Plant genes involved in root-nodule development on legumes

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SUMMARY

Rhizobium is able to induce the formation of a new organ on roots of leguminous plants, the root nodule, in which the penetrated bacteria fix atmospheric nitrogen. This process is initiated by specific lipo-oligosaccharides, called Nod factors, secreted by the bacterium. Nodule formation proceeds through distinct steps like infection thread formation and activation of mitotic activity in cortical cells. During these steps specific plant genes, nodulin genes, are induced and several of these have been identified and characterized. Nodulin genes are used now as markers to study Nod factor perception and signal transduction.

1. INTRODUCTION

The interaction of a compatible *Rhizobium* with its leguminous host-plant leads to the formation of a new organ on the plant root, in which the penetrated bacteria fix atmospheric nitrogen. The interaction is set in motion when bacteria are attracted by plant-secreted flavonoids and as a result start to produce specific lipo-oligosaccharides which in turn are recognized by the plant. Rhizobia attach to the plant root hairs. The root hair deforms, and the bacteria invade the plant by a newly formed infection thread growing through it. Simultaneously, cortical cells are mitotically activated giving rise to the nodule primordium. Infection threads grow towards the primordium and the bacteria are released into the cytoplasm of the host cells, surrounded by a plant-derived peribacteroid membrane (PBM). Thereupon the nodule primordium develops into a mature nodule, while the bacteria differentiate into their endosymbiotic form, bacteroids. At that stage bacteria synthesize nitrogenase which catalyses the reduction of N_2. Finally, the product of nitrogen fixation, ammonia, is exported to the plant. As occurs in the formation of other plant organs, in root nodule formation also a set of specific plant genes are involved, the so-called nodulin genes (van Kammen 1984). The early nodulin genes encode products which are expressed before the onset of nitrogen fixation and are involved in infection and nodule development. The products of late nodulin genes are involved in the interaction with the endosymbiont and in the metabolic specialization of the nodule.

Here, we focus on the early steps of the interaction between rhizobia and legumes and on the role of specific lipo-oligosaccharides secreted by rhizobia (as the availability of these signal molecules facilitates an effective dissection of the pathways underlying organogenesis in plants).

2. NOD-FACTOR STRUCTURE

The *Rhizobium* signal molecules that play a key role in the induction of the initial stages of nodulation are lipo-chitooligosaccharides, called Nod factors. The bacterial genes involved in Nod-factor synthesis are the *nod* (nodulation) genes. Of these genes only *nodD* is expressed constitutively. NodD has the ability to bind to specific flavonoids secreted by the roots of the host plant (Peters & Verma 1990), whereupon it becomes a positive transcriptional activator of the other *nod* genes. The latter encode enzymes involved in the synthesis of Nod factors.

The structure of the major Nod factor of *R. meliloti* was first resolved in 1990 (Lerouge *et al.* 1990) since then the structure of the Nod factors of most rhizobia has been determined (see, for example, Fisher & Long 1992; Spaink 1992; Dénarié & Cullimore 1993; Carlson *et al.* 1995).

In general, Nod factors consist of a backbone of three to five β-1,4-linked *N*-acetylglucosamines carrying a fatty acid at the non-reducing sugar residue. The factors can have various substitutions on both reducing and non-reducing terminal sugar residues.

nodA, *B* and *C* encode enzymes catalysing the synthesis of the Nod-factor backbone. NodC has homology to chitin synthases and therefore is the

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enzyme that most likely catalyses the synthesis of the chitin oligomer (Geremia *et al.* 1994). The latter is further modified by the action of NodB, which de-*N*-acetylates the terminal non-reducing end of the molecule (John *et al.* 1993). Finally, NodA transfers a fatty acid from an acyl carrier protein (Röhrig *et al.* 1994) at this position. The other Nod proteins are then involved in the synthesis or addition of the various substituents to the backbone. These substitutions determine host specificity as well as the biological activity of the molecules.

