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Symbiotic Signals in Early Stages of the Morphogenesis of Rhizobium-Induced Root Nodules*

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Introduction

Rhizobium bacteria induce root nodules on leguminous plants. This bacterium–plant interaction is specific in that for example *Rhizobium leguminosarum* bv *viciae* forms root nodules on *Vicia* plants but not on *Medicago sativa*, whereas the reverse is true for *R. meliloti*. Root nodules are highly structured nitrogen-fixing symbiotic organs, which are formed in a multistep process. After chemotaxis to and attachment at the plant root hairs, *Rhizobium* induces root hair deformation and curling, followed by the development of an infection thread which "grows" from the root hair tip into the cortex. After the induction of a meristem, the root nodule is formed in which the bacteria develop into nitrogen-fixing bacteroids. Mutants of the plant as well as of *Rhizobium* have been found which are effected in the different stages of root nodule morphogenesis (Vincent, 1980), indicating the existence in both organisms of genes for each step in nodulation. Root nodule formation is a very complicated process and exchange between both organisms of molecular signals is used to tune the sequence of events in nodulation. Symbiotic signals should induce or repress the activity of genes involved in nodulation or influence the activity of the gene products. To understand root nodule morphogenesis it is essential to identify these symbiotic signals of both, plant and bacterium. The aim of this paper is to summarize the recent advances in research on molecular signals involved in the early stages of root nodule morphogenesis with emphasis on the rhizobial signals.

Host specific induction of symbiotic root phenotypes

Two root phenotypes are often used in studies concerning root nodule formation

*Invited paper

and signal exchange. These are root hair deformation (Had) which is seen on all leguminous plants (Vincent 1980; Zaat et al., 1987a; Canter Cremers et al., 1986; Faucher et al., 1988) and the formation of Thick and short roots (Tsr), which is observed on *Vicia sativa* ssp. *nigra* (Van Brussel, 1986) and *Triolium repens* (Canter Cremers et al., 1986) plants. In general Had is induced when a *Rhizobium* species or biovar infects its specific host plant, whereas Tsr is formed on *V. sativa* ssp. *nigra* when it is infected with *R. leguminosarum* bv. *viciae*. The *Rhizobium nod*(ulation) genes required to induce these phenotypes are located on Sym(biosis) plasmids (see Long 1989 for a review). In a coculture of plants and rhizobia only part of the *nod* genes, *nodABC* and *nodD* of *Rhizobium*, are sufficient to induce Had and Tsr phenotypes on *V. sativa* ssp. *nigra* (Faucher et al., 1988). However, Had on *Medicago sativa* is only induced when in addition the *nodH* gene, one of the so called *hsn* (from host specific nodulation) genes of *R. meliloti*, is also present, demonstrating that the specificity of the interaction between *Rhizobium* and its plant host already takes place at this early stage.

Evidence for signal exchange between Rhizobium *and its host plants*

The existence of plant signals and bacterial signals was deduced from experiments in which the plants and the bacteria were grown separately on each others spent medium (Van Brussel et al., 1986). *V. sativa* ssp. *nigra* plants were grown axenically on liquid Jensen medium and after seven days *R. leguminosarum* bv. *viciae* cells were incubated in the medium, designated as root exudate. Subsequently the supernatant of this bacterial culture was filter-sterilized and used as growth substrate for axenic *V. sativa* ssp. *nigra* plants. After 7 days of growth the roots were examined for the symbiotic root phenotypes Had and Tsr and the results were positive. Plants grown on supernatants of bacterial cultures in medium without plant products, or plants grown on sterile plant root exudate in which no rhizobia had grown did not show the phenotypes (Van Brussel et al., 1986). This experiment showed the existence of plant signals in root exudate, which are required to induce the *Rhizobium* culture to produce the bacterial signal or signals called Tsr factor or Had factor, which induce Tsr- and Had phenotypes on *V. sativa* ssp. *nigra*. Similar data were obtained with *R. leguminosarum* bv. *trifolii* and *Trifolium repens* plants (Canter Cremers et al., 1986).

Regulation of the nod *genes and the bioassay for plant signals*

Three types of *nod* genes are distinguished on the Sym plasmid: (i) The regulatory gene *nodD*, which is constitutively expressed, (ii) The "common" *nodABCIJ* genes, which are inducible and functionally exchangeable between the different *Rhizobium* species and (iii) The *hsn* genes which are also inducible, and which are different in the

different *Rhizobium* species or biovars. Examples of *hsn* genes are those found in the *nodFEL* and *nodMN* operons of *R. leguminosarum* bv. *viciae* and genes in the *nodFEG* and *nodH* operons of *R. meliloti* (see Long 1989 for a review).

