether it is specifically released from protoxyleme poles is not known. Therefore it is unclear whether it contributes to the positioning of nodule primordia.

An other positive acting factor that could regulate cortical cell division is the product encoded by the early nodulin gene *ENOD40*. After Nod factor application this gene is rapidly induced in the region of the pericycle opposite a protoxyleme pole. Hence it expression pattern in the pericycle is complementary to that of ACC oxidase. *ENOD40* expression is activated markedly before the first cell divisions occur in the cortex (Minami *et al.*, 1996; T. Bisseling, pers. com.) and when cell divisions are induced, the gene is expressed in the dividing cells as well as in the pericycle (Kouchi & Hata, 1993; Yang *et al.*, 1993; Matvienko *et al.*, 1995). The role of *ENOD40* in stimulating cell division was shown by over-expression of *ENOD40* in *Medicago truncatula*, where it leads, even in the absence of Nod factor, to division of inner cortical root cells (Charon *et al.*, 1997).

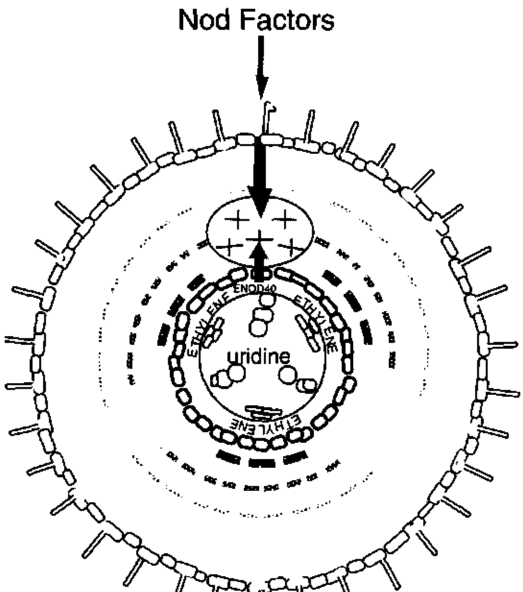


Figure 2. A model showing how the positioning of a nodule primordium could be established in a leguminous root. Both, positive and negative acting factors, contribute to the positioning. Ethylene, produced opposite the phloem poles, diffuses into the cortex and therefor inhibits division in the cortex especially in the area opposite the phloem poles. Nod factors trigger the expression of *ENOD40* in a region of the pericycle opposite xylem poles. The secreted ENOD40 peptide acts together with uridine as a positive signal leading to the mitotical activation of inner cortical cells opposite the xylem poles.

ENOD40 has been isolated from several legume as well as from some non-legume species. All *ENOD40* genes characterized so far contain two regions that are highly conserved. The 5' located box 1 contains a short conserved open reading frame encoding a small peptide of 10-13 amino acids. This peptide was shown to be present in soybean nodules (Van de Sande *et al*, 1996). In Ballistic introduction in *M. truncatula* roots of a DNA construct encoding this small peptide is sufficient to induce cell divisions (Charon *et al.*, 1997).

In Figure 2 a model is given how positioning of a nodule primordium could be established. Both, positive and negative acting factors, could contribute to this positioning. Ethylene, produced in the pericylce cells opposite the phloem poles, can diffuse into the cortex and therefore inhibits division in the cortex especially in the area opposite the phloem poles. Nod factors trigger the expression of *ENOD40* in a region of the pericycle opposite xylem poles. Whether ethylene plays a role in positioning *ENOD40* expression is unclear. The ENOD40 peptide could stimulate cell division in a non-autonomous manner. The pericylce cells secrete the peptide which than could be perceived by the cortical cells. Together with uridine as a positive signal, this may lead to the mitotical activation of inner cortical cells opposite the xylem poles.

Infection

The rhizobia enter the plant root via an infection thread. This is a tip growing tubular structure, initiated at the infection site in a curled root hair, that grows towards the dividing cortical cells. If the nodules are formed in the outer cortical cell layers, like in soybean (*Glycine max*), the infection thread grows through the root hair and can immediately invade the formed primordium. In contrast, in legumes in which nodules are formed in the inner cortex, e.g. pea, the infection thread has to cross several cortical cell layers before reaching the primordium. Before the infection thread traverses the outer cortical cells, the cytoplasm of these cells rearranges and forms a so-called pre-infection thread (PIT). Purified Nod factors are sufficient to induce PIT formation (Van Brussel *et al.*, 1992). A radial aligned file of cortical cells is formed, from which the nuclei have moved to the center and their cytoplasm and microtubules have an organization resembling phragmoplasts (Van Brussel *et al.*, 1992). This array of radial cortical cytoplasmic structures form the path by which the infection thread can traverse the cortical cells.

Within the infection thread, the bacteria are imbedded in a matrix and surrounded by a fibrillar wall. When the infection thread reaches the primordium bacteria are released from an unwalled tip of the infection thread, and via an invagination of the host cell membrane they enter the cytoplasm. Within the host cytoplasm the bacteria stay surrounded by the host membrane, and together they form a so-called symbiosome. The symbiosomes divide and the bacteria differentiate into their symbiotic form, the so-called bacteroids.

CONCLUSION

The communication between rhizobia and legumes has now been elucidated. Rhizobial Nod factors are a key step in this communication, since they are essential and in most cases sufficient to induce the (early) responses in the host plant. The host specific activity of Nod factors is depending on their structure and furthermore, they are active at low concentrations. Therefore, it is likely that they are perceived by receptor(s). However, such receptors have not been isolated, nor is it clear how the signals are transduced, but the above described experiments indicate that the involved mechanisms could be rather complex.

To unravel signal perception and transduction mechanisms, a genetic approach has shown to be successful. In *Arabidopsis thaliana* remarkable progress has been made in elucidating the perception and transduction mechanisms of classical phytohormones. For example for ethylene specific receptors and several components of the activated signal transduction pathway have been identified (for review see: Chang, 1997). To unravel Nod factor perception and transduction a genetic approach will also be essential. Pea has been intensively used for genetic studies in the past and this has led to the identification of many symbiotic mutants. Characterization of these mutants and subsequent cloning of the mutated genes could lead to a better insight how Nod factors are perceived. However, pea has a relatively big genome ($3.8\text{-}4.8.10^9$ basepairs per haploid genome; Ellis, 1993) by which positional cloning of the corresponding genes can be extremely labor intensive. Recently, macro-synteny between *Medicago truncatula* and pea has been demonstrated (D.R. Cook, pers. com.). Since *M. truncatula* has a relatively small genome ($0.5.10^9$ basepairs), and is easy to transform it has been selected as a model legume and recently H.S.F. has initiated a *Medicago* 'genomics' program. The occurrence of synteny together with the availability of molecular and genetic tools could be used to clone interesting pea genes, by the identification of the *M. truncatula* counterparts.

ENDOMYCORRHIZAE AND RHIZOBIAL NOD FACTORS BOTH REQUIRE SYM8 TO INDUCE THE EXPRESSION OF THE EARLY NODULIN GENES *PSENOD5* AND *PSENOD12A*

Catherine Albrecht, René Geurts, Frederic Lapeyrie and Ton Bisseling

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We report here that the pea early nodulin genes *PsENOD5* and *PsENOD12A* are induced during the interaction of pea roots and the endomycorrhizal fungus *Gigaspora margarita*. Using the pea nodulation mutant Sparkle-R25, which is mutated in *SYM8*, it is shown that SYM8 is essential for the induction of *PsENOD5* and *PsENOD12A* in pea roots interacting either with *Rhizobium* or the endomycorrhizal fungus *Gigaspora margarita*. Our results suggest that mycorrhizal signals activate a signal transduction cascade sharing at least one common step with the Nod factor activated signal transduction cascade.

INTRODUCTION

Leguminous plants can form an endosymbiotic association with bacteria of the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* (collectively referred to as rhizobia) as well as with endomycorrhizal fungi. Therefore, they provide the unique opportunity to identify host genes with a common role in endosymbioses (Duc *et al.*, 1989, Gianinazzi-Pearson *et al.*, 1991).

Both rhizobia and the mycorrhizal fungi improve the mineral nutrition of the host plant, which in exchange supplies the microorganisms with photosynthates. The rhizobia fix atmospheric nitrogen and provide the plant with ammonia, whereas mycorrhizal fungi facilitate the translocation of limiting nutrients, especially phosphorous, from the soil to the plant. The ability of plants to interact with mycorrhizal fungi is a very ancient phenomenon and widespread in the plant kingdom. 80% of the plant families are capable of forming arbuscular mycorrhizae (AM) and fossil data suggest that this symbiosis occurred more than 400 million years ago (Pirozynski & Dalpe, 1989; Simon *et al.*, 1993).

AM formation is rather aspecific since a single fungal species has the capacity to colonize many plant species and in addition a given plant can interact with different fungal species. In contrast, *Rhizobium*-induced nodulation is highly specific and, with the exception of *Parasponia*, it is restricted to leguminous plants. Within this family, *Rhizobium* species only nodulate a restricted set of host plants.

The molecular bases of the rhizobial symbiosis is partially elucidated. The interaction starts with the induction of the rhizobial nodulation genes (*nod*) by plant secreted-flavonoids, which leads to the synthesis of specific lipo chito-oligosaccharides, the Nod factors. The structure of these signal molecules varies depending on the *Rhizobium* species. Nod factors are essential and in most cases sufficient to trigger processes leading to nodule formation, e.g. induction of nodulin gene expression, root hair deformation and the formation of nodule primordia (for reviews see: Dénarié *et al.*, 1996; Long, 1996; Spaink, 1996). In contrast, the nature of the signals that set the mycorrhizal interaction in motion is unknown (for review see: Gianinazzi-Pearson, 1996).

When contacting the root surface, the fungal hyphae form an appressorium. Subsequent penetration of the root is mediated by hyphae that originate from these appressoria and that grow intercellularly towards the inner cortex. Upon reaching the inner cortex, fungal hyphae enter the cortical cells and differentiate into highly branched, structures known as arbuscules (AM). At this stage, the differentiated fungal hyphae are separated from the plant cytoplasm by a plant-derived perisymbiotiont membrane (Gianinazzi-Pearson, 1996).

Despite the obvious differences between the two symbioses, plant mutational analysis has shown that certain plant genes are essential in both AM and nodule formation. Such mutants (Nod$^-$, Myc$^-$) have been found in *Medicago truncatula*, alfalfa, faba bean, bean and pea (Duc *et al.*, 1989; Bradbury *et al.*, 1991; Sagan *et al.*, 1995; Shirtliffe & Vessey, 1996). Furthermore, it was shown that two nodulin genes *ENOD2* and *ENOD40*, that are activated during early stages of *Rhizobium* induced nodulation, are also induced during AM formation (Van Rhijn *et al.*, 1997). These observations, together with the ancient nature of AM formation, suggest that some of the plant processes leading to nodulation may have evolved from those already established for the fungal endosymbiosis (LaRue & Weeden, 1994). This raises the question whether Nod factor and mycorrhizal signal activated signal transduction cascades leading to the activation of early nodulin genes share common steps (Van Rhijn *et al.*, 1997).

To address this question it is essential to use early nodulin genes that are rapidly activated by Nod factors and are induced preferably in cells that are in direct contact with Nod factors. *ENOD2* does not seem to be very useful for such studies, since in Nod factor induced nodulation this gene is first activated when the nodule primordium forms the nodule parenchyma tissue (Van der Wiel *et al.*, 1990). This is a rather late response since it first occurs after several days. Furthermore, it is unknown whether Nod factors reach the primordium cells that develop into nodule parenchyma. Another reason that *ENOD2* is not very useful for Nod factor signal transduction studies is the fact that cytokinin can induce the expression of this early nodulin in existing root tissues within a few hours (Dehio & De Bruijn, 1992), while Nod factors are unable to do so. This suggests that Nod factor and cytokinin - which is known to be secreted by mycorrhizal fungi (Van Rhijn *et al.*, 1997) - induced expression of *ENOD2* might involve different mechanisms. The other early nodulin gene that is known to be induced in both interactions, *ENOD40*, is also not very attractive for Nod factor signal transduction studies since it is activated in the root pericycle and it is unknown whether Nod factors are transported to this tissue. Furthermore, this gene can be activated by chitin fragments (Minami *et al.*, 1996). Hence, this gene might be activated by fungal cell wall fragments, which makes it unclear whether its expression is of physiological meaning.

The *Rhizobium* infection related early nodulin genes *ENOD5* and *ENOD12* are more suitable to study Nod factor activated signal transduction. Both genes are rapidly activated in the epidermal root cells which are in direct contact with rhizobial Nod factors (Horvath *et al.*, 1993; Journet *et al.*, 1994; Vijn *et al.*, 1995; Heidstra *et al.*, 1997). Here we describe that the early nodulin genes *PsENOD5* and *PsENOD12A* are expressed during infection of pea (*Pisum sativum*) with AM fungus *Gigaspora margarita*. Therefore we used the induction of these genes as a tool to study whether the pathways used by mycorrhizal fungi and Nod factors share common steps. To answer this question we searched for a pea mutant that can block Nod factor and AM fungi-induced expression of *PsENOD5* and *PsENOD12A*.

In pea 4 loci (*SYM8*, *SYM9*, *SYM19* and *SYM30*) are known that are involved in the early steps of both endosymbiotic interactions (Weeden *et al.*, 1990; Gianinazzi-Pearson *et al.*, 1991, LaRue & Weeden, 1992, Balaji *et al.*, 1994, Borisov *et al.*, 1994, Gianinazzi-Pearson, 1996). Mutations in these genes block the penetration of mycorrhizal fungus into the root, but the fungus remains able to form appressoria (Gianinazzi-Pearson *et al.*, 1991, Balaji *et al.* 1994, Gollotte *et al.*, 1994). In the interaction with *Rhizobium* the *SYM8* mutant Sparkle-R25 is blocked at a very early step, since it has lost the ability to deform root hairs (Markwei & LaRue, 1992). In contrast, the *SYM9* mutant Sparkle-R72 and *SYM19* mutant Rondo-K24 remain able to form curled root hairs upon inoculation with *Rhizobium*, but neither infection threads nor nodule primordia are formed (Markwei & LaRue, 1992, Postma *et al.*, 1988). For *sym30* no detailed phenotype has been described. We choose to analyze the *SYM8* mutant Sparkle-R25, because it is blocked at an early stage of both endosymbiotic interactions. Here, we describe that in this mutant the induction of *PsENOD5* and *PsENOD12A* is blocked in both interactions. Thus SYM8 appears to be essential for Nod factor as well as for endomycorrhizal activated pathways leading to the induction of *PsENOD5* and *PsENOD12A*.

RESULTS

Phenotypic characterization of the interaction of *Gigaspora margarita* with wild type pea

To study the interaction of *Gigaspora margarita* and pea roots we first used mass inoculated plants. Ten days after inoculation, the hyphae are present at the surface of the root, appressoria have been formed and some hyphae have penetrated the outer cell layers of the root. Fifteen to twenty days after inoculation, hyphae have penetrated the outer and inner root cortex and at twenty days, intracellular colonization of the inner cortical cells by the fungus has started. At younger parts of the root, new infections occur by which different stages of mycorrhizal development are present simultaneously in a root system (data not shown). The advantage of a mass-inoculation procedure is that, ultimately, a major part of the root system is involved in the interaction with the fungus. Therefore, it is possible to obtain sufficient mycorrhizal plant material. However, since the infections do not occur simultaneously, it was impossible to obtain accurate information on the timing of gene expression. For this reason, we developed a spot-inoculation system to study the timing of plant gene expression during early stages of AM formation. Lateral roots of 10 day old pea seedlings grown on agar were spot-inoculated with spores of *G. margarita* (see materials and methods). About four days after inoculation a germ tube emerges from the spores and grows in a negative geotropic manner on the surface of the agar (Watrud *et al.*, 1978). The spores were positioned such that the initial contact between the germ tube and the elongation zone of the root occurs about 6 days after inoculation (see material and methods). Appressoria, from which new hyphae develop that invade the root tissue, are formed 2 to 4 days later. Intercellular colonization of the root cortex was observed during the four following days with the first arbuscules starting to develop 15 days after inoculation and this intracellular colonization of the inner cortex is well established 20 days after inoculation (Figure 1A, 1B, 1C).

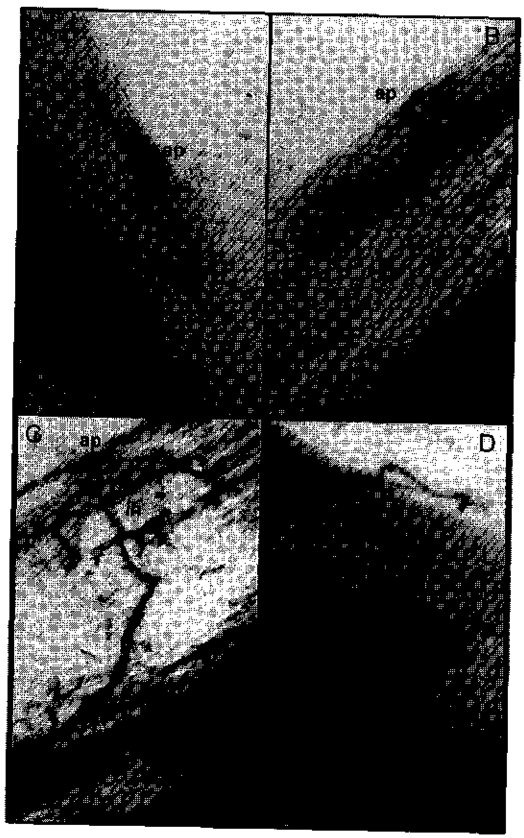

Figure 1. Spot-inoculation of wild type pea roots with the arbuscular mycorrhizal (AM) fungus *G. margarita*. The root was stained with trypanblue. **A.** ten days after inoculation, appressoria are formed and some hyphae starts to invade the outer root cortex. **B.** Fifteen days after inoculation, the intercellular colonization of the root cortex is well established. **C.** Twenty days after inoculation, the inner cortical cells are extensively colonized by the fungus. **D.** Spot-inoculation of Sparkle-R25 (*sym8*) roots with *G. margarita*. Twenty days after inoculation, appressoria are formed but root penetration and colonization of the root cortex does not occur.

Abbreviations: eh, external hypha; ap, appressorium; ih, intercellular hypha; a, arbuscule; x, xylem. Scale bar represent (a) 50 µm; (b) 100 µm; (c) and (d) 25µm.