In general rhizobia have the ability to interact only with a limited number of host plants. However some rhizobia, for example, *Rhizobium* NGR234, have a more promiscuous nature. *Rhizobium* NGR234 which can nodulate various tropical legumes, excretes 18 different Nod factors (Price *et al.* 1992). The production of this variety of factors has been thought to be the reason of its broad host range (Price *et al.* 1992).

3. INTERACTION WITH THE ROOT EPIDERMIS

As a result of legume root colonization, rhizobia induce deformation and curling of root hairs as well as the expression of several plant genes in the epidermis. It has been shown that purified Nod factors induce deformation of the root hairs at concentrations as low as 10^{-12} M, but root hair curling has not been observed (Lerouge *et al.* 1990; Spaink *et al.* 1991; Price *et al.* 1992; Sanjuan *et al.* 1992; Schulze *et al.* 1992; Mergaert *et al.* 1993; Heidstra *et al.* 1994). For *Vicia sativa* a fast semiquantitative root hair deformation assay has been developed. In this plant, the process is induced only in a small zone of the root, the susceptible zone, encompassing young root hairs that have almost reached their mature size (Heidstra *et al.* 1994). It starts with swelling of the root hair tips, which is already apparent within 1 h. Subsequently, new tip growth is initiated at the swollen tips, resulting in clearly deformed hairs within 3 h. An incubation period with Nod factors of about 10 mins is required to set the deformation process in motion (Heidstra *et al.* 1994).

The morphological changes in root hairs are preceded by a depolarization of the plasma membrane (Ehrhard *et al.* 1992), changes in the flux of calcium, efflux of protons, rearrangements of the actin filaments (Allen *et al.* 1994) and increased cytoplasmic streaming (Heidstra *et al.* 1994). These changes occur after 5–30 min and might be part of a series of events that eventually lead to root-hair deformation.

Several plant genes, whose expression is activated in the epidermis during nodulation, have been cloned. These have been used to study the mode of action of Nod factors. Examples are the early nodulins *ENOD5* (Scheres *et al.* 1990*b*) and *ENOD12* (Scheres *et al.* 1990*a*), that encode proline-rich proteins and *Msrip1* (Cook *et al.* 1995), encoding a peroxidase, which in uninoculated roots is expressed in the root pericycle. These three genes are induced in the epidermis within a few hours after application of Nod factors (Horvath *et al.* 1993; Journet *et al.* 1994; Cook *et al.* 1995). *Msrip1* is not involved in other steps of nodulation, whereas *ENOD12* and *ENOD5* are also activated during infection and nodule development (see §4,5).

The induction of *ENOD12* and *Msrip1* expression occurs in a relatively broad zone of the root. It starts just above the root tip, where root hairs have not emerged yet, and extends to the region containing mature root hairs (Pichon *et al.* 1992; Cook *et al.* 1995). Cytological studies showed that Nod factors elicit the expression of these genes in all epidermal cells (Journet *et al.* 1994; Cook *et al.* 1995) and a direct contact between Nod factors and epidermal cells is required (Journet *et al.* 1994). The activation is restricted to the epidermis, because in the hypodermal cell layer these genes are not expressed. Thus it is likely that within the susceptible zone, trichoblasts as well as atrichoblasts of the epidermis are responding to Nod factors.