All inducible *nod* operons are preceded by highly conserved 49 bp long nucleotide sequences, designated as *nod* boxes which form an essential part of the inducible *nod* promoters (Rostas et al., 1986; Schofield et al., 1986; Spaink et al., 1987a).

The regulation of the *nod* gene expression has been studied using plasmids in which *nod* region DNA fragments are fused to the promoter-less *Escherichia coli* structural *lacZ* gene. With these genetic constructs, designated reporter plasmids, *nod* gene expression can be monitored as β-galactosidase activity (Mulligan and Long 1985; Rossen et al., 1985; Innes et al., 1985; Wijffelman et al., 1986; Zaat et al., 1987b). This system can also be used as a bioassay for *nod* gene inducers. The expression of the inducible *nod* operons is under control of the *nodD* gene product which, in a form activated by root exudate, initiates transcription of these operons (Mulligan and Long 1985; Innes et al., 1985; Wijffelman et al., 1986; Spaink et al., 1986).

Molecular Signals in Plant root Exudate

The plant signals were originally assayed by using the induction of Had- and Tsr factor production of *Rhizobium*, which was detected with Had- and Tsr phenotypes on *Vicia*, as bioassay (Van Brussel et al., 1986). This bioassay was replaced by a more direct and more specific one, in which the induction of *nod* genes by plant signals present in root exudate was measured as β-galactosidase activity with the reporter plasmids described above. It appeared that *nod* gene inducers are flavonoid molecules (Peters et al., 1986; Redmond et al., 1986; Firmin et al., 1986; Kosslak et al., 1987; Zaat et al., 1989; Maxwell et al., 1989). Root exudates contain sets of different inducer molecules, the molecular composition of which is different for different plant species (Zaat et al., 1988a; Györgypal et al., 1989; Maxwell et al., 1989). It was also found that the NodD proteins of rhizobia with different host plant specificity differ in the set of flavonoids by which they are activated (Zaat et al., 1988a, 1989). Thus, the set of inducer molecules present in root exudate may determine host specificity (Spaink et al., 1987b; Horvath et al., 1987; Györgypal et al., 1988; Bender et al., 1988), although, most root exudates contain activators for more than one type of NodD protein. In the latter case host specificity is determined by other factors. In addition to the natural occurring inducers, a large number of commercially available flavonoids has been tested for *nod* gene inducing activity. These data revealed structural requirements of inducer molecules to be active with different NodD proteins, which have been described in detail by Zaat et al., (1989).

Symbiotic Signals of *Rhizobium*

The importance of the specificity of the bioassay

Historically, *Rhizobium* signals, though not mentioned as such, have been received more attention than plant signals, since, in contrast to plant signals, *Rhizobium* signals could be recognized by the phenotype Had (root hair deformation). As early as 1900, Hiltner (1900) discovered that *Rhizobium* contained substances capable of inducing Had. Up to now however the molecule or molecules responsible for the induction of this symbiotic phenotype have not been identified. This contrasts with the plant signals which had been identified very rapidly once a very specific bioassay, as described in the previous paragraph had been developed. Hence, one of the problems in the identification of the *Rhizobium* factors is that bioassays using the phenotypes Had or Tsr are rather impractical. They have to be carried out under axenic conditions and are time consuming. Moreover, in a number of cases Had and Tsr bioassays are not specific for symbiotic *nod* gene related factors of *Rhizobium*. These phenotypes can for instance, also be induced in *V. sativa* ssp. *nigra* plants with supernatants of high density cultures (10^9 – 10^{10} cells ml^{-1}) of *Rhizobium* strains that do not contain a Sym plasmid, or with supernatants of cultures of *Agrobacterium* or of a *Beyerinkia* strain (Van Brussel, unpublished results). The phenotype Had can also be induced in white clover plants by a *Rhizobium* strain cured of its Sym-plasmid (Canter Cremers et al., 1986). Ervin and Hubbell (1985) reported that almost every fraction of *Rhizobium trifolii* cells cultured in synthetic laboratorium medium can induce Had responses in white clover, although no *nod* gene inducers had been added to the medium. To make sure that only a symbiotic *nod* gene related *Rhizobium* factor is measured using Had and Tsr bioassays, every experiment must include numerous controls that demonstrate that aspecific responses do not take place. For the isolation of Had/Tsr factors that have symbiotic significance, this implies that the starting material has to be obtained under conditions that eliminate possible presence of products, which are not *nod* gene related and not flavonoid-dependent. Most investigations (Van Brussel et al., 1986; Canter Cremers et al., 1986; Zaat et al., 1988a; Faucher et al., 1988) have therefore been performed using symbiotic factor production methods with low bacterial concentrations (10^5 – 10^6 cells ml^{-1}). Under these conditions supernatants of cultures of *Rhizobium* with noninduced *nod* genes or of a *Rhizobium* without a Sym plasmid induce Had or Tsr phenotypes hardly or not at all. However, a remaining problem is that upon concentration of the crude symbiotic *nod* gene related factors, also the non specific factors are concentrated, and than, present in higher concentrations, do induce Tsr and Had.