Expression of *PsENOD5* and *PsENOD12A* during endomycorrhizal infection

Previously, it has been shown that the early nodulin gene *ENOD2*, that can be activated by both mycorhizal fungi and rhizobia, is also induced by cytokinin (Dehio & De Bruijn, 1992; Van Rhijn *et al.*, 1997). Therefore, we tested whether the infection related early nodulin genes *PsENOD5* and *PsENOD12A* can also be induced by cyotkinin. Pea roots were treated with 1 μM BAP and were harvested 24h later. No increase in the level of expression of either *PsENOD5* or *PsENOD12A* was induced, whereas a clear induction of *PsENOD2* could be observed (data not shown).

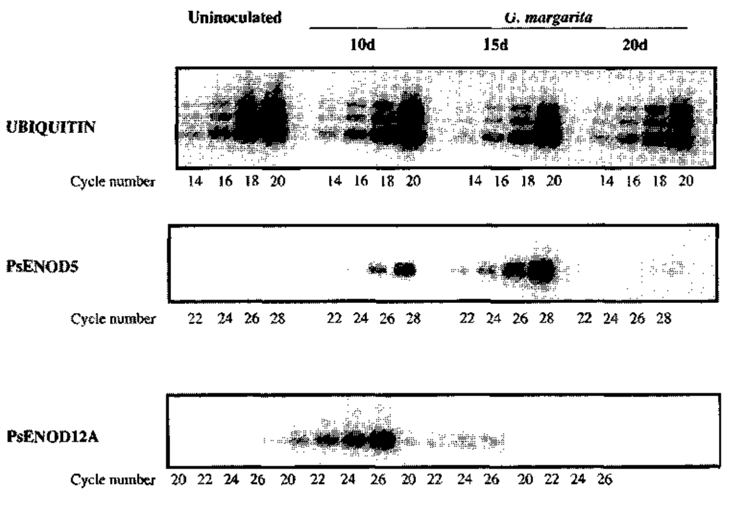

Figure 2. Induction of *PsENOD5* and *PsENOD12A* expression in *G. margarita* spot-inoculated pea roots. Infected root segments were collected 10, 15 and 20d after inoculation. Corresponding non-inoculated root segments were harvested after 10d. *PsENOD5* and *PsENOD12A* expression was analyzed by RT-PCR using total RNA. As a control, *Ubiquitin* mRNA was amplified. Under the conditions used, the amplification of *Ubiquitin* mRNA is exponential between 14 and 20 cycles, while *PsENOD5* amplification is exponential up to 28 cycles and *PsENOD12A* up to 26 cycles.

We investigated whether the expression of *PsENOD5* and *PsENOD12A* are induced by mycorrhizal fungi. Pea roots were inoculated with *G. margarita* spores using the spot-inoculation system. Root material was collected 10, 15, 20 and 30 days after inoculation. Total RNA was isolated and after reverse transcription *PsENOD5* and *PsENOD12A* cDNA was amplified by RT-PCR and ubiquitin mRNA was used as an internal standard. Amplification of *PsENOD5* and *PsENOD12A* cDNA resulted in fragments of 250 and 348 bp, respectively, which were not present when the reverse transcriptase step was omitted (results not shown). In the spot-inoculated plants, *PsENOD5* and *PsENOD12A* are both activated at an early stage of interaction, 10 days after inoculation. The *PsENOD12A* transcript is present at the highest concentration 10 days after inoculation, when appressoria are formed and hyphae develop to invade the cortex. *PsENOD5* mRNA is present at its maximum level 15d after inoculation, when the hyphae invade the root cortex. The level of both transcripts markedly decreases as the colonization proceeds (20 days after inoculation; Figure 2).

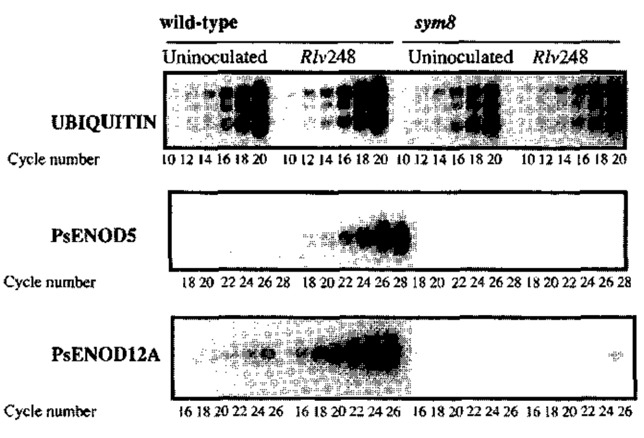

Figure 3: Induction of *PsENOD5* and *PsENOD12A* expression in root segments of *Pisum sativum* cv. Sparkle and the mutant Sparkle-R25 (*sym8*). *PsENOD5* and *PsENOD12A* expression was analyzed by RT-PCR using total RNA isolated from spot-inoculated root segments collected 24h after inoculation with *R.leguminosarum* bv *viciae* strain 248. As a control, *Ubiquitin* mRNA was amplified. Under the conditions used, the amplification of *Ubiquitin* mRNA is exponential between 14 and 20 cycles, while *PsENOD5* amplification is exponential up to 28 cycles and *PsENOD12A* up to 26 cycles.

These results show that the early nodulin genes *PsENOD5* and *PsENOD12A* are involved in early steps of AM formation in pea. The use of the spot-inoculation system, allowing a precise timing of the interaction, shows that these early nodulin transcripts accumulate at specific early stages of endomycorrhizal interaction, most likely the appressorium and invasion stage, respectively. Possibly due to the low level of expression of these genes, we were unable to detect their expression by *in situ* hybridization (data not shown).

PsENOD5 and *PsENOD12A* are not induced in the *sym8* mutant Sparkle-R25

Since the *SYM8* mutant Sparkle-R25 does not show any morphological response in the interaction with *Rhizobium* (Markwei & LaRue, 1992), we choose this mutant to determine whether the symbiotic induction of the early nodulins *PsENOD5* and *PsENOD12A* is affected.

First we studied the Myc⁻ phenotype of Sparkle-R25 by using the spot-inoculation method. Root segments were harvested 10 and 20 days after inoculation and stained for fungal hyphae. On this mutant *G. margarita* forms appressoria, but it does not penetrate the root (Figure 1D). Hence, the phenotype of Sparkle-R25 is similar as previously reported by Balaji *et al.* (1994), who used transformed root cultures.

To determine whether the Nod⁻ and Myc⁻ phenotype of the gamma radiation mutant Sparkle-R25 is caused by a single mutation we analyzed the Myc phenotype of two additional Sparkle*SYM8* Nod⁻ mutants, Sparkle-R19 (gamma radiation) and Sparkle-E140 (EMS) (Kneen *et al.*, 1994) by mass-inoculation with *G. margarita* (see material and methods). Roots were harvested and stained for mycorrhizal hyphae 30 days after inoculation. In the control plants, the cultivar Sparkle, the inner cortical cells of the mature part of the root contain many arbuscules. In contrast, *G. margarita* did not penetrate the roots of any of the *SYM8* mutants, but did form appressoria. Previously, a Nod⁻/Myc⁻ phenotype was observed for 2 other *SYM8* mutants, which were generated by EMS treatment of the pea cultivar Sprint (Borisov *et al.*, 1994). These results, together with ours, are strong evidence that a mutation in a single gene, *SYM8*, is responsible for the Nod⁻/Myc⁻ phenotype.

Expression of PsENOD5 and PsENOD12A in Rhizobium *spot-inoculated Sparkle-R25 roots*

To study whether Nod factors can still induce *PsENOD5* and *PsENOD12A* in a *SYM8* mutant, we have investigated the induction of expression of these genes in Sparkle-R25 roots spot-inoculated with *Rhizobium leguminosarum* bv *viciae* strain 248 or with the Nod factor NodRlv-IV(Ac,C18:4). Analyses of the RNA extracted from inoculated Sparkle-R25 roots showed that neither *Rhizobium* nor Nod factor induces the expression of *PsENOD12A* and *PsENOD5* while these genes are active in wild type Sparkle roots (Figures 3 and 4). Therefore, it seems probable that SYM8 is involved in Nod factor perception or transduction

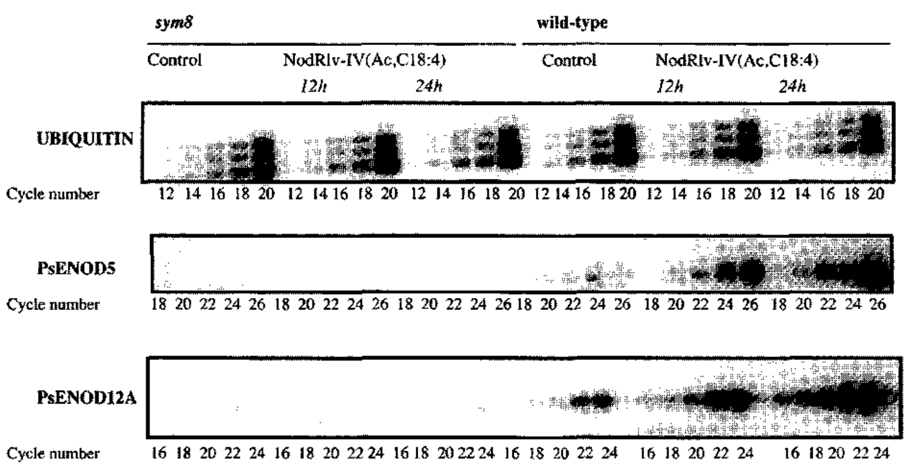

Figure 4. Induction of *PsENOD5* and *PsENOD12A* expression in root segments of *Pisum sativum* cv. Sparkle and the mutant Sparkle-R25 (*sym8*) after treatment with 10^{-9}M Nod factor (Nod Rlv-IV(Ac,C18:4)). *PsENOD5* and *PsENOD12A* expression was analyzed by RT-PCR using total RNA isolated from the susceptible zone collected 12 and 24h after treatment. As a control, *Ubiquitin* mRNA was amplified. Under the conditions used, the amplification of *Ubiquitin* mRNA is exponential between 14 and 20 cycles, while *PsENOD5* amplification is exponential up to 28 cycles and *PsENOD12A* up to 26 cycles.

Expression of PsENOD12A and PsENOD5 in G. margarita *spot-inoculated Sparkle-R25 roots*

To study whether Sparkle-R25 is also blocked in mycorrhizal induced *PsENOD5* and *PsENOD12A* expression, roots were spot-inoculated with *G. margarita* spores. As in

Rhizobium or Nod factor spot-inoculated Sparkle-R25 roots, *PsENOD5* and *PsENOD12A* expression was not induced (Figure 5). These results show that in both endosymbiotic interactions, SYM8 is essential for the induction of *PsENOD12A* as well as *PsENOD5.*

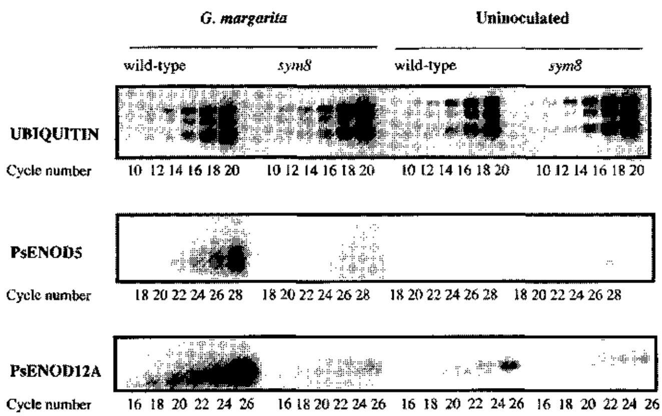

Figure 5. Induction of *PsENOD5* and *PsENOD12A* expression in root segments of *Pisum sativum* cv. Sparkle and the mutant Sparkle-R25 (*sym8*). *PsENOD5* and *PsENOD12A* expression was analyzed by RT-PCR using total RNA isolated from *G. margarita* spot-inoculated root segments collected 10d after inoculation. As a control, *Ubiquitin* mRNA was amplified. Under the conditions used, the amplification of *Ubiquitin* mRNA is exponential between 14 and 20 cycles, while *PsENOD5* amplification is exponential up to 28 cycles and *PsENOD12A* up to 26 cycles.

DISCUSSION

The early nodulin genes *PsENOD5* and *PsENOD12A* are induced in the host plant pea by the mycorrhizal fungus *G. margarita* at an early stage of AM formation as well as by rhizobial Nod factors. Since these genes are not induced by the pathogenic fungus *Fusarium oxysporum* (Scheres *et al.*, 1990a,b) the expression of these genes appears to be specific to the symbiotic interactions.

and are perceived by the same perception mechanisms, how could these signals trigger such different responses? Therefore it seems probable that the perception mechanisms for mycorrhizal signals and Nod factors are different, but that the used pathways have SYM8 in common.

MATERIALS & METHODS

Rhizobium leguminosarum

Rhizobium leguminosarum bv. *viciae* strain 248 was used in the described experiments and it was cultured in YEM medium (Jossey *et al.*, 1979).

Gigaspora margarita

A strain of *Gigaspora margarita* was maintained on *Plectranthus australis* growing in autoclaved Turface (OIL DRI SA, RFA). The plants were kept in a growth chamber with a day/night cycle of 16/8h at 20° C. They were watered with sterile deionized water and fertilized once a week with the following growth medium: $MgSO_4.7H_2O$ (3 mM), KNO_3 (1 mM), $CaCl_2.2H_2O$ (1.2 mM), NaFeEDTA (0.02 μM). Samples of the Turface were used as inoculum (crude inoculum) for the mass-inoculation experiments and for the purification of spores.

Spores were isolated from the Turface by wet sieving (Gerdeman & Nicholson, 1963) followed by centrifugation (30 s, 600 g) in a 60% sucrose solution. The supernatant is sieved (0.05mm) and the remaining spores were extensively washed with tap water. Ultimately, they are plated on 1% agar prepared in water and mature spores were selected. The spores were soaked in 0.05% (v/v) Tween 20 and 2% (w/v) chloramine T for 20 minutes and rinsed three time with a sterile streptomycin solution (200 mgl^{-1}). This treatment was repeated twice. The surface-sterilizedspores can be used immediately or can be stored in streptomycin solution (200 mgl^{-1}) for a few days at 4°C.

Plant culture and inoculation

Pisum sativum cv. Sparkle (pea) and the *SYM8* mutants Sparkle-R19, Sparkle-R25 and Sparkle-E140 were used (Markwei & LaRue, 1992, Kneen *et al.*, 1994). Pea seeds were surface sterilized with a commercial bleach solution for 15 minutes. After rinsing with tap water, they were treated for 15 minutes with 7% H_2O_2 and then washed five times in sterile water and placed on 1% agar. The seeds were germinated for three days at 20°C in the dark.

Spot-inoculation of Rhizobium and Nod factor

Germinated seeds were transferredto Petri dishes (145/20mm) in plant growth medium (PGM) ($CaCl_2.2H_2O$

(2.72 mM), $MgSO_4.7H_2O$ (1.95 mM), KH_2PO_4 (2.2 mM), $NaHPO_4.12H_2O$ (1.26 mM) and Fe(III)Citrate.$2H_2O$ (0.08 mM)) containing 1.5% agar. The dishes had a hole in the rim, allowing the shoots to grow out, while the roots grew on the (sterile) medium protected from light by aluminium foil. The seedlings were grown at 20°C with a day/night cycle of 16/8h. The lateral roots were spot-inoculated at the zone containing emerging root hairs with 0.2 µl rhizobial culture (A600 = 0.1) or 0.2 µl 10^{-9} M Nod factor (NodRlv-IV(Ac,C18:4)). The position was marked in the agar using sterile ink. Inoculated root segments of 5mm were harvested after 24h, immediately frozen in liquid nitrogen and stored at -80°C.

Mass-inoculation with Gigaspora margarita

Germinated pea seeds were inoculated with *Gigaspora margarita* by transplanting them in pots containing gravel mixed with crude inoculum (see above). They were maintained at 20°C with a day/night cycle of 16/8h. Pots were watered with deionized water and fertilized once a week as described above. Roots were harvested 10, 15, 20 and 30 days after inoculation, frozen in liquid nitrogen and stored at -80°C. To check for mycorrhizal formation, part of each root system was cleared for 3 minutes in 10% KOH (w/v), rinsed in 2% HCl for 3 minutes and stained with 0.1% trypanblue (w/v) for 10 minutes (Philipps and Haymans, 1970). Colonization of the roots was assessed with a light microscope.

Spot-inoculation with Gigaspora margarita

Pea plants were grown as described for the *Rhizobium* spot-inoculation system, but 1.5% agar PGM was replaced by $MgSO_4.7H_2O$ (3 mM), KNO_3 (1 mM), $Ca(NO_3)_3.4H_2O$ (1.2 mM), NaFeEDTA (0.02 µM) and agarose (0.5%). Lateral roots of 15 days old pea plants were spot-inoculated as follows: 5 to 10 spores of *Gigaspora margarita* were inserted just below the surface of the agar. The spores were positioned 1 cm before the root tip such that, about 6 days later, the growing germ tube can contact the elongation zone of the growing lateral roots (0.5 to 4 cm behind the tip). Root segments were harvested 10, 15 and 20 days after inoculation, frozen in liquid nitrogen and stored at -80°C. To check mycorrhizal formation, root segments were cleared, stained and assessed microscopically as described above.

Cytokinin treatment of uninoculated roots

Seven day old pea seedlings were transferredto sterile glass tubes containing PGM and 6-benzylaminopurine (BAP) at a concentration of 10^{-6}M. The shoots of the plants were growing outside the tubes and the system was kept in sterile condition by closing the tubes with a sterile cotton plug. The glass tubes were covered by aluminium foil. The plants were grown at the same conditions as described above. The roots were harvested 24h after treatment, frozen in liquid nitrogen and stored at -80°C.

RT-PCR to quantify *PsENOD2*, *PsENOD5* and *PsENOD12A* expression

Total RNA was isolated according to Pawlowski *et al.* (1994) followed by a DNaseI (Promega) treatment.

cDNA was made from 1 µg total RNA in a volume of 20 µl of 10 mM Tris/Cl pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 µg oligo dT12-18 (Pharmacia), 0.5 U RNA guard (Pharmacia) and 20 U AMV reverse transcriptase (Finzyme) for 10 min. at room temperature followed by 1 h at 42°C and 5 min. 95° C. The RT sample was then diluted to 100 µl. The PCR reactions were performed with 2 µl of the cDNA solution in 10 mM Tris/HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ 100 µM dNTPs, 50 ng primer each and 1 U Taq polymerase (Bhoeringer) in a total volume of 50 µl. *PsENOD2*, *PsENOD5*, *PsENOD12A* and ubiquitin were amplified using the PCR program 30 s 94°C, 30 s 58°C, 30 s 72°C by using the primers PsENOD2-f: 5'-GAAAAGCCCTCACCAAAGT-3', PsENOD2-r: 5'-TAAAAGGCATAACAAACAACC-3' PsENOD5-f: 5'-CGATACTATCGATGTAGTGG-3', PsENOD5-r: 5'-GACTGTAATTGACCTTCACC-3' to amplify *PsENOD5*; PsENOD12A-f: 5'-TCACTAGTGTTGTTCCTTGC-3', PsENOD12A-r: 5'-CCATAAGATGGTTTGTCACG-3' to amplify only *PsENOD12A* and UBIQ-f: 5'-ATGCAGAT$^{C}/_{T}$TTTGTGAAGAC-3', UBIQ-r: 5'-ACCACCACG$^{G}/_{A}$AGACGGAG-3' to amplify ubiquitin. The amplified DNA samples were separated on a 1.6% agarose gel and after alkaline blotting to a nylon membrane (Hybond-N+, Amersham) hybridized to ^{32}P-labelled *PsENOD2*, *PsENOD5*, *PsENOD12A* or ubiquitin DNA probe. All experiments were performed at least in duplo.