Assays scoring for root-hair deformation and the induction of early nodulin genes have been used to analyse the structural requirements of Nod factors to elicit responses in the epidermis. The length of the sugar backbone plays an important role. When roots are treated with Nod factors, molecules containing three or fewer sugars are rapidly generated by chitinases that are secreted by the plant. Such degradation products are at least 1000-fold less active in a deformation assay, than the Nod factors with the 4–5 glucosamine residues (Heidstra *et al.* 1994; Staehelin *et al.* 1994*b*). Also the importance of certain substitutions of the terminal sugar residues have been determined. For instance, an acyl group has to be present at the non-reducing end, because chitin molecules are not able to deform root hairs nor induce *MsENOD12* expression (Journet *et al.* 1994). However, the structure of the acyl group is not important because molecules with different fatty acid substitutions have a similar activity. Whereas the presence of an *O*-acetyl group at the reducing end is not required for root hair deformation (Ardourel *et al.* 1994), substitutions at the reducing end can have a dramatic effect. The sulfate moiety present in the Nod factors of *R. meliloti* is important for induction of root hair deformation and the elicitation of *MsENOD12* expression in alfalfa, the host of *R. meliloti*, because desulfation of the *R. meliloti* factors reduces their level of activity at least 1000-fold (Journet *et al.* 1994). On the other hand, the desulfation enable the factors to deform the root hairs of the *R. leguminosarum* bv. *viciae* host, vetch (Roche *et al.* 1991).

Thus Nod factors at concentrations as low as 10^{-12} M induce several responses in the root epidermis. If a receptor is involved in the elicitation of these responses, it has to recognize the length of the Nod factors and the substitutions at the reducing end. As the presence of an acyl moiety is essential, but its structure is not important, it is likely that this part of the molecule is not recognized by a receptor. Therefore, the acyl group might play a role in docking the Nod factors in the membranes, facilitating binding to a putative receptor.

4. (PRE-)INFECTION STRUCTURE FORMATION

After attachment of rhizobia to the root hair tips, tight curling occurs and bacteria become entrapped in these curls. Here, a local hydrolysis of the plant cell wall takes place (Callaham & Torrey, 1981; Van Spronsen *et al.* 1994), the plasma membrane invaginates and new plant cell wall material is deposited (for reviews see Bauer 1981; Newcomb 1981; Brewin 1991; Kijne 1992). In this way a tubular structure, the so-called infection thread, is formed by which the bacteria enter the plant.

The composition of the infection-thread wall is very similar to that of the plant cell wall, however, the possibility that it obtains specific properties by incorporation of certain nodulins cannot be ignored. The proline-rich early nodulins ENOD5 and ENOD12 are suitable candidates because cortical cells containing an infection thread express the corresponding genes (Scheres *et al.* 1990*a*, *b*). The bacteria in the infection thread are surrounded by a matrix which seems to consist of compounds secreted by both the plant and the bacteria. For example, a 95 kDa glycoprotein, normally present in the intercellular spaces of the root cortex, was shown to be localized in the infection thread matrix (Rae *et al.* 1992).

Concomitantly with infection thread formation, cortical cells are mitotically reactivated, forming the nodule primordia (see below). Infection threads grow towards these primordia and there bacteria are released into the cytoplasm. In legumes like alfalfa and pea that form indeterminate nodules, root inner-cortical cells give rise to nodule primordia. Hence the infection threads have to traverse the outer cortex before they reach these cells. Before infection-thread penetration, the outer cortical cells undergo morphological changes. The nuclei move to the centre, the microtubules and the cytoplasm rearrange and form a radially oriented conical structure that resembles a preprophase band, called the cytoplasmic bridge (Kijne 1992). The infection threads traverse the cortical cells through the radially aligned cytoplasmic bridges, which are therefore called preinfection threads (Van Brussel *et al.* 1992).

Although the preinfection thread forming outer cortical cells never divide, the induced morphological changes are reminiscent of those in cells that enter the cell cycle. *In situ* hybridization experiments (Yang *et al.* 1994), showed that narrow rows of outer cortical cells express the S phase specific histon H4 gene. A mitotic cyclin gene that is specifically expressed during the transition of G2 to M phase, is not activated. Hence the cells that form the preinfection thread, re-enter the cell cycle and become arrested in the G2 phase, whereas the inner cortical cells progress through the cell cycle and form the primordia. This shows that part of the infection process is derived from a common process, namely cell cycling. In some way, rhizobia have modified it and now exploit it for a completely different purpose, the infection of the root.

Although purified Nod factors induce preinfection thread formation, infection threads are not formed. Thus the bacteria seem to be required for the formation of infection threads.