Thus an important consideration in the choice of a bioassay is the specificity. Had on white clover, or Had and Tsr on *V. sativa* ssp. *nigra* are not very specific. In contrast, Had on *Medicago sativa* seems more specific since in coculture with

Rhizobium this plant only exhibits the Had phenotype if besides the *nodDABC* genes also the *hsn* gene *nodH* is present in the bacterial strain (Faucher et al., 1988; Banfalvi et al., 1989). However, so far no information has been published dealing with the possible induction of the Had phenotype by supernatants of high density cultures of *Rhizobium meliloti* strains without a Sym plasmid.

As the induction of mitosis in a protoplast culture of plant cells can be brought about by plant hormones, the bioassay for symbiotic factors described by Schmidt et al. (1988), which is based on this phenomenon, must also be classified among the non specific bioassays.

A more specific bioassay could be developed if the specific induction of plant genes by nodulating rhizobia or the specific products formed by these proteins could be used as bioassay. Plant proteins specifically induced upon infection with *Rhizobium* have been found in root nodules and are called nodulins (Scheres et al., 1990). The expression of one of these proteins, the early nodulin ENOD12 from pea, was analyzed and appeared to be expressed in root hairs within 24 hours after infection with *R. leguminosarum* bv. *viciae*, but not in root hairs of uninfected plants. The "common" *nod* genes and the *hsn* genes were required for ENOD12 expression. Thus ENOD12 expression can be used as a specific bioassay for the *hsn* genes related factor(s) (Scheres et al., 1990).

Recent experiments in our laboratory have provided other possible test systems. Root exudates of *V. sativa* ssp. *nigra* plants infected with *R. leguminosarum* bv. *viciae* appear to have a much higher *nod* gene inducing activity than exudates of uninfected plants. Thus this *Rhizobium* induces an increase in the production and/or excretion of *nod* gene inducers in *V. sativa* ssp. *nigra* plants. The induction of *Vicia* was specific for *R. leguminosarum* bv. *viciae* strains and required the presence of the *nodLEFDABCIJ* genes in this bacterium. Also, a supernatant of a culture of *R. leguminosarum* bv. *viciae* with induced *nod* genes appeared to have the same inducing effect on *Vicia*. Thus this *Rhizobium* produces (a) guest specific factor(s) in the culture supernatant which can be detected using the production or secretion of additional *nod* gene inducers as bioassay (van Brussel et al., submitted). Up to now we did not find activity in this bioassay under any other circumstances than described above. Therefore this bioassay is more specific than the bioassays using the Tsr or Had phenotypes of *Vicia*, and is it now possible to distinguish the guest specific *Rhizobium* factor(s) in normal *Rhizobium* cultures with $10^8 - 10^9$ cells ml^{-1} in a mannitol-nitrate minimal medium. We are currently using this bioassay to purify and subsequently identify the guest specific factor(s) of *R. leguminosarum* bv. *viciae*.