SYM2 OF *PISUM SATIVUM* IS INVOLVED IN A NOD FACTOR PERCEPTION MECHANISM THAT CONTROLS THE INFECTION PROCESS IN THE EPIDERMIS

René Geurts, Renze Heidstra, Az-Eddine Hadri, J. Allan Downie, Henk Franssen,
Ab van Kammen and Ton Bisseling

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In pea (*Pisum sativum*), up to 50 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 result 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 utilized 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 exemplified as follows: alfalfa (*Medicago sativa*) belongs to the cross-inoculation group that can be nodulated by *Sinorhizobium meliloti*, which produces Nod factors with a sulfate group at the reducing sugar (Lerouge *et al.*, 1990). In contrast, pea (*Pisum sativum*) is nodulated by *Rhizobium leguminosarum* biovar *viciae* (*R. l.* bv. *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 sulfation of the Nod factors in *S. meliloti* are introduced into *R. l.* bv. *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 depolarization of the root hair plasma membrane (Ehrhardt *et al.*, 1992; Felle *et al.*, 1995; Kurkdjian 1995), alkalization 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 recognized by host receptors (reviewed in: Geurts & 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 specific 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.* bv. *viciae* with its host plants, structural deficiencies of Nod factors due to a *nodE* mutation can be compensated by a protein, NodO. An inactive *nodE* will lead to 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 characterization of such mutants and corresponding genes will improve our understanding of Nod factor perception. In pea, several mutants have been identified as being affected in 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.* bv. *viciae* strains (Lie, 1984). A single bacterial nodulation gene, *nodX*, was shown to confer the ability to nodulate plants harboring this *SYM2*^{A} allele from 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, surprisingly, determined by the *R. l.* bv. *viciae* strain used as inoculum. For example, the *R. l.* bv. *viciae nodX-* strains 248 and PF2 form nodules on heterozygotic *SYM2*^{A}*SYM2*^{C} plants, whereas a similar *nodX-* strain PRE does not (Lie, 1984; Kozik *et al.*, 1995). Strikingly, *R.l.* bv. *viciae* strains 248 and PF2 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.* bv. *viciae* strain 248 into strain PRE, leading to an increase of Nod factor production, did not change the dominant nature of *SYM2*^{A} in heterozygous *SYM2*^{A}*SYM2*^{C} plants (Kozik *et al.*, 1995).

Here we report on the role of *SYM2*^{A} in Nod factor perception. Our experiments show that *R.l.* bv. *viciae* strains lacking *nodX* are specifically arrested in the infection process in their interaction with *SYM2*^{A} harboring peas. Furthermore, we show that *SYM2*^{A} mediated control of infection thread growth is affected by *nodO*. By analyzing the efficiency of Nod factor degradation we showed that SYM2^{A} does not strongly enhance Nod factor degradation.

RESULTS

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

Since the *R.l.* bv. *viciae* strain used as inoculum determines which *SYM2* allele is dominant, it is possible that those strains which can nodulate heterozygous Rondo-$SYM2^A SYM2^C$ plants may have extra genes compared with those strains that cannot. To address this, we analyzed the nodulation behavior of a pSym-cured derivative of *R.l.* bv. *viciae* strain 248 containing large cloned *nod* gene regions from the Sym plasmid pRL1JI (Figure 1). The cured strain carrying pIJ1089 retained the characteristics of *R.l.* bv. *viciae* strain 248 in that it nodulated heterozygous Rondo-$SYM2^A SYM2^C$ and homozygous Rondo-$SYM2^C SYM2^C$ plants (Table I). Although the equivalent strain carrying pMP225 did nodulate Rondo-$SYM2^C SYM2^C$ plants, it could not nodulate heterozygous Rondo-$SYM2^A SYM2^C$ plants. The major difference between pMP225 and pIJ1089 is that pIJ1089 is about 9 kb larger and the *nodO, rhiABC* and *rhiR* operons are contained within the additional DNA. It follows that a gene or genes within this region of DNA determine whether the bacteria can nodulate heterozygous $SYM2^A SYM2^C$ plants.

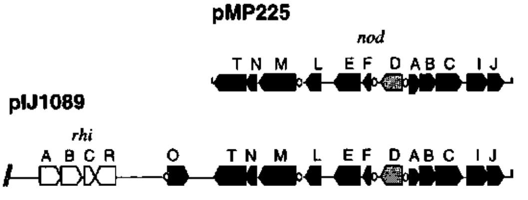

Figure 1. Map of the *nod-rhi* gene region of the *R.l.* bv. *viciae* Sym-plasmid pRL1JI cloned in pMP225 (Spaink *et al.*, 1987) and pIJ1089 (Downie *et al.*, 1983). The *nod* genes are indicated as black arrows with the open circle indicating a *nod* box promoter. The constitutively expressed *nodD*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

To establish which of the known genes in the additional 9 kb region of pIJ1089 is required for nodulation of heterozygous $SYM2^A SYM2^C$ plants, we analyzed nodulation of *R.l.* bv. *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 I). This demonstrates that *nodO* is essential for nodulation of heterozygous Rondo-$SYM2^A$ $SYM2^C$ plants by *R.l.* bv. *viciae* strain 248.

Table I. Nodulation behavior of *R.l.* bv. *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	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.

A role for *nodO* can also be seen in the nodulation of homozygous Rondo-$SYM2^A SYM2^A$ plants. *R.l.* bv. *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.* bv. *viciae* strain 248 carrying *nodX* (Table II). This demonstrates that in the compatible interaction with $SYM2^A$ containing plants (i.e. when NodX-acetylated Nod factors are made), *nodO* is not essential for nodulation.

$SYM2^A$ mediated response

We attempted to examine the differences in Nod factor induced responses in the incompatible interactions of *R.l.* bv. *viciae* strains 248 and 248*nodO*$^-$ and the compatible interaction of strain 248*nodX* on Rondo-*SYM2*^{A} *SYM2*^{A} plants. Root hair deformation and *ENOD12* induction are both responses in the epidermis which do not demand stringent Nod factor structure requirements (Horvath *et al.*, 1993; Journet *et al.*, 1994). We found it to be extremely difficult to analyze 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.* bv. *viciae* strains 248, 248*nodO*$^-$ and 248*nodX*. The level of *ENOD12* mRNA in the root hairs was determined by RT-PCR after 24 hours (Figure 2). In spite of the inability of *R.l.* bv. *viciae* strains 248 and 248*nodO*$^-$ to nodulate homozygous *SYM2*^{A} plants, they trigger *ENOD12* expression to a similar level as the compatible strain 248*nodX*.

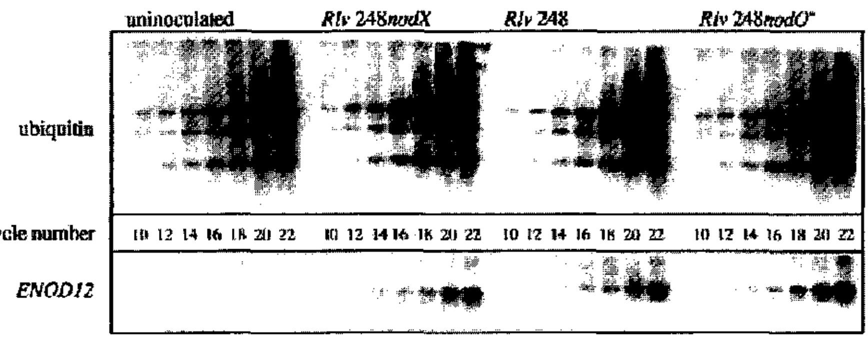

Figure 2. Induction of *ENOD12* expression in root hairs of Rondo-*SYM2*^{A}*SYM2*^{A}. *ENOD12* expression was analyzed by RT-PCR using total RNA isolated from root hairs collected 24 hours after inoculation with *R.l.* bv. *viciae* strain 248, 248*nodO*$^-$ or 248*nodX*. As a control *Ubiquitin* mRNA was amplified. Under the conditions used the amplification of *Ubiquitin* mRNA is exponential between 12 and 16 cycles, while *ENOD12* amplification is exponential up to 22 cycles.

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.* bv. *viciae* strain 248*nod*X 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.* bv. *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 II), but the cells never appeared to be infected. Figure 3 shows a cross section of a Rondo-*SYM2*^{A}*SYM2*^{A} root segment, spot-inoculated with *R.l.* bv. *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.* bv. *viciae* strain 248*nodO*$^-$ appeared to be smaller than those formed by strain 248 (data not shown). This might explain why a slightly reduced number of primordia are found in plants inoculated with *R.l.* bv. *viciae* strain 248*nodO*$^-$. Infection thread formation was only rarely found (less than 10% of the cases, Figure 4), and these infection threads could only be detected in the epidermis and never grew into the inner cortical cell layers (Table II).

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.* bv. *viciae* strains lacking *nodX* formed a notably reduced number of infection threads while formed infection threads got arrested in the epidermis.

Table II. 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.

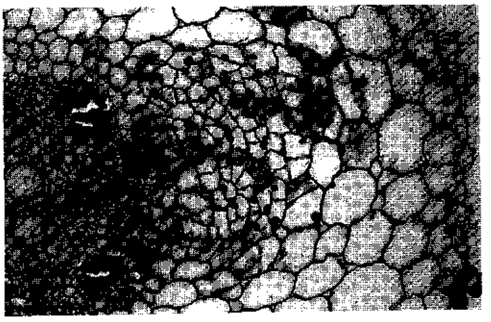

Figure 3. Nodule primordium formation on Rondo-*SYM2^A SYM2^A* by *R.l.* bv. *viciae* strain 248*nodO*⁻. A cross section (7 μm) is shown of a Rondo-*SYM2^A SYM2^A* root segment, 10 days after spot-inoculation with *R.l.* bv. *viciae* strain 248*nodO*⁻. A nodule primordium is formed in the inner cortex but the cells are not infected since there is no infection thread formed.

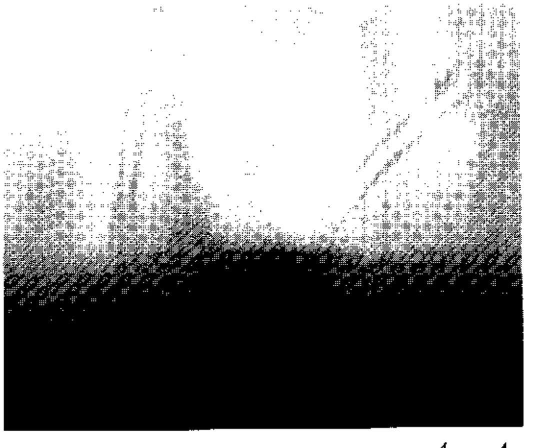

Figure 4. Infection thread formation in the epidermis of Rondo-*SYM2^A SYM2^A* by *R.l.* bv. *viciae* strain 248*lacZnodO*⁻. Root segments were collected 10 days after spot-inoculation with *R.l.* bv. *viciae* 248*lacZnodO*⁻ and infections were scored by staining the roots to detect ß-galactosidase activity. Infection threads were rarely found in the epidermis and where never detected in the root cortex.

SYM2^A is specifically active during the first days of the interaction

Based on studies with *S. 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 activity of $SYM2^A$ we made use of the temperature sensitive nature of the phenotype of $SYM2^A$ peas (Kozik *et al.*, 1995). At the permissive temperature (26°C) the number of nodules formed by *R.l.* bv. *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 III).

Table III. 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.

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.* bv. *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.* bv. *viciae* strain 248 with a similar efficiency as strain 248*nodX* (60-80%). *R.l.* bv. *viciae* strain 248*nodO*⁻ was also able to nodulate, but the number of successful infections was lower than in the compatible interaction with strain 248*nodX* (Table V).

To determine how far an infection thread develops within 3 days at 26°C, we spot-inoculated Rondo-$SYM2^A SYM2^A$ roots with *R.l.* bv. *viciae* strains 248, 248*nodO*⁻ and 248*nodX* harboring 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.* bv. *viciae nodX*-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 active in the first cell layer..

Table IV. Temperature shift experiments with spot-inoculated Rondo-*SYM2^A SYM2^A*

Rhizobium strain	1 day at 26°C		2 days at 26°C		3 days at 26°C	
	spots	nodules	spots	nodules	spots	nodules
248nodX	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.

SYM2^A does not enhance Nod factor degradation

In theory it is possible that *R.l.* bv. *viciae* strains harboring *nodX* are able to nodulate *SYM2^A* containing plants because the NodX modification might provide protection against Nod factor degrading activity encoded by *SYM2^A* (Firmin *et al.*, 1993). This hypothesis implies that Nod factors that do not harbor an acetyl group at their reducing end are less stable on *SYM2^A* harboring peas when compared to *SYM2^C* harboring peas. This hypothesis was tested by comparing the degradation of the labeled pentameric Nod factor [14C]NodRlv-V(Ac,C18:4) by the near isogenic lines Rondo-*SYM2^C SYM2^C* and Rondo-*SYM2^A SYM2^A*. Two, 5 day old, seedlings were incubated for 1, 3, 8 and 24 hours respectively in the presence of radioactive Nod factor after which the medium and the roots were collected. After extraction with *n*-butanol the root and medium extracts were analyzed by TLC and the presence of Nod factor and its degradation products was determined by quantification of the radioactivity. The rate of Nod factor degradation was not significantly different between Rondo-*SYM2^C SYM2^C* and Rondo-*SYM2^A SYM2^A* (Figure 5). Furthermore, the tetra-, tri- and di-meric Nod factor derivatives were formed in similar amounts (data not shown). Thus, the presence of the *SYM2*^{A} allele does not significantly enhance degradation of Nod factors lacking the NodX-mediated acetyl group. Comparison of the degradation activity of both genotypes of NodX acetylated Nod factors showed similar results (data not shown), indicating that *SYM2*^{A} is unlikely to be involved in a general breakdown of Nod factors.

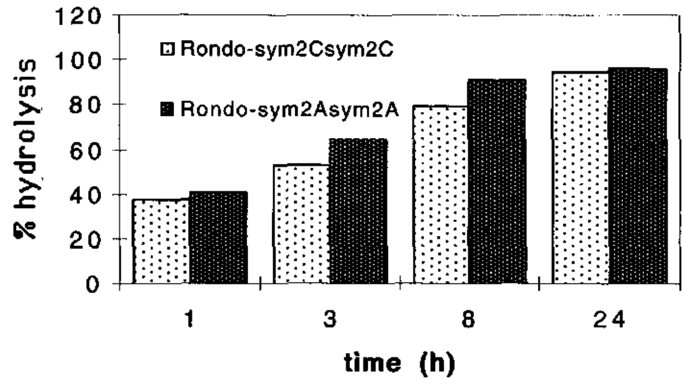

Figure 5. Degradation of [14C]NodRlv-V(Ac,C18:4) by roots of Rondo-*SYM2*^{C}*SYM2*^{C} and Rondo-*SYM2*^{A}*SYM2*^{A}. Two 5 day old pea seedlings were incubated in 4 ml medium with 25,000 cpm (6 x 10-7 M) labeled Nod factor for 1, 3, 8 and 24 hours. Shown are the average data of an in duplo performed experiment. 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.

DISCUSSION

Here we show that Nod factors produced by *R.l.* bv. *viciae* strains lacking *nodX* are perceived in the incompatible interaction with *SYM2*^{A} harboring peas, where they induce Nod factor specific responses. *R.l.* bv. *viciae* strains lacking or harboring *nodX* induce with a similar efficiency the expression of the early nodulin gene *ENOD12* in the epidermis and the formation of a nodule primordium in the inner cortex. The *SYM2*^{A} allele appears to confer a very specific block in formation and growth of infection threads in the epidermis. When rhizobia produce the NodX substituted Nod factors infection thread formation takes place efficiently on *SYM2*^{A} harboring plants.

NodO stimulates Nod factor induced infection

The bacterial NodO protein can fully compensate for the lack of NodX-mediated substitution on Nod factors of *R.l.* bv. *viciae* strains in the interaction with heterozygous *SYM2*^{A} *SYM2*^{C} plants, whereas only a partial compensation is achieved in the interaction with homozygous *SYM2*^{A}*SYM2*^{A} plants. Previously, it was shown that *nodO* can compensate for the absence of the highly unsaturated acyl chain (C18:4) in Nod factors from *R.l.* bv. *viciae* strain 248*nodE*$^{-}$ (Economou *et al.*, 1994). *R.l.* bv. *viciae* strain 248*nodE*$^{-}$*nodO*$^{-}$ is seriously hampered in nodule formation on pea, while *R.l.* bv. *viciae* strain 248*nodE*$^{-}$*nodO*$^{+}$ has similar abilities to induce nodule formation as the wild type strain (Economou *et al.*, 1994). These studies demonstrated that NodO can stimulate nodule formation, but it remained unclear which step(s) of nodulation were affected. Since the *SYM2*^{A} allele confers inhibition of infection thread growth, which can partially be overcome by *nodO*, it can be concluded that NodO at least stimulates the infection process. Hence it is well probable that in *R.l.* bv. *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.* bv. *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 (Figure 4). Furthermore, using the temperature sensitive nature of the *SYM2*^{A} phenotype, we showed that this gene has its effect only during the first days of the interaction. Taken together these data strongly suggest that *SYM2*^{A} is active in the epidermis, but it is unable to confer a block upon nodulation once the infection thread has reached the cortical cells.

strains and plasmids used in this study are listed Table V. The plasmids pMP225, pIJ1089, pXLGD4, pMW1071 and pMW2102 were transferred to *R.l.* bv. *viciae* strains using triparental mating with pRK2013 as a helper plasmid (Ditta *et al.*, 1980). The *R.l.* bv. *viciae* strains 248*nodO⁻*, 248*rhiA⁻* and 248*rhiR⁻* were made by crossing in derivatives 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).