5. INDUCTION OF CELL DIVISIONS

During mitotic reactivation of root cortical cells by rhizobia, genes that control the progression through the cell cycle, like *cdc2* and mitotic cyclins, are induced (Yang *et al.* 1994). Additionally, several nodulin genes are expressed, allowing a distinction between nodule primordia and root or shoot meristems. Examples of such nodulin genes are *ENOD12* (Scheres *et al.* 1990*a*), *Gm93* (Kouchi *et al.* 1993), *ENOD40* (Kouchi *et al.* 1993; Yang *et al.* 1993; Matvienko *et al.* 1994) and *MsPRP4* (Wilson *et al.* 1994). These genes are activated in all cells of the primordia. Furthermore, *ENOD40* is also induced in the region of the pericycle opposite to the dividing cortical cells (Kouchi *et al.* 1993; Yang *et al.* 1993). In contrast, the early nodulin gene *ENOD5* (Scheres *et al.* 1990*b*) is transcribed only in primordial cells that contain rhizobia and therefore most likely the expression of this gene is related to the infection of primordial cells.

Nod factors are sufficient for mitotic reativation of the cortical cells (Spaink *et al.* 1991; Truchet *et al.* 1991; Relic *et al.* 1993) and in some host plants even genuine nodules are formed (Truchet *et al.* 1991; Mergaert *et al.* 1993; Stokkermans and Peters, 1994). The early nodulins *ENOD12* and *ENOD40* were shown to be activated in such primordia (Vijn *et al.* 1993).

In vetch, activation of the cortical cells is elicited only by factors carrying a highly unsaturated acyl group (C18:4) at the non-reducing terminal sugar (Van Brussel *et al.* 1992). In alfafa and soybean, changes in chain length or saturation of the acyl moiety of Nod factors affect their behaviour. Activation of the cortex is observed but they are less efficient in inducing nodule formation in alfalfa (Ardourel *et al.* 1994), whereas in soybean emerging nodules are not even observed (Stokkermans *et al.* 1994). It is not clear yet whether this reduction in activity is due to the change of the length of the fatty acids or to their degree of desaturation.

Interestingly, only specific cortical cells are susceptible to Nod factors. In tropical legumes such as soybean, outer cortical cells are mitotically activated. In temperate legumes like pea, vetch and alfalfa, the inner cortical cells and especially those located opposite protoxylem poles divide. It has been postulated for decades that the susceptibility of cortical cells is conferred by an arrest in the G2 phase. But by using cell phase specific genes as probes in *in situ* hybridization experiments this was proven not to be the case. Using a histon4 probe it was shown that only narrow rows of cortical cells are activated by *Rhizobium*. At present, it is not known how the susceptibility in such narrow rows of cells is controlled. These rows of susceptibile cells are located opposite to protoxylem poles. Already in 1973 it was found by Libbenga *et al.* that an alcoholic extract or the stele could induce cell division in explants of the pea root cortex in the presence of auxin and cytokinine. The substance

responsible for this activity, the so-called stele factor, has been purified meanwhile. It is supposed to be released from the protoxylem poles and to render the cortical cells to be susceptible to Nod factors (J. Kijne, personal communication).

To unravel the mechanism by which Nod factors elicit cortical cell divisions, studies on compounds that can mimick their mitogenic activity were performed. Two lines of evidence, strongly suggest that Nod factors cause a change in the auxin/cytokinin balance. Compounds (NPA, TIBA) that block polar transport of auxin (Hirsch *et al.* 1989; van de Wiel *et al.* 1990) induce the formation of nodule-like structures on alfalfa, as Nod factors do. An *E. coli* strain carrying a plasmid containing the zeatin gene, involved in cytokinin synthesis, can partly complement nod mutants of *R. meliloti* (Cooper *et al.* 1994) leading to the formation of nodule-like structures. Early nodulin genes are expressed in both cases.