Guest specificity and the nod *genes of* Rhizobium *required*

Two types of experiments can be done to detect the effect of symbiotic signals on plants: (i) "coculture" experiments in which plants and *Rhizobium* are both present in

the same culture, and (ii) experiments in which only the plant is present and the effects on the plants of bacterial culture supernatants or bacterial fractions are tested. The results in both types of experiments are different. For example Had and Tsr phenotypes are both induced in coculture of *V. sativa* ssp. *nigra* and a *Rhizobium* strain containing only the *nodDABC* genes and no other Sym plasmid located *nod* genes. When, however, a sterile supernatant of a culture of the same strain with induced *nod* genes is added to the same plants, the Tsr phenotype is not induced and only slight root hair deformation is observed. The reason for this difference, which does not occur when in addition to the *nodDABC* genes also the *hsn* genes of *R. leguminosarum* bv. *viciae* are present in *Rhizobium*, is not known. However care has to be taken when conclusions concerning excreted *soluble* factors of *Rhizobium* are drawn from coculture experiments. Thus the soluble *nod* gene related Tsr factor of *R. leguminosarum* bv. *viciae* has only been found in culture supernatants when the common *nod* genes as well as the *hsn* genes were present in this bacterium (Van Brussel et al., 1986; Zaat et al., 1987a). Hence the *soluble nod* gene related factor must be reckoned to the guest specific factors. Furthermore, we have seen in the preceding paragraph that the production of the soluble *R. leguminosarum* bv. *viciae* factor which induces the production and/or excretion of *nod* gene inducers by *V. sativa* ssp. *nigra* plants, requires the presence of the Sym plasmid in *Rhizobium*. The *nod* genes which are needed for production in *Rhizobium* culture remain to be determined. However, since in coculture the *nodLEFDABCIJ* genes are required, it can be expected that at least these genes are needed for the production in *Rhizobium* culture. Thus also the production of this factor requires the *hsn* genes and hence must be considered as a guest specific factor.

A soluble *nod* gene related factor present in a culture of *R. meliloti* bacteria with induced *nod* genes causes Had on *Medicago* sativa plants. A Tn5 mutation in the *hsn* gene *nodH* causes the disappearance of this effect (Faucher et al., 1988). Introduction of the *hsn* genes of *R. meliloti* in the *R. leguminosarum* bv. *viciae* strain 248 suppresses the presence of Tsr factor (which affects *V. sativa* ssp *nigra*) in culture supernatants of this strain and causes the appearance of Had factor for *Medicago sativa*. The *hsn* genes of *R. meliloti* responsible for this effect are *nodH* and *nodQ* (Faucher, 1989a). Thus the production of *R. meliloti* symbiotic factor also requires *hsn* genes and therefore is guest specific.

As the *nod* gene promoters do not function in *Escherichia coli*, *nod* gene expression in this bacterium can only be obtained if these promoters are replaced by *E. coli* promoters. Recently, Banfalvi et al. (1989) found indications for the production of a *nodABC* genes related soluble factor produced by an *E. coli* strain containing the *nodABC* genes of *R. meliloti* cloned behind an *E. coli* promoter. All three mentioned *nod* genes are required for the production of this factor, which is present in the supernatant of the *E. coli* culture and induces the Had phenotype on *Trifolium repens*

but not on *Medicago sativa*. When, in addition, an *E. coli* promoter preceded *hsn* gene, the *nodH* gene, is introduced in this *E. coli* strain, its culture supernatant causes Had on *Medicago sativa* as well as on *Trifolium repens*. The data show that the common *nod* genes, *nodABC*, alone code for the production of (a) factor(s) and induce(s) the symbiotic root phenotype Had on *Trifolium repens* but that for Had factor production on *Medicago sativa* additional *hsn* genes are required. This confirms the guest specificity of the *R. meliloti* Had factor.

Schmidt et al. (1988) found (a) factor(s) in the cytosol of strains of *R. meliloti*, which contain all the *nod* genes except the *nodBC* genes or except the *nodC* gene. The factor(s) was (were) detected by induction of mitosis in a plant protoplast culture. The cytosol of the strain lacking only the *nodC* gene was more active in the assay than the cytosol of the strain lacking the *nodBC* genes, indicating according to the authors that the NodA protein is involved in generating a factor which is converted by the NodB protein into a more active compound. To obtain root hair deformation with a bacterial cytosol fraction, *nodAB* genes are not sufficient and an additional *nodC* gene is required (Schmidt et al., 1988; Banfalvi et al., 1989). Since in the experiments of Schmidt et al. (1988) the *hsn* genes are always present, the factors produced may not be encoded by the common *nodAB* genes alone. Thus it cannot be decided whether this factor is "common" or guest specific.