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

Rhizobium strains	Relevant characteristics	Reference
248	*R.l.* bv. *viciae* strain containing pRL1JI	Josey *et al.* (1979)
248*nodX*	248 carrying pMW1071 or pMW2102	Kozik *et al.* (1995);this study
248^c	(1391) strain 248-Rif^R cured of its Sym plasmid pRL1JI	Schlaman *et al.* (1992)
248^c.pMP225	1391 carrying pMP225	This study
248^c.pIJ1089	1391 carrying pIJ1089	This study
248*nodO⁻*	1391/pRL1JInodO_{94}::Tn5	This study
248*rhiA⁻*	1391/pRL1JIrhiA_4::Tn5	This study
248*rhiR⁻*	1391/pRL1JIrhiR_1::Tn5	This study
248*nodO⁻nodX*	1391/pRL1JInodO_{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/pRL1JInodO_{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. bv. 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)

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) 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 ($A600$ = 0.5). The position was marked in the agar using sterile ink.

For the quantification of infection threads *lacZ* containing *R.l.* bv. *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% glutaraldehyde + 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 (Wehrheim, Germany).

RT-PCR to quantify ENOD12 expression

Pea plants were cultured as described by Bisseling *et al.* (1978). Four day old seedlings were inoculated (1 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 DNaseI (Promega) treatment. cDNA was made from 2.5 µg total RNA in a volume of 20 µl of 10 mM Tris/Cl pH 8.8, 50 mM KCl, 5 mM MgCl2, 1 mM dNTPs, 1 µg oligo dT12-18 (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 µl of the cDNA solution in 10 mM Tris/Cl pH 8.3, 50 mM KCl, 2.5 mM MgCl2 100 µm dNTPs, 50 ng primer each and 1 U Taq polymerase (Bhoeringer) in a total volume of 50 µl. *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-f: 5'-TCACTAGTGTTGTTCCTTGC-3', psENOD12A-r: 5'-CCATAAGATGGTTTGTCACG-3' to amplify only *psENOD12A*, and UBIQ-f: 5'- ATGCAGAT$^{C}/_{T}$TTTGTGAAGAC-3', UBIQ-r: 5'-ACCACCACG$^{G}/_{A}$AGACGGAG-3' to amplify Ubiquitin. The amplified DNA samples were separated on a 1.6% agarose gel and after alkaline blotting to a nylon membrane (Hybond-N+, Amersham) hybridized to 32P-labelled *psENOD12A* or *ubiquitin* DNA probes.

Degradation of Nod factors

The Nod factor NodRlv-V(Ac,C18:4) was labeled with [14C]acetate using the Nod factor overproducing *R.l.* bv. *viciae* strain 248c.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 hours. 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).

RESTRICTION OF HOST RANGE BY THE *SYM2^A* ALLELE OF AFGHAN PEA IS NON-SPECIFIC FOR THE TYPE OF MODIFICATION AT THE REDUCING TERMINUS OF NODULATION SIGNALS

THE DATA PRESENTED IN THIS CHAPTER ARE PART OF THE PUBLICATION:
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***Rhizobium leguminosarum* bv. *viciae* strains producing lipo-chitin oligosaccharides (LCOs) that are *O*-acetylated at the reducing terminus are required for nodulation of wild pea cultivars originating from Afghanistan which possess the *SYM2^A* allele. The *O*-acetylation of the reducing sugar of LCOs is mediated by the bacterial *nodX* gene which presumably encodes an acetyltransferase. We found that for nodulation on Afghan pea cultivars and *SYM2^A*-introgression lines the *nodX* gene can be functionally replaced by the *nodZ* gene of *Bradyrhizobium japonicum*, which encodes a fucosyltransferase which fucosylates the reducing terminus of LCOs.**

Within the cross-inoculation group of *Rhizobium leguminosarum* bv. *viciae* (*R. l.* bv *viciae*) a cultivar specificity exists in that some primitive pea (*Pisum sativum*) cultivars originating from the Middle East (e.g. Afghanistan, Iran, Turkey, Israel; however, here collectively called Afghan peas), are not nodulated by the ordinary European and North American strains, but require *R. l.* bv *viciae* strains from the Middle East for the symbiosis (Govorov 1928; Govorov 1937; Lie 1978). The resistance of Afghan peas to nodulation was found to be controlled by the *SYM2^A* allele, which is involved in early stages of the infection process (Lie 1984; Kozik *et al.* 1995; Geurts *et al.* 1997). *R.. l.* bv. *viciae* strains able to nodulate Afghan peas were isolated first from soils of Turkey, e.g strain TOM (Winarno & Lie, 1979), and later from different geographic regions of the world (Denmark, China, India, Morocco, Yugoslavia, Russia) (Ma & Iyer, 1990). It was shown that the ability to nodulate Afghan peas in strain TOM is conferred by the *nodX* gene, which is located downstream of the *nodABCIJ* genes, indicating a gene-for-gene relationship (Davis *et al.*, 1988; Geurts *et al.*, 1997). The function of the host-specific gene *nodX*, which is present in all *R. l. bv viciae* strains nodulating Afghan peas, is to *O*-acetylate lipo-chitin oligosaccharides (LCOs; also called Nod factors) at their reducing terminus (Firmin *et al.*, 1993). As a consequence, it has become clear that the acetylation of the reducing terminus of Nod factor of *R. l.* bv. *viciae* is necessary to achieve infection on *SYM2^A*-harboring peas, leading to successful nodulation (Firmin *et al.*, 1993; Kozik *et al.*, 1995; Geurts *et al.*, 1997).

In order to test the structural requirements of Nod factors for nodulation of peas containing the *SYM2^A* allele, we have constructed a set of strains carrying additional *nod* genes on separate plasmids. As a uniform background for the introduction of *nod* genes *R. l.* bv. *viciae* strain 248 was used, which nodulates European peas (homozygote *SYM2^C*) efficiently, but fails to nodulate pea lines homozygote for $SYM2^A$. The following genes were introduced into strain 248 on plasmids of different incompatibility groups: the *nodX* gene from *R. l.* bv. *viciae* strain TOM and the *nodZ* gene from *Bradyrhizobium japonicum,* which links a fucosyl group to the reducing terminus of LCOs. The presence of introduced *nod* genes in transconjugant strains was in all cases confirmed by thin layer chromatography (TLC) of ^{14}C-labelled LCOs as previously described (López-Lara *et al.,* 1995; Spaink *et al.,* 1995) and by PCR (data not shown).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevantcharacteristics	Sourceorreference
R. l. bv viciae		
248	R.l. bv.viciae wild type	(Josey *et al.* 1979)
248 nodO::Tn5	1391 carrying pRL1JInodO_{94} ::Tn5	(Geurts *et al.* 1997)
Plasmids		
pRL1JI	Sym plasmid of R.. l. bv.viciae strain 248	(Johnston *et al.* 1978)
pRK2013	IncColE1, Tra^+, Km^R	(Ditta *et al.* 1980)
pMP2450	IncP, contains pA-nodZ, Tc^R	(López-Lara *et al.* 1996)
pMW1071	IncP, contains pA-nodX, Tc^R	(Kozik *et al.* 1995)

Abbreviations: Tc^R, Rif ^{R}, and Km^R: tetracycline, rifampicin, and kanamycin resistance, respectively; pA, promoter of *nodA* gene of *R. l.* bv *viciae*; Inc, plasmid incompatibility group; *nodO94*::Tn5, Tn5 mutation in the *nodO* gene; Tra^+, region of conjugation transfer. Rhizobial strains were grown on B^- medium (van Brussel *et al.,* 1977). Transconjugants were selected on B^- media supplemented with 2 mg/l tetracycline for IncP plasmids.

To test whether the transconjugant strains are able to nodulate $SYM2^A$ harboring peas, the two Afghan pea lines L2150 (also known as cv. Afghanistan) and L6559 and the $SYM2^A$ introgression line 37(1)2 were inoculated in a gravel based nodulation assay (Raggio and Raggio, 1956). Line 37(1)2 resulted from crossing of the European line NGB1238 with the Afghan line L2150, followed by 6-7 selfcrosses with selection of plants with nodulation-minus phenotype upon inoculation by European strains. Nodules were scored three weeks after inoculation (Table 2). The *nodX*-containing strains induced nodules on all pea lines tested. Surprisingly, strains that contained the nodZ gene also were able to elicit nodules on *SYM2^A* harboring peas.

To determine the relative number of bacteria harboring plasmids inside the nodules, we have isolated bacteria from nodules and tested the frequency of antibiotic resistance. About 70-80% of isolated clones were resistant to the tested antibiotics. Since the IncP plasmids used in this study are lost relatively rapid in the absence of antibiotics (data not shown) these results indicate that plasmids carrying *nodX* or *nodZ* genes conferred a selective advantage during the infection process.

Table 2. Number of nodules on wild type Afghan and *SYM2^A* introgression pea lines inoculated with isogenic *R. l.* bv. *viciae* strains.

R..l. bv. viciae strain/plasmid	Afghan pea line L2150	Afghan pea line L6556	Introgression line 37(1)2
248	0	0	1±1
248.pMW1071 (nodX)	9±3	9±2	16±8
248.pMP2450 (nodZ)	8±2	7±2	5±1

A minimum of 6 plants was grown in a gravel-based assay. For this assay seeds of pea (*Pisum sativum* L.) were surface - sterilized for 5-7 min in concentrated sulfuric acid, thoroughly washed several times with sterile water and allowed to germinate on minimal medium solidified with agar. Three days old seedlings were transferred into sterile 5 - liter glass beakers filled with red gravel and watered with Raggio nutrient solution (Raggio & Raggio, 1956). Each pea plant was inoculated with 500 ml of a suspension of the freshly grown rhizobia in Jensen medium (van Brussel *et al.,* 1982) diluted up to an OD620 value of 0.1.

The gene *nodO* encodes a secreted protein that is not involved in LCO synthesis or secretion, but, it may partially compensate the lack of genes participating in LCO modification (Downie and Surin, 1990; Economou *et al.,* 1994; Sutton *et al.,* 1994; Van Rhijn *et al.,* 1996). Wild type strain 248, harboring an active *nodO* gene, sporadically triggers infections on *SYM2^A* introgression lines, leading to the formation of functional nodules (Table 2), whereas a *nodO* mutant is absolutely unsuccessful in triggering successful infections (Geurts *et al.,* 1997). We have tested whether *nodO* contributes to the nodulation ability of the strains producing fucosylated Nod factors. Therefor we introduced the plasmid pMP2450 carrying the *nodZ* gene into strain 248 with a defective *nodO* gene. The analysis of the NodO effect was performed by using a nodulation assay in which, instead of gravel, the pea plants were grown on perlite. In this assay the number of nodules obtained is higher than on gravel, facilitating the detection of a *nodO⁻* related phenotype. The cultivar (cv.) Rondo (homozygote for $SYM2^C$) and the $SYM2^A$ introgression line Rondo-A5.4.3 were inoculated with the strains 248, 248.pMW1071 (*nodX)* and 248.pMP2450 *(nodZ)* as well as with their *nodO*::Tn5 counterparts. Near isogenic lines cv. Rondo ($SYM2^C$) and the backcross line Rondo-A5.4.3 ($SYM2^A$) were described by Kozik *et al.* (1995). Introgression line Rondo-A5.4.3 resulted from crossing of pea L2150 (cv. Afghanistan) to European cv. Rondo *(*$SYM2^C$*)*, with subsequent 3 backcrosses to cv. Rondo. This line contains less introgressed DNA of Afghan line L2150 when compared to line 37(1)2.

Table 3.Nodule formation on near isogenic pea lines upon inoculation with *Rhizobium* strain harboring additional *nod* genes.

R. l. bv. viciae strain/plasmid	Rondo-A5.4.3 ($SYM2^A$)	cv. Rondo ($SYM2^C$)
248	2±1 (n=8)	50±4 (n=8)
248.pMW1071(*nodX*)	51±4 (n=8)	50±5 (n=8)
248.pMP2450 (*nodZ*)	50±4 (n=8)	48±2 (n=7)
248nodO::Tn5	0 (n=18)	46±2 (n=18)
248nodO::Tn5.pMW1071(*nodX*)	51±4 (n=18)	41±3 (n=18)
248nodO::Tn5.pMP2450(*nodZ*)	28±2 (n=18)	45±3 (n=17)

Deviations are given for the number of plants indicated. For this assay pea seeds were surface sterilized (15 min. commercial bleach, 15 min. 7% H_2O_2, thoroughly washed several times with sterile water) and sown in modified Leonard jars, which consist of a plastic (coffee)beaker of about 100 ml filled with perlite (Lie *et al.*, 1988). This beaker is put into a 360 ml preservation jar, which serves as the reservoir for the nutrient solution (Fahraeus, 1957). A foam plastic wick is inserted through a slit made in the bottom of the beaker. Before use the Leonard jars were kept for 5 days at 70°C. After sowing, the pea seeds were inoculated with 2 ml freshly grown rhizobia of OD_{620}=0.1, and covered with a layer of sterilized, fine gravel to prevent contamination.

Nodules were scored three weeks after inoculation (Table 3). From the results of nodulation experiments it is apparent that in the presence of *nodO* there is no difference in nodulation efficiency between the *nodX* or the *nodZ* harboring strain; 248*nodZ* nodulates the Rondo $SYM2^A$ introgression line A5.4.3 as efficient as cv Rondo ($SYM2^C$). In the absence of *nodO*, the *nodZ* harboring strain was also able to elicit nodules on Rondo-A5.4.3, although at a slightly lower frequency when compared to 248*nodO*::Tn5.pMW1071(*nodX*) (Table 3). Therefore, we can conclude that the presence of a fucosyl decoration at the C6 position of the reducing terminal glucosamine of the Nod factors is sufficient to overrule the block on nodulation independently from *nodO*.

In this work we have shown that fucosylation of the reducing terminus of Nod factors confers on the bacteria an ability to nodulate peas carrying the *SYM2*^{A} allele. The mechanisms of Nod factor perception by a leguminous host plant remain unclear. Basically, there could be two possibilities how a plant perceives LCOs with different modifications. Firstly, differently decorated LCOs may fit to different plant receptors. In this case, the stringent requirements to LCO structure should be dictated by more than one receptor. Alternatively, different Nod factors might be recognized by the same receptor but their stability may vary depending on the host plant. There is evidence that decorations of Nod factor backbone such as *nodH*-mediated sulfation, *nodEF*- mediated acylation and others may improve their stability against plant chitinases which cause degradation of LCO molecules (Staehelin *et al.*, 1994). Our results show that in case of Afghan peas (*SYM2*^{A} allele) the requirements for LCO structures are not very strict, since apparently a fucosyl group can functionally replace the structurally different *O*-acetyl group for infection and nodulation. This observation is not in favor for the hypothesis of involvement of the modifications of the reducing terminus for specific receptor-ligand interaction, but it rather seems to support the second possibility- increased stability of LCOs towards plant chitinases. On the other hand, studies on the degradation rate of monoacetylated Nod factors by European and Afghan peas did not reveal any differences in degrading activity between root exudates of *SYM2*^{A}- and *SYM2*^{C}- containing lines, suggesting that Afghan peas do not possess specific chitinase activity which destroy monoacetylated LCOs faster than doubly acetylated LCOs. To get a better insight into the mechanisms of host range restriction by Afghan peas, it would be interesting to compare in more detail (preferably *in situ*) the relative stability of mono- and double-acetylated Nod factors towards degradation by plant enzymes in *SYM2*^{A} and *SYM2*^{C} homozygous backgrounds.

THE PEA MUTANT XIM-1 SHOWS A *NODX* INDEPENDENT NODULATION PHENOTYPE WITH *RHIZOBIUM LEGUMINOSARUM* BV *VICIAE*

René Geurts, Olga Kulikova, Marja Moerman and Ton Bisseling

The in nature found rhizobial strains which are compatible with *SYM2*^{A} harboring peas contain an additional *nod* gene; *nodX*. This gene *O*-acetylates the reducing terminus of the bacterium secreted Nod factors. In an incompatible interaction with *R. leguminosarum* bv *viciae* strains secreting Nod factors that lack the proper substitution, infection thread formation is initiated, but these threads get arrested in the root epidermis. We have isolated a mutant (Xim-1) of *SYM2*^{A} containing pea, which shows a *nodX* independent nodulation. In case this is a 'knock out' mutation it implicates that *SYM2*^{A} peas have in addition to a control at infection thread initiation, a mechanism controlling infection thread growth. The activity of the latter mechanism depends on the structure of the reducing terminal sugar residue of Nod factors. The mutation in Xim-1 has eliminated this mechanism, resulting in a *nodX* independent nodulation phenotype.

Bacteria of the genera *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium* (here collectively called rhizobia) secrete lipo-chitin oligosaccharides (LCOs), called nodulation (Nod) factors, when they grow in the vicinity of the root system of their leguminous host plants. Nod factors consist in general of 4 or 5 1,4-linked D-glucosamine units, of which the terminal non-reducing residue is mono-N-acylated. The structure of the acyl chain can vary between different *Rhizobium* species or biovars. Furthermore, the terminal glucosamine units can have species, biovar or even strain specific decorations (For review see: Carlson *et al.*, 1994).

Nod factors play a pivotal role in the symbiotic relation of *Rhizobium* bacteria and their host plants. They redirect the developmental fate of root cortical cells, which form nodule primordia. Furthermore, Nod factors play a crucial role in the infection process. *Rhizobium* bacteria enter the root of their host plant via an inward growing tubular structure, called infection thread. The formation of infection threads shows a very stringent dependence on Nod factor structure. The structure of the acyl chain as well as the presence specific substitutions at the terminal glucosamine residues are essential for infection thread formation (Ardourel *et al.*, 1994; Geurts *et al.*, 1997). For example, in the interaction between *Sinorhizobium meliloti* and alfalfa (*Medicago sativa*) only bacteria secreting a Nod factor carrying two double unsaturated bounds in the acyl chain, which is 16 C-atoms in length (C16:2), and an acetyl group at the non-reducing and an sulfate at the reducing terminal sugar, efficiently form infection threads (Ardourel *et al.*, 1994). Strikingly, other epidermal responses like the deformation of root hairs or the expression of the Nod factor inducible early nodulin gene *ENOD12* are not affected. This has led to the hypothesis that in the root epidermis more than one Nod factor perception mechanism will be present (Ardourel *et al.*, 1994).