At least four early nodulin genes are induced when cortical cells divide and the question is whether such nodulins can establish a phytohormone change. The early nodulin gene *ENOD40* that is induced by Nod factors in the root pericycle, as well as in the dividing cortical cells has a phytohormone effect in the non-legume tobacco. This effect was studied in a protoplast assay in which the correlation between efficiency of cell division and auxin concentration is monitored. Tobacco protoplasts expressing legume *ENOD40* gene divide efficiently at high auxin concentration, whereas in the control protoplasts this level of auxin suppresses their ability to divide (Pawlowski *et al.* 1995).

The induction of *ENOD40* in the pericycle precedes the first cortical divisions. Hence Nod factors or secondary signals generated after the interaction with the epidermis, elicit *ENOD40* expression in the pericycle, followed by a mitotic activation of the cortical cells. Because *ENOD40* is sufficient to cause a phytohormone-like effect in tobacco, we hypothesize that *ENOD40* expression in the pericycle of legume roots can cause a change in the cytokinin/auxin ratio or perception in the cortex, leading to cell division. Alternatively, *ENOD40* might inhibit the diffusion of plant compound(s) which normally supress(es) cell division. In that case the cortical cells become susceptible to Nod factors or second messengers generated in the epidermis and subsequently divide.

ENOD40 cDNA clones has been isolated from different legumes, but only in the soybean cDNAs, a long ORF could be found (Kouchi *et al.* 1993, Yang *et al.* 1993; Crespi *et al.* 1994; Matvienko *et al.* 1994). The lack of homologue ORFs within the cDNA clones from other legumes has led to the hypothesis that this gene is active on the RNA level (Crespi *et al.* 1994; Matvienko *et al.* 1994).

6. NOD-FACTOR PERCEPTION AND SIGNAL TRANSDUCTION

In the previous paragraphs, it was described that Nod factors induce responses in three different tissues of the root, namely epidermis, cortex and pericycle. Nod factors are active at low concentrations and their biological activity on a certain host is controlled by the presence of certain substitutions. These data suggest that Nod factors are recognized by a receptor in the host plant. However, whether Nod factors directly interact with all three responding tissues or whether for example, the interaction of Nod factors with epidermal cells results in the generation of second messengers that after diffusion/transport trigger the responses in the inner tissues is unclear.

The induction of the different responses has different Nod-factor structural requirements. For instance, for the induction of root hair deformation the structure of the acyl group at the non-reducing end is not important, whereas the induction of infection thread formation in the same hairs requires a very specific structure, because Nod factors carrying the highly unsaturated acyl group are needed (Ardourel *et al.* 1994). This has led Ardourel et al. (1994) to postulate that at least two different Nod-factor receptors are present in the epidermis: the 'signalling receptor' involved in the induction of root hair deformation and cortical cell division and the 'uptake receptor' that is only activated by molecules with a very specific structure, to initiate the infection process (Ardourel *et al.* 1994).

The existence of a 'signalling' and an 'uptake' receptor is supported by studies in the pea gene *sym2* that controls nodulation. *sym2* originates from the wild pea variety Afghanistan. Afghanistan peas and cultivated peas containing *sym2* nodulate only after inoculation with *R. leguminosarum* bv. *viciae* strain carrying an additional *nod* gene, namely *nodX*. *NodX* catalyses the *O*-acetylation of *R. leguminosarum* bv. *viciae* Nod factors at the reducing end (Firmin *et al.* 1993). R.leguminosarum bv. *viciae* lacking *nodX* induces root hair deformation and even cortical cell divisions. However, the ability to induce infection thread formation is strongly reduced. Therefore, Sym2 is a good candidate for an 'uptake receptor' which interacts with the NodX modified Nod factors.