In pea (*Pisum sativum*) the gene *SYM2*, identified in various primitive pea ecotypes (e.g. ecotype Afghanistan), proved to be involved in controlling infection thread formation in relation to Nod factor structure (Geurts *et al.*, 1997). Plants containing the *SYM2* gene of the ecotype Afghanistan ($SYM2^A$) can be efficiently infected, and subsequently nodulated, by *Rhizobium leguminosarum* biovar *viciae* (*R. l.* bv *viciae*) strains that harbor the nodulation gene *nodX* (Kozik *et al.*, 1995). This bacterial gene encodes an *O*-acetyl transferase that acetylates the reducing end of pentameric Nod factors (Firmin *et al.*, 1993). However, the host range restriction due to $SYM2^A$ does not show a very strict relation with the nature of the decoration at the reducing glucosamine subunit of the Nod factors. *R. l.* bv *viciae* strains harboring the fucosyltransferase encoding *nodZ* gene of *Bradyrhizobium japonicum*, by which they secrete Nod factors with a fucosyl group at the reducing terminus, can also infect $SYM2^A$ harboring peas (Ovtsyna *et al.*, 1998).

R.l. bv *viciae* strains producing Nod factors without a modification at the reducing terminus only rarely form infection threads on $SYM2^A$ harboring plants (Geurts *et al.*, 1997). As a consequence $SYM2^A$ plants nodulate only very poorly when such strains are used as an inoculum, leading to an incompatible interaction. Such an incompatible interaction showed to be temperature sensitive (Kozik *et al.*, 1995; Geurts *et al.*, 1997). High temperatures (26°C) during the first days of the interactions significantly increase the number of infected nodules. Furthermore, additional rhizobial genes influence the incompatibility. Genetic analysis of the *nodX* (and *nodZ*) lacking *R. l.* bv *viciae* strain 248 showed that the bacterial *nodO* gene is contributing to the few successful interactions of this strain on $SYM2^A$ harboring peas (Geurts *et al.*, 1997). NodO is a secreted protein which is neither involved in Nod factor production nor in secretion, but it is postulated to be integrated into the plasma membrane of the host cell, where it could form ion channels (Sutton *et al.*, 1994). Its complementation abilities for Nod factor structure deficiency it not unique for the reducing end terminus. In *R. l.* bv *viciae* NodO can also complement for other structural deficiencies like the lack of unsaturated bounds in the acyl chain (Economou *et al.*, 1994).

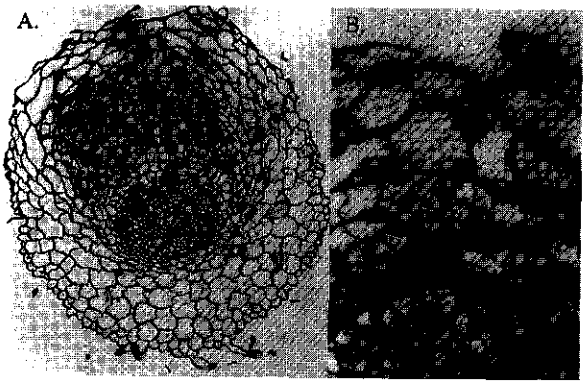

Figure 1. A cross section of a root of the Rondo-*SYM2*^{A} line A.5.4.3 (Kozik *et al.*, 1995), 10 days after spot inoculation with *R. l.* bv. *viciae* strain 248. The inner cortical cells divided to form a nodule primordium, which however stayed uninfected since the infection thread is aborted in the first cortical cell (**A**). In this cell the cytoplasm has been rearranged and forms a so-called pre-infection thread (Van Brussel *et al.*, 1992). This structure is normally used by the infection thread to traverse the cell. However, here its growth has stopped in the middle of a hypodermal cell (**B**).

Previously, we reported that in an incompatible interaction between *SYM2*^{A} carrying peas and *R. l.* bv *viciae* strains occasionally aborted infections could be observed (Figure 1; Geurts *et al*; 1997). This suggests that the incompatibility is not due to an inefficient initiation of infection, but rather that the plant controls the growth of already initiated infection threads. This model is depicted in figure 2. Infection thread formation is initiated upon recognition of Nod factor; visualized in the model with an arrow. However, infection thread growth is restrained by the hypothesized feed back mechanism. *R. l.* bv *viciae* can overrule this block by an unknown mechanism, which requires properly decorated Nod factors. If the bacterium is unable to produce these Nod factors, only a minority of the initial infection sites will lead to a successful infection.

The hypothesis of a negative control mechanism on infection thread formation can be tested by mutational analysis. A mutation in a component of the feedback mechanism should eliminate its infection thread growth controlling activity, resulting in successful infections with *R. l.* bv *viciae* stains producing sub-optimal Nod factors. To test this hypothesis we performed EMS mutagenesis on *SYM2*^{A} harboring peas and searched for *nodX* independent nodulation mutants. 10.000 seeds of the Rondo-*SYM2*^{A} line A.5.4.3 (Kozik *et al.*, 1995) were treated with 0.1% EMS for 8 hours and multiplied in soil. The M2 population of 60.000 individuals was grown in gravel, and inoculated with the *R. l.* bv. *viciae* strain 248 to select for nodulating plants. In total 6 potential mutants were isolated. One of these potential mutants, named Xim-1 (*nodX* independent *SYM2^A* nodulation) was further analyzed. At least one backross with the parental line Rondo-A5.4.3 and two rounds of self-fertilization where performed. Nodulation assays (Lie *et al.*, 1988) showed a stable *nodX* independent nodulation phenotype. Inoculation with *R. l.* bv *viciae* 248 or 248*nodO*::Tn5 led on average of 51±7 respectively 50±6 nodules (table I).

To determine whether a single locus is causing the Nod+ phenotype, Xim-1 was crossed with the Rondo-*SYM2^A* line A.5.6.9. (Kozik *et al.*, 1995). The F2 population of this cross (n= 69) segregates in a 1 to 3 ratio of Nod- plants and Nod+ plants (17 Nod- : 52 Nod+ plants) when inoculated with *R. l.* bv. *viciae* strain 248, which shows that only a single locus is causing the *nodX* independent nodulation phenotype that acts dominant when strain 248 is used.

Table 2. Number of nodules in cv. Rondo, Rondo-A.5.4.3 (*SYM2^A*) and the mutant Xim-1 inoculated with isogenic *R. l.* bv. *viciae* strains.

R. l. bv *viciae* strain	cv. Rondo (*SYM2^C*)	Rondo-A.5.4.3 (*SYM2^A*)	Xim-1
248	57±5	2±3	51±7
248.pMW2102 (*nodX*)	61±7	57±6	53±8
248nodO::Tn5	50±6	0	49±5

Deviations are given for the number of plants indicated. For this assay pea seeds were surface sterilized (15 min. commercial bleach, 15 min. 7% H_2O_2, thoroughly washed several times with sterile water) and sown in modified Leonard jars (Lie *et al.*, 1988). After sowing, the pea seeds were inoculated with 2 ml freshly grown rhizobia of OD_{620}=0.1.

The fact that a suppressor mutation of the *SYM2^A* phenotype could be identified, shows that *SYM2^A* peas contain an infection thread growth suppressing mechanism, of which the activity is Nod factor structure dependent (figure 2). In wild type peas harboring *SYM2^A*, e.g. Rondo-A5.4.3, this suppressor mechanism is overruled when *R. l.* bv. *viciae* produces Nod factors with an additional group at the reducing glucosamine residue. In the Xim-1 mutant this specific decoration of Nod factors is no longer required showing that the negative control mechanism is ineffective.

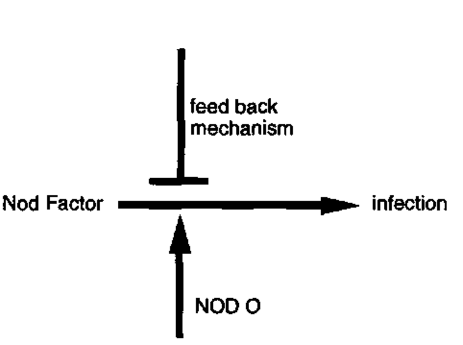

Figure 2. A model how rhizobal infection thread formation could be regulated in *SYM2*[A] harboring plants. It implies that the growth of infection threads, initiated by Nod factors, is controlled by a negatively acting feedbackmechanism. This infection thread controlling mechanism can be overruled by Nod factors with an appropriate decoration at the reducing terminal sugar residue; e.g. acetate or fucosyl. Furthermore other factors like the bacterial NodO protein have the ability, to overrule the infection thread controlling mechanism at least partially.

Whether the Xim-1 is mutated in a previously characterized *SYM* gene remains unclear. However, since the mutation in Xim-1 leads to a extended host range its is unlikely that the mutated gene is allelic to a *SYM* gene previously identified by mutational analysis, since such phenotype has not been reported. However, it can not be excluded that the *SYM2* gene is mutated.

Strikingly, the *xim-1* mutation shows a dominant nature. Standard explanations for such phenotype include the hypothesis that the encoded protein is part of a complex, which is poisoned by the mutant form. Alternatively, the mutated protein could be in a permanent active conformation.

Characterization of the ethylene perception mechanism in *Arabidopsis thaliana* showed that the identified ethylene receptors negatively regulate ethylene response, through inactivation of these proteins upon perception of ethylene (Hua & Meyerowitz, 1998). Since Nod factor structure is involved in the incompatibility mechanism controlling infection thread growth, it is possible that the XIM-1 protein is involved in a mechanism that senses the structure of Nod factors in the infection site, by which this pathway becomes inactivated. This model implies that infection thread formation involves Nod factor activity at two stages: the initiation of an infection thread as well as the suppression of the incompatibility response. Whereas a relatively wide range of Nod factors initiate the formation of infection threads, suppression of the incompatibility response requires a Nod factor with a more specific structure.

THE USE OF DIFFERENTIAL RNA DISPLAY TO ISOLATE CDNA BASED MARKERS LINKED TO THE *SYM2* LOCUS OF PEA

René Geurts, Olga Kulikova and Ton Bisseling

The *SYM2* gene of pea (*Pisum sativum*) is involved in controlling *Rhizobium* Nod factor induced infection tread formation. Cloning of this gene could give insight how leguminous host plants perceive Nod factors. However, a positional cloning approach requires a detailed genetic map position of the gene of interest. Here, we describe the use of differential RNA display to identify cDNA-based markers linked to the *SYM2* locus. By comparing root hair RNA of two near isogenic pea lines 4 different cDNAs linked to *SYM2* locus could be identified. One of these genes, encoding a putative receptor-kinase, showed tight linkage to *SYM2*. This gene, *W62*, is analyzed in more detail.

INTRODUCTION

The *SYM2^A^* allele that occurs in various primitive pea (*Pisum sativum*) ecotypes, e.g. Afghanistan pea, is involved in the mechanism by which *Rhizobium leguminosarum* bv *viciae* (*R. l.* bv *viciae*) infects the root. In some way, *SYM2^A^* activity depends on the structure of the Nod factor secreted by the bacterium (Geurts *et al.*, 1997, this thesis). Nod factors consist of a mono- acylated 1,4-linked D-glucosamine tetra- or pentameric oligomer and they play a pivotal role in the symbiotic interaction of the bacterium and its host plants (for review see: Dénarié *et al.*, 1996; Long, 1996). Successful infection of *SYM2^A^* harboring peas is achieved by *R.l.* bv *viciae* strains producing Nod factors with an acetyl decoration at the reducing sugar unit (Firmin *et al*, 1993). However, the gene responsible for this specific Nod factor acetylation, *nodX*, is not widely spread among strains of *R. l.* bv *viciae*. Strains lacking *nodX* are unable to infect *SYM2^A^* harboring peas, whereas they induce other responses, like nodule primordium formation (Geurts *et al.*, 1997).

Cloning of the *SYM2* gene could give more insight in the host mechanism that controls the infection process in a Nod factor structure dependent manner. Genes with a known phenotype can be isolated e.g. by positional cloning or transposon tagging. Since a transposon tagging system is not available for pea, positional cloning seems to be the most suitable approach. This approach requires a detailed map of the region where the gene of interest is located. The *SYM2* locus is located in linkage group I in a cluster of about 40 cM containing several other genes that are involved in nodulation (Weeden *et al.* 1990, 1996, Ellis, 1993, Kozik *et al.*, 1995, 1996, Kozik, 1996). However, the available molecular map does not contain sufficient markers in this region to start a chromosome walk.

DNA fingerprinting methods, e.g. Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995), have successfully been used to create a more detailed genetic map. However, since the pea genome is relatively big, $3.8\text{-}4.8.10^9$ basepairs per haploid genome (Ellis, 1993), it can be expected that markers generated by AFLP mainly will involve repetitive DNA. Such markers will be difficult to use in chromosome walking and to avoid this problem, cDNA based markers may be used. Several methods to identify sequence polymorphisms in cDNAs are available (Liang & Pardee, 1992; Welsh *et al.*, 1992; Hannappel *et al.*, 1995; Bachem *et al.*, 1996). Here, we describe the use of differential RNA display (Liang & Pardee, 1992) to identify cDNA based markers linked to the pea locus *SYM2*. This method was chosen for two reasons: First, differential display visualizes mainly 3'-untranslated regions of mRNAs, which in general is the most polymorphic region. Second, small amounts of starting material can be used. This allowed the comparison of root hair RNA of two near isogenic lines which, in theory, genetically differ only in the region around the *SYM2* locus. Root hair RNA is used, since it is probable that the SYM2 gene is expressed in the root epidermis (Geurts *et al.*, 1997).

By using differential display we identified 4 different genes, which are expressed in root hairs, and genetically linked to the *SYM2* locus. One of these genes, that showed the tightest linkage to SYM2, encodes a putative receptor-kinase. This gene, *W62*, is analyzed in more detail.

Results

Differential RNA display

The near isogenic pea lines used for the RNA display studies are cv. Sparkle and the Sparkle-*SYM2*^{A} introgression line BC-*sym2*. The latter was made by crossing cv Sparkle with Afghanistan L2150 followed by 6 back crossings with cv Sparkle (Kneen *et al.*, 1984, Thymnekh *et al.*, 1995b). Therefore, the genome of BC-*sym2* contains, in theory, less than 1% of Afghanistan DNA. We assumed that the *SYM2* gene is expressed in root hairs of uninoculated plants and therefore we isolated root hairs of 5 days old seedlings. Taking into account the theoretical considerations of Bauer *et al.* (1993) for a saturating screen and assuming that about 15,000 genes are expressed in root hair containing cells, at least 25 upstream primers in combination with 12 oligo(dT)12 anchor primers should be used in order to visualize 30,000 bands, representing 95% of all mRNAs. RNA was isolated from Sparkle and BC-*sym2* and this was used to synthesize cDNA with 12 different anchor primers. The 12 cDNA samples were used as template in PCR reactions containing the corresponding anchor primer, an arbitrary decamere primer and α-^{33}P labeled dATP (Liang & Pardee, 1992, Material & Methods). The PCR reaction mix was separated on a denaturating 6% acrylamide gel, and subsequently autoradiographed (see Material & Methods). All reactions were performed in duplo. By using 30 arbitrary decamere primers, more than 30,000 bands representing mRNAs were visualized. In total 145 polymorphisms were found in duplo. The corresponding cDNA fragments were isolated from the gel.

Confirmation of polymorphism by RFLP analysis

Since the differential RNA display technique generates a relatively high number of false positive fragments (Bauer *et al.*, 1993, Callard *et al.*, 1994), we tested the polymorphism of the differential cDNA by RFLP analysis using genomic DNA of Sparkle, BC-*sym2* and the donor line Afghanistan L2150. Initially, we used the uncloned differential cDNA fragments. However, most of the PCR products contained more than one (similar sized) DNA fragment, leading to complex hybridization patterns in the RFLP analyses (data not shown). Therefore the isolated cDNA fragments were cloned. This was done for 65 randomly chosen fragments of the in total 145 isolated. By using dot-blot analysis the number of different clones obtained for each isolated band was determined. This number varied between 1 and 5. All unique clones were used as probe in a RFLP analysis using genomic DNA of Sparkle, Afghanistan and BC-*sym2* digested with HeaIII, RsaI, Sau3AI or TaqI respectively and in a few cases also BamHI, EcoRI or HindIII digested genomic DNA was used. A certain cDNA band was selected for further study if one of the cloned fragments showed a RFLP between cv Sparkle and BC-*sym2*. For 37 of the 65 selected cDNAs a RFLP between Sparkle and Afghanistan was observed. 17 out of these 37 showed a RFLP between Sparkle and BC-*sym2*. These latter 17 clones represent Afghanistan genes present in BC-*sym2*, but this gene does not necessarily have to be linked to the *SYM2* locus.

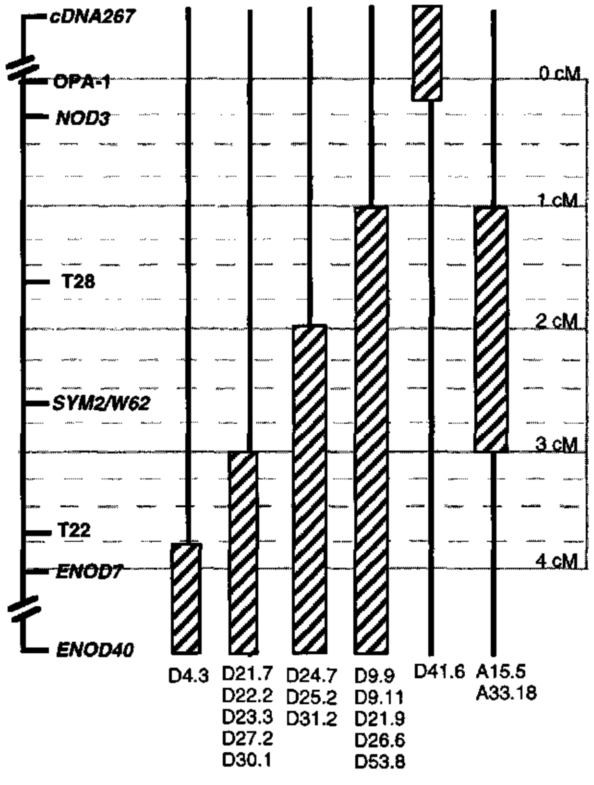

figure 1. Genetic map of the region around the pea *SYM2* locus. Also shown are the recombinant inbred lines (RILs; lines marked with 'D') and the Rondo-*SYM2*^{A} intogression lines A15.5 and A33.18 (Kozik, 1996). The RILs were selected by identifying cross-over events between the *NOD3* locus and the *SYM2* flanking markers OPA-1 and *ENOD7*, which map approximately 4 cM apart from each other (Kozik, 1996, see Material & Methods). These RILs were used to determine the map position of the by differentialRNA display identified cDNAs. The markers T22, T28 and W62 showed to be located between OPA-1 and *ENOD7*. T22 and T28 map approximately 1.3 respectively 0.8 cM apart from *SYM2*, whereas *W62* showed to be tightly linked to this locus.