A biochemical approach to isolate (the) Nod-factors receptor(s) is feasible as high quantities of purified Nod factors, as well as chemically synthesized ones (Nicolaou *et al.* 1992), are available. A first report (Bono *et al.* 1995) on Nod-factor binding proteins has revealed the occurrence of a binding protein (NFBS1) in the microsomal fraction of root extracts from alfalfa. However, the affinity of this binding protein for its ligand is lower than the concentration at which Nod factors are active. Furthermore, it binds to sulfated and non-sulfated factors in a similar way, whereas factors lacking the sulfate group are hardly active on alfalfa. Therefore, it is unlikely that this protein is the Nod-factor receptor.

The availability of a root-hair deformation assay, the plant genes that are activated by the Nod factors, as well as methods to inject root hairs, makes it possible to unravel the signal-transduction cascades that are activated after perception of Nod factors. These tools have been developed only recently and therefore our knowledge on Nod factor transduction is still in its infancy. Additional studies are required to determine the relevance of the Nod factor induced changes like

ion fluxes, membrane depolarization, and rearrangements of the actin filaments, in the signal transduction pathways. Furthermore, it will now become possible to test whether a rhizobial protein, NodO, indeed accelerates Nod factor induced signal transduction as it has recently been postulated (Sutton *et al.* 1994).

7. NOD FACTORS AND NON-LEGUME PLANTS

At present, genetic approaches to unravel Nod-factor perception and transduction are restricted to a few legumes like pea, soybean and *Medicago*. Unfortunately, the first two species are recalcitrant to molecular genetic strategies leading to cloning of genes. Therefore it might be essential to develop new legume model systems (Barker *et al.* 1990; Handberg & Stougaard 1992) or to explore the potential of non-legumes systems as *Arabidopsis*, to study the mode of action of Nod factors. The latter at first sight is illogical, but a few observations show that Nod factors are recognized by non-legumes. For example, expression of rhizobial *nod* genes in tobacco affects the development of these plants (Schmidt *et al.* 1993). A mutated carrot cell-line that has lost the ability to form somatic embryos can be rescued by Nod factors (De Jong *et al.* 1993). In addition, Nod factors trigger the alkalinization of tomato suspension culture medium (Staehelin *et al.* 1994*a*). Consequently, Nod-factor receptors could occur in non-legumes. However in all these systems, it is possible that Nod factors are recognized not only by a specific receptor. In the tomato suspension-culture studies for instance, Nod factors mimic the activity of chitin fragments but they are at least 30 times less active than chitin fragments (Baureithel *et al.* 1994). Therefore, it is more probable that in this case they are recognized by a putative chitin receptor (Baureithel *et al.* 1994). On the other hand, the best support for the occurence of a Nod-factor receptor in non-legumes, is given by the fact that a non-legume, *Parasponia*, can form nodules only with *Rhizobium* with functional *nod* genes (Marvel *et al.* 1987). This strongly suggests that as in legumes, nodule development is initiated by Nod factors, implicating that (at least) in one non-legume Nod-factor receptor(s) occur.

8. CONCLUDING REMARKS

Nod factors induce a number of cell-specific responses to different tissues of legume roots. The same molecules can elicit responses in non-legumes, indicating that these signals mimick endogenous plant molecules involved in different aspects of development (de Jong *et al.* 1993). Furthermore, expression of *nod* genes in plants leads to phenotypical changes (Schmidt *et al.* 1993). These data lead to the tentative conclusion that Nod factors are related to a new class of plant growth regulators. Nodule formation is a process resembling other developmental programs. The infection thread formation for instance recalls typical cell-tip growth, a process that gives rise to root hairs, trichomes and the extending pollen tube. During infection, the bacteria also make use of the cell-cycle machinery, resulting in the preinfection thread formation in the outer cortex, where the cells are arrested in G2, or the formation of nodule primordia in the inner cortex, where the cells proceed with the cell cycle. In addition, most nodulin genes are not nodulins *sensu stricto* as they are expressed also in other plant tissues where they probably function as in nodules. Therefore it is plausible to suggest that nodule formation is a process that has evolved from processes common among the dicots. Based on examination of spontaneously formed alfalfa nodules it has been hypothesized that legume nodules have developed from carbon storage organs (Joshi *et al.* 1993).

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