Mapping of selected cDNA clones

To determine whether the 17 selected positive clones are linked to the *SYM2* locus a set of 15 Recombinant Inbred Lines (RILs) was constructed, which genetically dissect the region around *SYM2* (figure 1, see also Materials & Methods). In each of the RILs a cross-over event had occurred in the region between the *SYM2* flanking markers *PsENOD7* and *OPA-1*, which are approximately 4 cM apart (Kozik, 1996). So, in the RIL population on average 4 cross-over events per cM have occured.

The 15 RILs, their parental lines A.54 and Nod3, and the Rondo-*SYM2*^{A} introgression lines A.5.4.3, A.5.6.9, A.15.5 and A.33.18 (Kozik *et al.*, 1995; Kozik, 1996; figure 1) where used for RFLP analyses. Ten of the 17 cDNAs mapped in linkage group I around the *SYM2* locus, whereas the other 7 clones represent other regions of BC-*sym2* containing Afghanistan DNA. One of the 10 clones, W62.8, showed tight linkage with *SYM2*, since no cross-over between both loci had occurred in neither the RILs nor in the *SYM2*^{A} introgression lines. Two clones, T22 and T28 are flanking the *SYM2* locus, respectively for 1.3 and 0.8 cM (figure 1). The remaining 7 clones T3, T7, T12, T16, T17 T20 and T21 showed linkage with the marker *PsENOD7*. It is likely that these cDNA markers are located between *PsENOD7* and the more distal marker *PsENOD40* (Kozik, 1996). Strikingly, these 7 clones showed identical hybridization patterns, suggesting that they are cDNAs of the same gene. Sequencing of these clones showed that this was indeed the case (data not shown).

W62 encodes a putative receptor-kinase

W62.8 is 308 bp long and does not contain an open reading frame. Therefore it is likely that it represents a 3'-untranslated region. To determine the full sequence, a pea root hair cDNA library was screened with the W62.8 fragment as probe. Several clones were isolated and the longest clones were 2.2 kb long. Sequencing of several clones indicated that the isolated cDNAs originated from two (homologous) genes. One gene showed 100% homology with W62.8 and was named *W62*. The second gene showed some sequence polymorphisms. The latter gene was named *H62* (*HOMOLOG62*). Full size cDNA clones of *H62* and *W62* of cv Rondo harbored an open reading frame encoding a protein of respectively 620 and 617 amino acids, which have a similarity of 87.6%. A comparison of both protein sequences with all available sequences in the databases (NCBI blast) showed that the C-terminal part (300 amino acids) of both, *H62* and *W62*, is highly homologous to plant serine/threonine kinases, including the 11 subdomains characteristic for protein kinases (data not shown). *H62* and *W62* contain a N-terminal signal peptide of 19 amino acids. Furthermore, they both contain a stretch of 16 hydrophobic amino acids (amino acids 233-249) which could serve as transmembrane spanning domain. Therefore it can be hypothesized that both, *H62* and *W62,* are transmembrane receptor kinases. Strikingly, the putative extracellular domains (amino acids 20-232) do not show any significant homology with protein sequences available in the data base (NCBI-BLAST). It is also this region that is most polymorphic (82.5% similarity).

Only W62 is tightly linked to SYM2

As described above, linkage of *SYM2* and W62.8 has been studied by RFLP analysis. Since *H62* and *W62* are highly homologous, it can not be excluded that in stead of *W62*, *H62* is linked to *SYM2*. To discriminate between both genes, gene specific primers were designed (not shown). By digesting the amplified *H62* and *W62* products with either AluI (*H62*) or HaeIII (*W62*) the Rondo and Sparkle alleles could be distinguished from the Afghanistan allele. By using genomic DNA from the RIL population as well as $SYM2^A$ introgression lines it was shown that *W62* is tightly linked to $SYM2^A$, and *H62* is not.

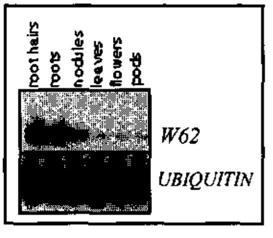

Figure 2. RT-PCR analysis of W62 expression in different tissue types. 25 cycles of PCR were performed using *W62* specific primers on cDNA made from RNA isolated from root hairs, roots, nodules, leaves, flowers or pods of Rondo. As a control, *Ubiquitin* mRNA was amplified (12 cycles).

W62 is expressed in Roots and Root hairs

W62 is isolated from root hair RNA. To determine whether the gene is also expressed in other tissues, RNA from root hairs, roots, nodules, leaves, flowers and pods of cv Rondo was isolated. Expression of *W62* in these tissues was determined by RT-PCR by performing 25 cycles by using specific primers. No expression could be detected in the leaves, flowers and pods, whereas *W62* mRNA is clearly present in root hairs as well as roots (figure 2). In nodule RNA *W62* is present at a markedly lower level than in roots. Therefore it can not be excluded that the observed *W62* expression in nodules is caused by contamination of the isolate with pieces of root tissue.

DISCUSSION

By using differential RNA display, we identified 4 different root hair cDNAs of genes that are closely linked to the *SYM2* locus of pea. This demonstrates that RNA display in combination with introgression lines is a useful method to identify cDNA based markers that are linked to a gene of interest.

In the saturating screen using root hair RNA of the near isogenic pea lines cv Sparkle and the Sparkle-*SYM2*^{A} introgression line BC-*sym2* 145 polymorphic cDNAs were identified. This number is within the range of what could be expected, considering that in root hair containing cells approximately 15.000 genes are expressed, of which in BC-*sym2* probably 1% originates from Afghanistan.

Since the RNA display method generates in general a relatively high number of false positives, the identified polymorphisms require further analysis. We used RFLP analysis to determine whether the identified cDNAs were encoded in BC-*sym2* by genes originating from Afghanistan pea. By using 4 different restriction enzymes we could discriminate between the Afghanistan and Sparkle allele for 37 out of 65 cDNAs. Seventeen of those 37 indeed originated from Afghanistan DNA present in BC-*sym2*.

A major problem in identifying positive clones, is the identification of an RFLP between cv Sparkle and Afghanistan pea, which can be used for mapping. To overcome these problems, more polymorphic lines could be used. Alternatively, the number of differential cDNA from which the poylmorphisms has to be confirmed could be reduced by using lines with a smaller intogressed region.

One clone, W62.8, is tightly linked to the *SYM2* locus. Sequence comparisons showed that the *W62* gene encodes a putative receptor kinase. Strikingly, the putative extracellular receptor part does not show homology with other known (putative) receptor domains.

To clone *SYM2, W62* can serve as a starting point for a chromosome walk, since the gene is probably located within 0.5 cM of the *SYM2* locus. To perform chromosome walking a Yeast or Bacterial Artificial Chromosome (YAC or BAC) library would be required. Alternatively, the synteny between leguminous species could be used. Recently, macro synteny between *Medicago* spp. and pea throughout pea linkage group I was demonstrated (D.R. Cook, pers. com.). Since it is probable that *SYM2* is functioning in a more general mechanism controlling infection thread growth (this thesis), it is likely that *Medicago* spp. harbor a similar gene. The cDNA based molecular markers isolated by RNA display could be used to analyze the level of synteny around *SYM2* between pea and *Medicago truncatula*. If the degree of synteny shows to be relatively high, chromosome walking could be performed in *M. truncatula*, since it has a relatively small genome ($5x10^8$ bp) from which a BAC library is available (D.R. Cook, pers. com.).

MATERIAL & METHODS

Plant material

The differential RNA display is performed with cv Sparkle and the Sparkle-*SYM2^A^* introgression line BC-*sym2* (Kneen *et al.*, 1984, Temnykh *et al.*, 195b). For mapping of the isolated cDNAs a RIL population (see bellow) and the Rondo-$SYM2^A$ introgression lines A5.4.3, A5.6.9 (both Kozik *et al.*, 1995), A15.5 and A33.18 were used. The latter two lines are constructed from A.5.4.3 by performing an additional backcross and selection for a cross-over event between $SYM2^A$ and the RAPD marker OPA-1 (Kozik, 1996).

The RIL population is constructed by crossing the Rondo-$SYM2^A$ introgression line A54 with the Rondo EMS mutant Nod3 (Jacobsen *et al.*, 1984). A54 is generated by using Rondo as the recurrent parent for 4 cylces of back crosses and selecting for the $SYM2^A$ phenotype as well as for *SYM2* flanking markers of Afghanistan orgin. Nod3 was chosen for constructing a RIL population since it was shown previously that the *NOD3* locus is, like *SYM2*, located in linkage group I of pea (Temnykh *et al.*, 1995a, 1995b). From the F2 population of 882 plants, the hypernodulating individuals were selected (in total 190) upon inoculation with *R.l.* bv. *viciae* strain 248.pMW1071(*nodX*) (Kozik *et al.*, 1995). These 190 plants, homozygous for the recessive *nod3* allele, were screened for the *SYM2* flanking markers OPA-1 and *PsENOD7* (Kozik, 1996, Kozik *et al.*, 1996; figure 1) to select the individuals that contain the Afghanistan alleles for either one or both markers. 14 of them contained $PsENOD7^A$, whereas one plant contained $OPA\text{-}1^A$. None of the hypernodulating plants harbored both *SYM2* flanking markers of Afghanistan origin, confirming that *NOD3* is linked to *SYM2*. The 15 plants harboring one of the markers of Afghanistan origin were selfed and F3 individuals homozygous for this marker were inoculated with *R.l.* bv *viciae* strain 248 to determine which of this 15 lines harbor the $SYM2^A$ allele of Afghanistan origin. 8 lines containing $PsENOD7^A$ (D9.9, D9.11, D21.9, D24.7, D25.2, D26.6, D31.2 and D53.8) formed only a restricted number of nodules (0-10 nodules), whereas the remaining lines (D4.3, D21.7, D22.2, D23.3, D27.2, D30.1 and D41.6) showed a hypernodulating phenotype. This indicates that the 8 lines D9.9, D9.11, D21.9, D24.7, D25.2, D26.6, D31.2 and D53.8 harbor besides the *nod3* allele also the $SYM2^A$ allele of Afghanistan origin. This result was confirmed by crossing the 15 lines with the $SYM2^A$ line A5.6.9 to determine the nodulation behavior of the F1 plants upon inoculation with *R. l.* bv *viciae* strain 248.

Differential RNA display

Root hairs were harvested from 5 day old seedlings of cv Sparkle and BC-*sym2* (Gloudemans *et al.*, 1989). Total RNA was isolated according to Pawlowski *et al.* (1994) followed by a DNaseI (Promega) treatment. cDNA is synthesized by using 200 ng oligo(dT)12 anchor primers. The anchor consists out of 2 additional bases to the 3'-end of the oligo(dT)12. The cDNA population will be divided in 12 cDNA populations (AA, AC, AG, AT, CA, CC, CG, CT, GA, GC, GG and GT). Before cDNA synthesis the RNA was heated for 5 min. at 70°C followed by 3 min on ice. cDNA was made from 0.25 µg total RNA in a volume of 20 µl of 50 mM Tris/Cl pH 8.8, 75 mM KCl, 3 mM $MgCl_2$, 20 µM dNTPs, 10 mM DTT, 200 ng of the appropriate anchor primer, 17 U RNA guard (Pharmacia) and 300 U M-MuLV reverse transcriptase (GIBCO-BRL) at 35°C for 1 hour, followed by 5 min. 95°C.

For the PCR reaction 2 ul of the cDNA is used. The PCR reaction is performed in 10 mM Tris/Cl pH 8.3, 50 mM KCl, 1.25 mM $MgCl_2$, 2 µM dNTPs, 0.1 µl ^{33}P-dATP, 0.5 U Taq polymerase (Boehringer) containing 40 ng of arbitrary decamere primer (50-70% GC content) and the 200 ng of the appropriate anchor primer. The reaction mixture is heated for 5 min at 94°C followed by 40 cycles of 30 s 94°C, 1 min 38°C (ramp of 3s/°C from 38°C to 72°C) and 30 s 72°C, where after 5 min 72°C.

The PCR mixture is vacuum dried and resolved on resolved in 5 ul formamide containing 10 mM EDTA pH8.0, 0.1% xylene cyanol and 0.1% bromophenol blue. The PCR products are separated on denaturing 6% acrylamide gel. After electrophoreses the gel is transferred to 3MM paper, dried on a slab gel dryer (15 min, 80°C), and subsequently autoradiographed.

Differential cDNAs are isolated form the acryl amide gel (including the 3MM paper) and collected in siliconized eppendorf tube. The fragments are incubated in 100 µl TE (pH9.0), 10 min at room temperature, and subsequently boiled for 20 min. The supernatant is collected, filtered trough a glass wool filter and subsequently the DNA is ethanol precipitated in the presence of 0.3M Na-acetate (pH5.2), 5 µg glycogen (1 mg/ml) and redissolved in 10 µl H_2O. 4 ul of the isolated cDNA is used as template in a PCR reaction containing 10 mM Tris/Cl pH 8.3, 50 mM KCl), 2.5 mM, $MgCl_2$, 0.1 mM dNTPs, 1 U Taq polymerase (Boehringer) and the appropriate decamere (40 ng) and anchor primer (100 ng) in a final volume of 50 µl. The same PCR program as the initial differential RNA display reaction is performed. The reaction mixture is separated on a 1.5% agarose gel. DNA fragments are isolated from gel using a gel extraction kit (Qiagen) and cloned by using the pGEM-T vector (Promega). Dot blot analysis as described by Callard *et al.* (1994) was used to determine the number of fragments cloned.

RFLP analysis

Restriction enzyme digestion, gel electrophoresis, Southern blotting and filter hybridization (Hybond-N+ membrane, Amersham) are performed under standard conditions (Sambrook *et al.*, 1989).

Root hair library screening

The pea root hair cDNA library was constructed by Strategene in λZAPII vector system, using poly(A)+ RNA isolated from root hairs of cv Finalle uninoculated and inoculated with *R. leguminosarum* bv *viciae*. The sreeing was performed according to the manual of Strategene.

RT-PCR

Total RNA from several tissues was isolated according to Pawlowski *et al.* (1994) followed by a DNaseI (Promega) treatment. cDNA was made from 2.5 µg total RNA in a volume of 20 µl of 10 mM Tris/Cl pH 8.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 µg oligo dT_{12-18} (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 µl of the cDNA solution in 10 mM Tris/Cl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ 100 µM dNTPs, 50 ng primer each and 1 U Taq polymerase (Bhoeringer) in a total volume of 50 µl. The PCR program 30 s 94°C, 30 s 58°C, 30 s 72°C by using the primers UBIQ-f: 5'-ATGCAGAT$^C/_T$TTTGTGAAGAC-3', UBIQ-r: 5'-ACCACCACG$^G/_A$AGACGGAG-3' to amplify Ubiquitin and specific primer for *W62* (not shown) was used. 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 *W62* or ubiquitin DNA probes.

LEGUME NODULATION AND MYCORRHIZAE FORMATION; TWO EXTREMES IN HOST SPECIFICITY MEET

Catherine Albrecht, René Geurts and Ton Bisseling

(1998) *EMBO J.* (in press)

INTRODUCTION

Most higher plants have the ability to form arbuscular endomycorrhiza (AM); a symbiotic association of the plant root with fungi belonging to the order of *Glomales*. These fungi grow towards the inner cortical cells of the root where they differentiate into highly branched structures, the so-called arbuscules (Figure 1). In AM symbiosis, the fungus also forms hyphae outside the plant and these provide a connection between soil and the inner part of the plant and facilitate the uptake of nutrients like phosphate (for reviews see: Gianinazzi-Pearson, 1996; Harrison, 1997).

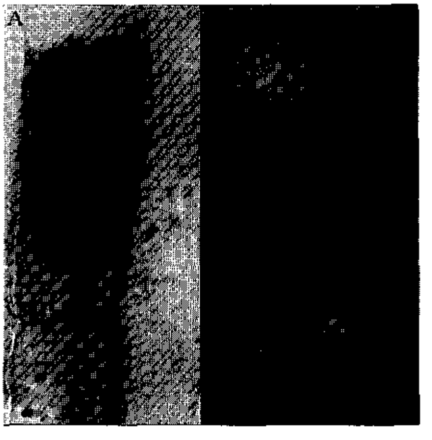

Figure 1. Pea root cortex infected by the mycorrhizal fungus *Glomus intraradices* (**A**) and a *Rhizobium leguminosarum* bv *viciae* induced infection thread in a vetch root hair (**B**). The AM fungus has entered the root intercellularly and it has formed an intercellular arbuscule (Picture A; trypanblue staining). In contrast, *Rhizobium* enters its host plant intracellularly via an infection thread (Picture B, Vetch root hair with an infection thread containing *R. leguminosarum* bv *viciae* bacteria expressing GFP (Spaink *et al.*, 1998)).

In contrast to AM formation, only a few plant species have the ability to interact symbiotically with bacteria of the genera *Azorhizobium, Bradyrhizobium, Rhizobium* and *Sinorhizobium* (here collectively called *Rhizobium*). This interaction is almost completely restricted to leguminous plants and results in the formation of a completely new organ, the root nodule. In these nodules the bacteria are hosted intracellularly and there they find the proper environment to reduce atmospheric nitrogen into ammonia, a source of nitrogen that can be used by the plant (for reviews see: Mylona *et al.*, 1995; Long, 1996).

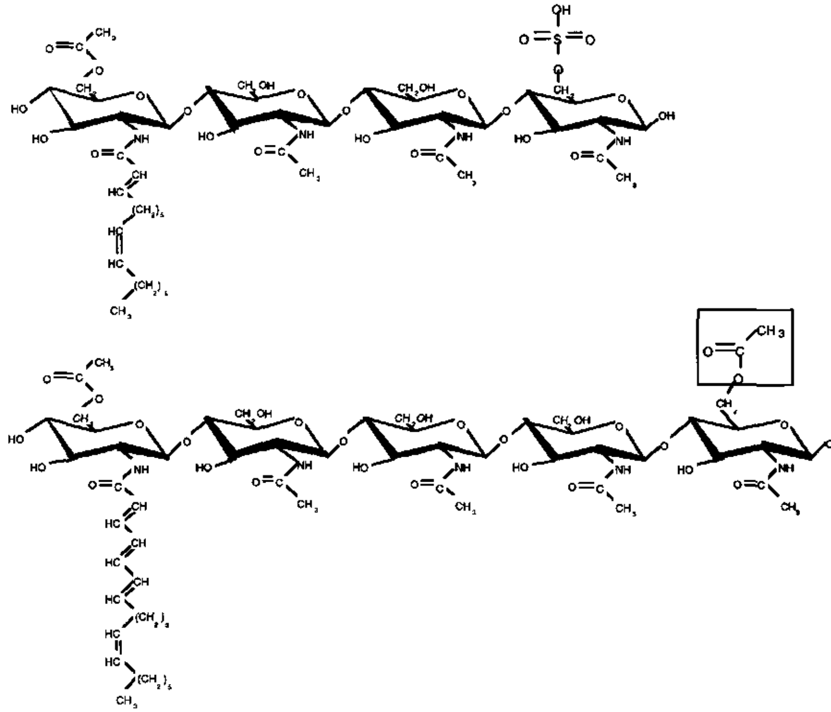

Figure 2. The major Nod factor produced by *Sinorhizobium meliloti* (top) and one of the *Rhizobium leguminosarum* bv *viciae* secreted factors (bottom). The major differencebetween both Nod factors concerns the specific decoration at the reducing terminal sugar unit and the structure of the acyl chain. The *S. meliloti* Nod factor contains 4 glucosamine units, an acyl chain of 16 C-atoms in length with two unsaturated bounds, an acetyl group at the non-reducing and a sulfate group at the reducing terminal sugar residue (Lerouge *et al.*, 1990). In contrast, *R. leguminosarum* bv *viciae* produces a mixture of factors that contains several major compounds. The length of the glucosamine backbone is 4 or 5 units carrying an acyl chain of 18 C-atoms either with 1 or 4 unsaturated bounds. These Nod factors can be *O*-acetylated at the non-reducing terminal sugar residue (Spaink *et al.*, 1991). Pentameric Nod factors can be partially acetylated at their reducing terminal sugar residue (gray box) when the bacterium contains the *nodX* gene, whereas in the absence of this *nod* gene no substitution is present (Firmin *et al.*, 1993).

At first glance the interactions of plants with rhizobia and AM fungi seem to have little in common. The induced morphological responses of the host plants are different. Furthermore, both interactions are extremes in terms of host specificity. Whereas in AM formation there is very little host specificity, the *Rhizobium*-legume interaction is highly specific. However, genetic studies have shown that several common steps are involved in establishing these symbioses (Duc *et al.*, 1989; Bradbury *et al.*, 1991; Gianinazzi-Pearson, 1996). Furthermore, some host genes are induced during the initial steps of both interactions. In this overview, first the *Rhizobium*-plant interaction is described with an emphasis on factors that determine the specificity of the interaction. In the second part, AM formation is described as well as the common aspects of both symbioses.

RHIZOBIUM INDUCED NODULE FORMATION

Nod factors

Root nodule formation involves growth responses in the epidermis as well as cortex of the root. This implies that the bacteria redirect the development of fully differentiated plant cells. The bacterial signals that set this in motion are the so-called nodulation (Nod) factors. Nod factors of the different *Rhizobium* species have a common basic structure; a β-1,4-linked N-acyl-D-glucosamine backbone of mostly 4 or 5 units, containing a fatty acid at the non-reducing terminal sugar (Figure 2, for review see: Carlson *et al.*, 1994; Long, 1996).

The bio-synthesis of the basic Nod factor structure is catalyzed by the bacterial NodA, NodB and NodC proteins. NodC is an N-acetylglucosaminyl-transferase and catalyses the synthesis of the chitin oligomer and controls the length of this backbone. The terminal non-reducing glucosamine unit of this oligomer is deacetylated by NodB, and subsequently substituted with an acyl chain by NodA. Several other Nod proteins, which can be specific for a certain *Rhizobium* species, modify a terminal sugar residue or determine the nature of the acyl chain (Carlson *et al.*, 1994). These modifications determine the biological activity and host specificity (see below) of Nod factors. As an example, Nod factors produced by *Sinorhizobium meliloti* (previously named *Rhizobium meliloti*) and *Rhizobium leguminosarum* biovar *viciae*, are shown in figure 2. The major difference between both Nod factors concerns the presence of a sulfate group at the reducing terminal sugar of the *S. meliloti* factor and the structure of the acyl chain.

Since bioactivity of Nod factors is controlled by their structure it is very likely that they are recognized by receptors of the host. However, such receptors have not been cloned. Biochemical studies showed that a few Nod factor binding proteins occur, but it is not yet clear whether this are Nod factor receptors (Bono *et al.*, 1995; Niebel *et al.*, 1997).

Nodulation process

Nod factor secreting rhizobia induce shepherd crook like curling of root hairs within 1-2 days after inoculation (Figure 1). *Rhizobium* uses the microenvironment within such curl to establish an infection site. They locally degrade the plant cell wall and enter the root hair via invagination of the plasmamembrane (Turgeon & Bauer, 1985). Vesicles are directed to the invaginated membrane, leading to the formation of an 'inward tip growing' tubular structure, the so-called infection thread (Figure 1). In general, Nod factors are not sufficient to trigger root hair curling and infection thread formation, but they play a crucial role in the infection process, since infections can only be initiated when the bacteria secrete specific Nod factors (Ardourel *et al.*, 1994; Geurts *et al.*, 1997).

Results obtained with bioassays provided some insight in the mechanism by which Nod factors alter the growth pattern of root hairs. Such studies have most extensively been done with vetch (*Vicia sativa*). Vetch root hairs that respond morphologically to the application of Nod factor have almost terminated growth. The morphological changes start with swelling of the root hair tip, which occurs within one hour after Nod factor application (Heidstra *et al.*, 1994). This swelling is the result of isotropic growth and it is accompanied by the formation of a calcium gradient at the plasma membrane and requires proteins synthesis (Vijn *et al.*, 1995; De Ruijter *et al.*, 1998). At these swollen tips, new tip growth is initiated and the cytoarchitecture of the resulting outgrowth shows a strong resemblance with that of normal growing root hairs. Such studies show that Nod factors can re-induce (tip) growth in root hairs. However, it remains unclear how Nod factor secreting bacteria can redirect tip growth in such way that shepherd crook like curls are formed. Furthermore, it remains to be solved whether/how the bacteria exploit and modify this growth process for infection thread formation.

Nod factor induced growth responses in root hairs are preceded by rapid physiological changes. These involve a rapid influx of calcium into the hairs (Gehring *et al.*, 1997; Felle *et al.*, 1998). Shortly after this calcium flux, an opposite directed flux of chloride ions occurs, which is accompanied by a depolarization of the root hair membrane (Ehrhardt *et al.*, 1992; Felle *et al.*, 1998). These processes are followed by an alkalinization of 0.2-0.3 pH units of the root hair cytoplasm (Felle *et al.*, 1996). Several minutes after the application of Nod factors, a regular oscillation of cytoplasmic calcium occurs around the nucleus (Ehrhardt *et al.*, 1996). Whether and how these physiological changes are involved in the alteration of growth of hairs is unknown.

Nod factors can mitotically activate clusters of cortical cells by which nodule primordia are formed. Which cortical cells will form a nodule primordium is determined by the host plant. Primordia are mainly formed opposite the proto-xylem poles and furthermore, the host species determines whether inner or outer cortical cells are involved in primordium formation. When the nodule primordia are formed in the outer cortical cell layers, like in soybean (*Glycine max*), the infection thread grows through the root hair and can immediately invade the primordium. In contrast, in legumes -e.g. pea (*Pisum sativum*) and *Medicago* species- in which nodules are formed in the inner cortex, the infection thread has to cross several cortical cell layers before reaching the primordium. The cortical cells that will be traversed by an infection thread, have reallocated their nuclei to their center from where microtubules and cytoplasmic strands are positioned anticlinically to the advancing infection thread (Van Brussel *et al.*, 1992). By using cell cycle phase specific markers it has been shown that these cells have entered the cell cycle but became arrested in G2, which indicates that the found cytological structure resembles a phragmoplast (Yang *et al.*, 1994). A radial array of cortical cells containing such phragmoplast-like structures provides a track for the infection thread to support its growth and to guide it to the primordia. The formation of phragmoplast-like structures during infection, shows that rhizobia recruit and modify a common process, namely cell division, and use this for a completely different purpose, the infection process.

When the infection thread reaches the primordium, the bacteria are released, and enter the cytoplasm via an invagination of the host cell membrane. Within the host cytoplasm the bacteria stay surrounded by a host membrane, and together they form a so-called symbiosome that divides. In this way the bacterial surface is never in direct contact with the plant cytoplasm. Upon infection the nodule primordia form simultaneously a meristem as well as the different tissues that form a nodule. In most species nodules are macroscopically visible 4-7 days after inoculation. The meristems maintain their mitotic activity, at least during a substantial part of the lifetime of a nodule, and they add cells to the different nodule tissues by which the organ grows.

HOST SPICIFICTY

Nod factor controlled host specificity

An intriguing property of the *Rhizobium*-legume interaction is its host specificity. Most rhizobia have a very narrow host range. For example *S. meliloti* is able to interact with *Medicago,* e.g. alfalfa (*Medicago sativa*), *Trigonella* and *Melilotus* species, whereas *R. leguminosarum* bv *viciae* forms nodules on species of the genera *Pisum* (e.g. pea), *Vicia* (e.g. vetch), *Lathyrus* and *Lens*.

In most cases host specificity is controlled at several levels but often synthesis and structure of Nod factors play a prominent role. The rhizobial genes encoding the enzymes involved in the biosynthesis of Nod factors are activated when the bacteria grow in the vicinity of the root system of their host plants. There, rhizobia sense signal molecules -in general flavonoids- secreted by the host. These molecules activate the constitutively formed transcriptional regulator(s) NodD that induces the expression of the other *nod* genes of which most are involved in the biosynthesis and secretion of Nod factors. The nature of the flavonoids secreted by the host can play a key role in controlling host specificity. For example, *Rhizobium etli* and *Rhizobium loti* secrete Nod factors with an identical structure, but their NodD's are only activated by elicitors secreted by their specific host plant. By introducing a gene encoding a constitutively active NodD protein these bacteria obtained a broader host range and could infect each others host plant (Cardenas *et al.*, 1995, Lopez-Lara *et al.*, 1995).

The length of the glucosamine backbone, the structure of the acyl chain and specific substitutions at the terminal sugar residues of the produced Nod factors all can contribute to the ability of the bacterium to nodulate their host plants. Changes in Nod factor structure, either by *nod* gene mutations or introduction of *nod* genes of other *Rhizobium* species, mostly results in a decreased nodulation potential of such strain, but in some cases it obtains the ability to nodulate non-host plants. For example, mutating the sulphotransferase encoding gene *nodH* of *S. meliloti*, which is involved in sulphation of the reducing sugar terminus of the Nod factor (Roche *et al.*, 1991), results in the inability of such strain to interact with alfalfa, but it has gained the ability to interact with vetch (Debellé *et al.*, 1988; Faucher *et al.*, 1989). Such experiments demonstrate that the plant discriminates the different *Rhizobium* species by recognizing their specific Nod factors. Some rhizobia have overcome this restriction by producing a great variety of Nod factors; e.g. *Rhizobium* sp. NGR234 (Price *et al.*, 1992), which can nodulate a broad host range encompassing legume species of more than 110 genera (Freiberg *et al.*, 1997) as well as the only non-legume known to establish a symbiosis with rhizobia, *Parasponia andersonni.*

Strict regulation of bacterial entry

Of all Nod factor controlled responses bacterial entry appears to be most stringently controlled. Infection thread initiation/growth in the root epidermis will only occur efficiently when the rhizobia produce Nod factors with a specific structure, whereas other responses do depend less on Nod factor structure. For example, *S. meliloti* strains mutated in either *nodL*, leading to an absence of the *O*-acetylation of the non-reducing terminal sugar residue, or mutated in *nodFE*, leading to the absence of specific unsaturated bounds in the acyl chain, are both seriously hampered in the infection process, whereas other Nod factor induced plant responses are not affected (Ardourel *et al.*, 1994).

A host gene that is specifically involved in controlling infection is *SYM2* (Geurts *et al.*, 1997). *SYM2* has first been identified in the wild pea ecotype Afghanistan (*SYM2*^{A}), where it inhibits nodulation of *R. leguminosarum* bv *viciae* strains lacking *nodX*. NodX *O*-acetylates pentameric *R. leguminosarum* bv *viciae* Nod factors at the reducing terminal sugar residue (Firmin *et al.*, 1993; figure 2), which does not harbor a specific substitution in the absence of *nodX* (Spaink *et al.*, 1991). Thus the activity of *SYM2* depends on the structure of the Nod factors secreted by the infecting rhizobia. However, the correlation between infection thread formation on *SYM2*^{A} harboring peas, and Nod factor structure is not very strict since *nodZ* of *Bradyrhizobium japonicum*, which *O*-fucosylates the reducing sugar unit of pentameric Nod factors, can in part replace *nodX* (Ovtsyna *et al.*, 1998).

In the incompatible interaction of *SYM2*^{A} harboring peas with *R. leguminosarum* bv *viciae* strains secreting Nod factors that lack the proper substitution, infection threads are formed, but they get arrested in the root epidermis. However, occasionally the incompatible interaction results in a successful infection leading to a nodule (Geurts *et al.*, 1997). This suggest that incompatibility is not due to inability to induce infection thread formation, but rather it is due to a defect in bypassing a negative acting mechanism controlling infection thread growth. Compatible strains appear to have this ability by producing a specifically decorated Nod factor.

The regulation of infection thread initiation/growth at different stages shows analogies with the regulation of pollen tube growth in self-incompatible plants. After initiation of pollen tube formation, the continuation of their growth is controlled by a self-incompatibility system. This mechanism is based on pollen-pistil recognition and aims to avoid self-fertilization of plants (for review see: Hiscock *et al.*, 1996). It seems probable that the incompatibility mechanism controlling infection thread growth also will involve the recognition of epitopes at the infection thread membrane. Since this incompatibility mechanism depends on Nod factor structure it is possible that the host senses the structure of Nod factors secreted by the bacteria inside the infection thread when the infection thread grows in the epidermal cell. The model implies that the infection thread formation involves Nod factor activity at two stages; the initiation of an infection thread as well as the suppression of the incompatibility response. Whereas the formation of infection threads is initiated by a relatively wide range of Nod factors, suppression of the incompatibility response requires a Nod factor with a more specific structure.

Alternatively, it is possible that Nod factors can induce the infection response at variable levels depending on the structure of the Nod factor. This would imply that the ability of the plant to block the growth of infection threads is lower when the infection response is higher.

Besides Nod factors, also other components can facilitate infection thread growth. For several plants it was shown that deficiencies in Nod factor structure, by which infections are hampered, can be complemented, in part, by the rhizobial NodO protein (Economou *et al.*, 1994; Geurts *et al.*, 1997; Vlassak *et al.*, 1998). NodO is a secreted protein, which is not involved in Nod factor production or secretion (Economou *et al.*, 1990). It has been shown that NodO is able to bind calcium and it can integrate into artificial membranes where it forms ion channels (Economou *et al.*, 1990; Sutton *et al.*, 1994). Therefore it has been postulated that it will form ion channels in the host plasma-membrane as well, where it could contribute to the suppression of the incompatibility mechanism or the induction of the infection responses.

Other host proteins involved in the regulation of infection thread growth are lectins. In the root, lectins are present in relatively low amounts and are localized on the external surface of elongated epidermal cells and on the tips of developing root hairs (Diaz *et al.*, 1995a; Van

Rhijn *et al.*, 1998). Introduction of the pea lectin into white clover (*Trifolium repens*) showed to increase nodulation by its host strain *R. leguminosarum* bv *trifolii* (Diaz *et al.*, 1995b). Strikingly, expression of heterologous lectins does also facilitate infection by non-host rhizobia, showing that lectins -in analogy to NodO- decrease the threshold level for the infection response (Diaz *et al.*, 1989, 1995b; Van Rhijn *et al.*, 1998). How this is achieved is not exactly known, however, data obtained with a lectin mutated in the carbohydrate binding site shows that this protein is unable to extend the host range and can also no longer facilitate attachment of the bacterium to the root hair surface (Van Rhijn *et al.*, 1998).

ENDOMYCORRHIZAL SYMBIOSIS

In nature, most plants do not simply have roots, but instead they have mycorrhizae; the symbiotic association of a fungus and plant roots. Arbuscular mycorrhiza (AM) are by far the most common root endosymbiotic association and are formed between the roots of most higher plants and fungi belonging to the order of *Glomales*. AM fungi are obligate biotrophs and strictly dependent on their host plant for their survival. Like in the *Rhizobium*-legume interaction, this symbiosis is set in motion by the exchange of signals between the two symbionts, although the nature and the mechanism of action of these molecules are unknown. Exudates of a host root, especially (iso) flavonoids, enhance spore germination, and elongation and branching of hyphae (Nair *et al.*, 1991; Giovannetti *et al.*, 1993). At the root surface the hyphae form swollen structures, named appressoria. The formation of appressoria is initiated upon contacting the cell wall of a root epidermal cell. In contrast appressoria are not formed when contacting cortical or vascular cell walls, indicating that the fungus recognizes specific epitopes present in the cell wall of root epidermal cells (Nagahashi & Douds, 1997).

The appressoria become firmly attached to the root epidermis and subsequently new hyphae develop which will enter the root. Depending on the host plant this can occur either intercellular or intracellular. Since AM involving intercellular infection -the so-called Arum type- is predominantly found in cultivated herbs, it became more frequently studied than the AM involving intracellular infection; the so called Parish type (for review see: Smith & Smith, 1997). Therefore, we will focus in this review on the Arum type interaction in which the fungus enters the root between two epidermal cells. The plant accommodates the invasion of the fungus by secreting new cell wall material that surrounds the infecting hyphae. In the inner cortex, the fungus invades cells and there they differentiate into highly ramified structures the so-called arbuscules (Figure 1). These structures are thought to facilitate the exchange of nutrients between both organisms.

Although arbuscules occur intracellular, they never are in direct contact with the cell cytoplasm. A perifungal membrane, originating from the plant plasma membrane invaginates and surrounds the arbuscules. During the formation of arbuscules, the plant cell becomes cytoplasmic dense, it's vacuole fragmentates, and the number of Golgi bodies increases. Furthermore, the nucleus moves to a more central position in the cell (Balestrini *et al.*, 1992).

When the mycorrhizal fungi colonize the roots and appressoria have been formed, the fungus rapidly enters the cortex. Upon entry of the root arbuscules are formed within a few days (Albrecht *et al.*, 1998). Arbuscules have a similar morphology as haustoria; the feeding structures which are formed by several pathogenic fungi during a compatibe interaction. During both haustorium and arbuscle formation plant defense responses are induced, but only at a low level. For these reasons it seems probable that the haustorium and arbuscle formation involve similar mechanisms.

In contrast to the *Rhizobium*-legume interaction, there is very little host specificity in AM symbioses. A fungus can interact with a diverse range of host plant species, whereas a certain host plant can interact with several fungal species. However, several plant families can be considered as 'nonmycorrhizal' or 'rarely mycorrhizal'; e.g. the *Brassicacea.*

COMMON GENES ARE INVOLVED IN MYCORRHIZAE AND NODULE FORMATION

The morphological responses that take place in the epidermal and cortical cells when roots become infected by rhizobia or AM fungi, respectively, seem at first sight to involve unrelated processes. However, molecular and genetic studies show that the infection processes are strikingly similar. Several genes have been identified that are induced during both symbiotic interactions; e.g. the early nodulin genes *ENOD2, ENOD40* (Van Rhijn *et al.*, 1997), *ENOD5, ENOD12* (Albrecht *et al.*, 1998), the leghemoglobin gene *VFLb29* (Frühling *et al.*, 1995), and the aquaporin encoding gene *NOD26* (Wyss *et al.*, 1990). However, the most convincing evidence that the infection processes used by both microsymbionts involve common steps came from studies with legume mutants that have lost the ability to form nodules. A large proportion of the nodulation resistant mutants, are also completely resistant to AM fungi, while their interaction with soil pathogens has not been affected (for reviews: Gianinazzi-Pearson, 1996; Harrison, 1997). In pea, 4 genes have been identified that are essential for early steps of both the rhizobial and mycorrhizal interactions. Mutants of three of these genes, *sym8*, *sym9* and *sym19*, have been studied in more detail at a cytological level. These mutants are unable to form an infection thread (LaRue & Weeden, 1994) and although AM fungi still can form appressoria on these Nod-/Myc- mutants they fail to develop intercellular hyphae (Gianinazzi-Pearson, 1996). This shows that rhizobial and mycorrhizal infection involves common mechanisms.

DO SIGNALS FROM AM FUNGI AND RHIZOBIAL NOD FACTORS ACTIVATE COMMON SIGNAL TRANSDUCTION PATHWAYS?

The availability of marker genes that are activated during both symbiotic interactions as well as host mutants blocked in the infection by either microsymbiont, provided the means to study whether the induction of the infection related genes involved common mechanisms. Here we will focus on two early nodulin genes; *ENOD12* and *ENOD40.*

ENOD12

ENOD12 is the best studied marker gene for early rhizobial Nod factor induced responses. The gene is induced in cells involved in or getting prepared for infection by rhizobia (Scheres *et al.*, 1990; Journet *et al.*, 1994). Using a spot inoculation assay it was shown that in the AM interaction *ENOD12* also is activated when the fungus infects the roots (Albrecht *et al.*, 1998). Since the encoded protein might be a cell wall component it could be part of the matrix secreted by the host that surrounds the microsymbionts.

Studies with transgenic *Medicago* plants, carrying the promoter of the *ENOD12* gene in front of the β-glucuronidase reporter gene (*GUS*), it was shown that the *ENOD12* inducing activity of Nod factors can be mimicked by mastoparan, a compound that is supposed to activate G-proteins (Pingret *et al.*, 1998). Furthermore, studies using various putative PLC antagonists suggest that inositol phosphate signaling plays a role. Whether inositol phosphate signaling plays a role in *ENOD12* induction by AM fungi is unknown. However, *ENOD12* is neither induced by Nod factors nor by mycorrhiza in the root epidermis of a pea *sym8* mutant

(Albrecht *et al.*, 1998). Therefore, it is probable that the signal transduction cascades leading to *ENOD12* expression, which are activated by rhizobial Nod factors and mycorrhiza, have at least SYM8 in common.

ENOD40

Like *ENOD12*, the early nodulin gene *ENOD40* also is activated by both, *Rhizobium* and AM fungi (Van Rhijn *et al.*, 1997). In the *Rhizobium* interaction this gene is first expressed in pericycle cells opposite the proto-xyleme poles within a few hours after Nod factor application and markedly before the first cell divisions occur in the root cortex (W.C. Yang and T. Bisseling, unpublished results). Later, when cell divisions are induced, *ENOD40* is also expressed in the dividing cells (Kouchi & Hata, 1993; Yang *et al.*, 1993). Strikingly, the expression in the pericycle opposite xyleme poles, is complementary to the expression pattern of ACC synthase, the gene encoding the enzyme catalyzing the last step of the biosynthesis of ethylene (figure 3). Since ethylene is an inhibitor of cortical cell division, the localized expression of ACC synthase contributes to the positioning nodule primordia (Heidstra *et al.*, 1997). Overexpression of *ENOD40* as well as ballistic introduction of an *ENOD40* expression construct induces cell divisions in the root inner cortex (Charon *et al.*, 1997). Hence the local induction of *ENOD40* expression in the region of the pericycle opposite the phloem poles could provide additional positional information for nodule primordium formation.

ENOD40 has been isolated from several legumes as well as from some non-legume species. All these *ENOD40* genes contain two regions that are highly conserved. However, only the 5' located box 1 contains a short conserved open reading frame encoding a small peptide of 10-13 amino acids. Ballistic introduction in *M. varia* roots of a DNA construct encoding this small peptide is sufficient to induce cortical cell divisions (Charon *et al.*, 1997), but experiments that showed that this small peptide conferred to tobacco protoplasts tolerance to high auxin are under debate (Van de Sande *et al.*, 1996; Schell *et al.*, 1998).

The question remains how *ENOD40* is induced by the AM fungus. Since the gene also can be activated by cytokinin (Minami *et al.*, 1996) and it was shown that AM fungi produce cytokinin, it can be postulated that this hormone induces *ENOD40* in the AM interaction (Van Rhijn *et al.*, 1997). Also, it has been proposed that Nod factors cause an increase in the level of cytokinin in root tissues (Cooper & Long, 1994), and this could explain why *ENOD40* is induced by both microsymbionts (Van Rhijn *et al.*, 1997). However, it seems probable that in addition to cytokinin at least one other molecule in the *ENOD40* activating cascade is in common. Nod factors can not induce *ENOD40* in the alfalfa Nod-/Myc- mutant MN NN1008, whereas cytokinin is able to do so (Bradbury *et al.*, 1991; W.C. Yang & T. Bisseling unpublished). This suggests that MN NN1008 is mutated in a gene active upstream of cytokinin. Furthermore it is blocked in Nod factor activated calcium spiking which is induced within minutes after Nod factor application (Ehrhardt *et al.*, 1996). This suggests that the mutated gene is probably involved in an early step of the activated signaling cascades.

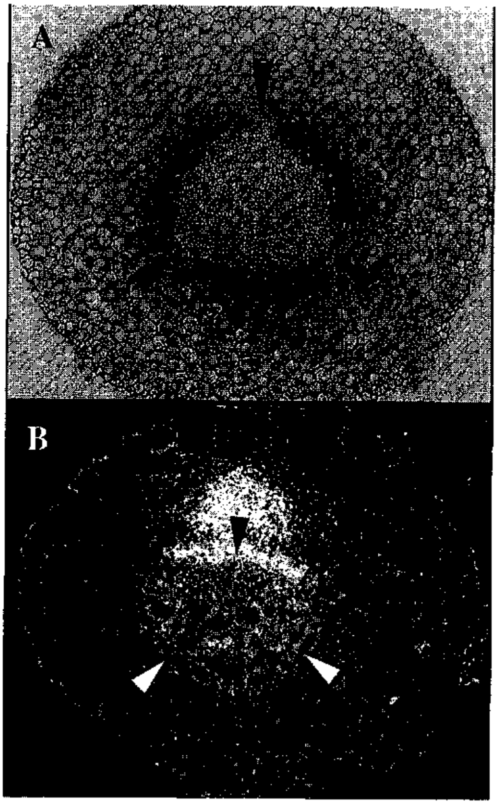

Figure 3. A. Accumulation of ACC oxidase mRNA in the regions of the pericycle opposite the phloem poles of an uninoculated pea root, visualized by in situ hybridization with a DIG labeled ACC oxidase antisence RNA (Picture A; Heidstra *et al.*, 1997). **B.** Induction of *ENOD40* in the pericycle of the root opposite protoxyleme poles by *Rhizobium leguminosarum* bv *viciae* Nod factor 2 days after application (Vijn *et al.*, 1993). *ENOD40* mRNA is visualized by *in situ* hybridization of with 35S labeled *ENOD40* antisence RNA (dark field).The protoxyleme poles are marked by an arrow head.

CONCLUSION

The above reviewed studies have made clear that common host genes are involved in the rhizobial and mycorrhizal interaction. This finding has an important implication since in contrast to *Rhizobium*, AM fungi have the ability to interact with a wide range of higher plants. Assuming that the mechanisms by which AM fungi infect their various hosts are similar, it implies that *SYM* and *ENOD* genes, required for the interaction of legumes with both micro-symbionts, are probably widespread in the plant kingdom. Present studies with transgenic rice (*Oryza sativa*) are consistent with this idea (Reddy *et al.*, 1998). These studies show that a *Medicago ENOD12* promoter in transgenic rice can be activated by rhizobial Nod factors, demonstrating that a signal transduction cascade involved in the activation of this leguminous promoter is present in rice. In legumes SYM8 is essential for the induction of *ENOD12* either by rhizobial Nod factors or AM fungi. Therefore it is likely that this gene will be present in non-legumes (e.g. rice) as well. Although, with the exception of *Parasponia andersonni*, non-leguminous plants are unable to establish a symbiosis with *Rhizobium*, they seem to harbor a perception mechanism by which Nod factors can be recognized. Obviously this perception mechanism is not maintained by non-legumes to recognize rhizobial Nod factors. The natural ligands for this perception mechanism are unknown. However, since its activation leads to *ENOD12* transcription it is worthwhile to study whether molecules of AM fungi are natural ligands. Although the function of the non-leguminous perception mechanism is not clear, it seem probable that it has a wide spread occurrence, and that the Nod factor perception mechanism of legumes has evolved from it.

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SAMENVATTING

Rhizobium bacteriën zijn in staat de wortels van vlinderbloemige planten binnen te dringen en daar de vorming van een nieuw orgaan, de wortelknol, te induceren. De fysiologische condities in deze knol zijn zodanig dat de bacteriën in staat zijn moleculaire stikstof te reduceren tot ammonia, een stikstofbron die de gastheerplant kan gebruiken. In ruil hiervoor voorziet de plant de bacterie van suikers.

De vorming van wortelknollen vereist dat de ontwikkeling van een aantal corticale wortelcellen wordt geherprogrammeerd. Deze cellen verliezen hun oorspronkelijke bestemming van corticale cel, en gaan delen om zo een knolprimordium te vormen, dat zich vervolgens kan differentiëren tot een functionele knol.

Naast de vorming van een wortelknol, dient de plant ook toe te laten dat de *Rhizobium* bacteriën de wortel binnen dringen. De bacterie nestelt zich in een door hem geïnduceerde wortelhaarkrul, waarna de wortelhaarcel het binnendringen van de bacterie mogelijk maakt door middel van invaginatie van de plasmamembraan. De bacterie bevindt zich dus niet in het cytoplasma van de cel, maar wordt omgeven door een membraan en celwandmateriaal. Deze structuur, infectiedraad genoemd, leidt de bacterie naar het knolprimordium.

Bij zowel knolprimordiumvorming als infectie, speelt het door *Rhizobium* geproduceerde signaalmolecuul, genaamd nodulatie factor (Nod factor), een prominente rol. Nod factoren bestaan uit een chitine-oligomeer van 4 of 5 eenheden, met daaraan een vetzuur gekoppeld. Verder kunnen er verschillende groepen aan de terminale glucosamine-eenheden aanwezig zijn, bijvoorbeeld een acetaat-, sulfaat- of fucose-groep, welke van groot belang zijn voor de biologische activiteit van het molecuul. Welke zijgroepen aanwezig zijn, is afhankelijk van de bacterie soort.

Dit proefschrift concentreert zich rond de vraag hoe vlinderbloemige planten *Rhizobium* Nod factoren herkennen. De diversiteit aan responsen welke door Nod factoren bij de gastheerplant worden geïnduceerd impliceert dat de perceptie- en het signaaltransductie-mechanisme nogal complex kan zijn. Om inzicht hierin te verkrijgen is voor een genetische benadering gekozen. Aangezien van de erwt reeds tientallen symbiotische mutanten zijn geïsoleerd, heeft het onderzoek zich met name op deze plantensoort geconcentreerd.

SYM8

Van een erwtenmutant gemuteerd in een Nod factor-receptor zou men kunnen verwachten dat deze geen enkele respons vertoont op de toevoeging van Nod factoren. Hoewel niet alle beschikbare mutanten tot in detail zijn gekarakteriseerd, is wel duidelijk dat sommige geen morfologische respons vertonen na inoculatie met *Rhizobium*. Een van deze mutanten is de erwtenlijn Sparkle-R25, welke gemuteerd is in het *SYM8* gen. Zowel de inductie van celdelingen als de initiatie van een infectieplaats is geblokkeerd bij Sparkle-R25. Wat opvalt, bij deze en sommige andere 'vroege' mutanten, is dat, naast de *Rhizobium* symbiose, ook de endomycorrhizae symbiose is verstoord. Dit is een symbiose tussen de wortel van een plant en een schimmel. De schimmel penetreert de wortel vaak intercellulair, en soms, als *Rhizobium*, intracellulair. Echter nooit worden er celdelingen geïnduceerd. Ook bestaat er nagenoeg geen gastheerspecificiteit en vormen naast vlinderbloemige ook de meeste andere hogere plantensoorten mycorrhizae. Mutanten als Sparkle-R25 duiden er op dat mycorrhizae schimmels en *Rhizobium* gebruik maken van een gelijksoortig mechanisme om een gastheer te infecteren. Onderzoek beschreven in dit proefschrift laat zien dat dit inderdaad het geval is. De 2 genen *PsENOD5* en *PsENOD12A* waarvan de expressie door Nod factoren wordt geïnduceerd blijken ook door een mycorrhizae schimmel te worden geactiveerd. Verder wordt aangetoond dat de expressie van beide genen is geblokkeerd in Sparkle-R25. Dus het *SYM8* gen is zowel in de *Rhizobium* als de mycorrhizae symbiose betrokken bij een signaaltransductieweg leidend tot de expressie van *PsENOD5* en *PsENOD12A*. Echter sinds er geen bewijs is dat mycorrhizae schimmels Nod factor-gelijkende moleculen maken, en aangezien de morfologische responsen geïnduceerd door Nod factoren en mycorrhizae schimmels nogal verschillen, is het niet waarschijnlijk dat het *SYM8* gen betrokken is bij de perceptie van Nod factoren.

SYM2

Door de nodulatie eigenschappen van wilde erwten-variëteiten met cultuurerwten te vergelijken is er in het verleden een gen geïdentificeerd, genaamd *SYM2*, waarvan de activiteit afhankelijk is van de Nod factor-structuur. Erwtenplanten die het *SYM2* gen van het ecotype Afghanistan bevatten ($SYM2^A$) kunnen alleen genoduleerd worden als *Rhizobium* een Nod factor met een acetaat- dan wel fucose-groep aan het reducerende-uiteinde produceert. Voor nodulatie van cultuurerwten, zonder *SYM2*^{A}, zijn deze decoraties aan de Nod factor niet vereist. Aangezien het *SYM2* gen een relatie vertoond met Nod factor-structuur, is het fenotype van *SYM2*^{A} bevattende lijnen in detail gekarakteriseerd. Het blijkt dat *Rhizobium* bacteriën die geen Nod factoren met een additionele acetaat- of fucose-groep produceren, niet in staat zijn het infectieproces efficiënt te induceren. Andere Nod factor geïnduceerde processen zoals corticale celdelingen en *PsENOD12A* expressie vinden wel plaats. Dit duidt er op dat de inductie van infectiedraadvormingen groei onafhankelijk van de overige processen door de gastheerplant wordt gecontroleerd.

Door een *SYM2*^{A} bevattende lijn te mutageniseren, is een mutant geïdentificeerd, genaamd Xim, waarin de blokkade op infectiedraadvorming door incompatibele *Rhizobium* bacteriën is opgeheven. Dit duidt er op dat deze blokkade in *SYM2* planten een actief proces is, welke door een mutatie in Xim is uitgeschakeld.

Klonering van *SYM2* kan inzicht verschaffen in hoe gastheerplanten het infectieproces reguleren in relatie tot Nod factor-structuur. De klonering van een gen waarvan alleen het fenotype bekend is kan gebeuren via 'transposon tagging' of via een positionele klonering. Aangezien in erwt geen transposon systeem voorhanden is, is in theorie alleen een positionele klonering mogelijk. Dit vereist echter een gedetailleerde genetische kaart van het genoom rond het *SYM2* locus. Om deze te verkrijgen is een differentiële RT-PCR screen uitgevoerd op twee bijna isogene lijnen die alleen verschillen in het gebied rond het *SYM2* gen. Om de mogelijkheid open te houden het *SYM2* gen zelf op deze wijze te kloneren is wortelhaar RNA als uitgangsmateriaal gebruikt, aangezien het waarschijnlijk is dat het *SYM2* gen in wortelharen tot expressie komt. Op deze wijze zijn 4 cDNAs geïdentificeerd waarvan de corresponderende genen genetisch gezien gekoppeld zijn aan het *SYM2* locus. Een van deze genen, genaamd *W62*, is met de beschikbare segregerende populaties en een geconstrueerde set van 'Recombinant Inbred Lines' genetisch niet te scheiden van het *SYM2* locus. Het door *W62* gecodeerde eiwit heeft sterke homologie met plant gecodeerde serine/threonine-receptorkinases, en komt alleen in wortelharen en wortels tot expressie. *W62* is, in theorie, een potentiële kandidaat om *SYM2* te zijn. Echter, het hier gepresenteerde onderzoek geeft hierover nog geen uitsluitsel. In ieder geval kan *W62* fungeren als startpunt voor een positionele klonering van het *SYM2* gen.

CURRICULUM VITAE

René Geurts is geboren op 30 januari 1968 te Brunssum. Aan het Rombouts college te Brunssum is in 1984 het M.A.V.O. en 2 jaar later het H.A.V.O. diploma gehaald. In 1986 is in Wageningen een studie begonnen aan de R.H.A.S. (afdeling laboratorium onderwijs). Na het behalen van de propedeuse is in 1987 de overstap naar de Landbouwuniversiteit gemaakt om een studie plantenveredeling met een cel/genetische oriëntatie te beginnen. Deze is in 1992 succesvol afgesloten met als hoofdvakken bio-chemie en moleculaire biologie. Vanaf september 1992 is bij de leerstoelgroep Moleculaire Biologie van de Landbouwuniversiteit Wageningen promotieonderzoek uitgevoerd onder leiding van dr. T. Bisseling en professor dr. A. van Kammen. Eerst als Onderzoeker in Opleiding en later als onderzoeksmedewerker aan de Landbouwuniversiteit. Het onderzoek werd gefinancierd door een Pionier-subsidie van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (N.W.O.) welke was toegekend aan dr. T. Bisseling. Het onderzoek beschreven in dit proefschrift zal, gefinancierd door de 'Human Frontiers Science Organisation', de komende 4 jaar worden gecontinueerd.