In conclusion two types of soluble *nod* gene related *Rhizobium* factors have been described: (i) guest specific factors, different for the different *Rhizobium* species, which in *Rhizobium* or *E. coli* require the presence of the common *nod* genes and one (*nodH* in *E. coli*) or more *hsn* genes and, (ii) a "common" factor, which can be produced by an *E. coli* strain containing the *nodABC* genes. Why this common, *nodABC*-related factor cannot or only in very low concentrations be found in *Rhizobium* culture supernatants is not known. One of the possibilities is that this factor is only produced in low concentration by the *Rhizobium* strain but, owing to an other type of regulation, in a higher concentration by the *E. coli* strain.

Chemical properties

The chemical identity of symbiotic signals that influence plant cell growth and morphogenesis is very important for the understanding of the root nodule morphogenesis and for plant morphogenesis in general. Up to now very little information is available about these signals. The data that exist are based on indirect evidence which will be given below.

Since the Tsr factor is not produced any more by *R. leguminosarum* bv. *viciae* when cloned *hsn* genes of *R. meliloti* are present in this bacterium, and instead a *R. meliloti* Had factor is formed, Faucher et al. (1988, 1989a) proposed as a working hypothesis that the Tsr factor is modified into a *Medicago sativa* specific Had factor. Furthermore, since the production of these symbiotic signals requires the *nodAB*(*C*) genes in the

producing strain, Banfalvi et al. (1989) suppose that both these symbiotic signals are related. This is in accordance with the fact that the genetically well defined symbiotic signals of *Rhizobium*, that are related to known *nod* genes, are heat stable and that their molcular weights are smaller than 5000 Da as determined by ultrafiltration (Faucher et al., 1989a; Banfalvi et al., 1989; Van Brussel et al., unpublished). The *nodAB* related factor of *R. meliloti* even passes through a filter with a 1000 Da cut-off (Schmidt et al., 1988). This was not the case with the guest specific factors of *R. meliloti* and *R. leguminosarum* bv. *viciae* (Faucher et al., 1989a). Thus for the moment the experimental evidence fits with the model of Faucher et al. (1988, 1989a) that the *nodABC* genes are involved in generating a low molcular weight compound or compounds, which are converted by the products of the *hsn* genes of *Rhizobium* in guest specific factors.

One of the possibilities, namely that the flavonoid inducer molecules are converted by *Rhizobium* into the bacterial symbiotic signal (Van Brussel et al., 1986) has been investigated in our laboratory. In these experiments we used a hybrid *nodD* gene which codes for a constitutive inducing NodD protein, i.e. that induces the *nod* operons without requiring activation by flavonoid inducer molecules (Spaink et al., 1989). When this hybrid *nodD* gene is present in *R. leguminosarum* bv. *viciae*, Tsr- and Had factor were produced in its culture in absence of flavonoids (Zaat 1988b). The factor responsible for the increased *nod* gene inducing activity in the *V. sativa* ssp. *nigra* exudate was also produced (Van Brussel et al., submitted). Thus it is unlikely that flavonoid molecules are the precursors of these *Rhizobium* symbiotic signals. Banfalvi et al. (1989) came to the same conclusions based on the fact that Had factor is produced in absence of flavonoids by an *E. coli* strain containing the cloned *nodABC* genes.

Recently Faucher et al. (1989b) extracted the *R. meliloti* Had factors produced by cultures with induced *nod* genes with ethylacetate and butanol. Subsequent purification of the factors was carried out by HPLC chromatografy with a C18 reversed phase column and a water/acetonitrile gradient. They found 2 peaks which were absent when the factor producing *R. meliloti* strain contained a Tn5 mutation in the *nodA* or the *nodC* genes, showing that the common *nod* genes are required for the compounds responsible for the 2 peaks. When the same bacterium contained a Tn5 mutation in the *nodH* or the *nodQ* gene, both peaks were less prominent and a third peak with more hydrophobic properties appeared, providing biochemical evidence that both of these *hsn* genes are involved in the modification of the extracellular factors. NMR and mass spectrometry data also indicated that these factors are of low molecular weight.

Future Prospects

Since the symbiotic signals of *Rhizobium* are being studied by a number of groups and since more specific bioassays are available now, we expect that the chemical

structure of these signals will be elucidated in the near future. The role of the symbiotic signals of *Rhizobium* in root nodule morphogenesis can then be determined and their guest specific properties studied. As the knowledge of the first plant signal has been an important tool in the study of rhizobial *nod* gene regulation, we expect that knowledge of the Rhizobium signals will be of great help in the study of the regulation of plant genes involved in nodulation. It remains to be determined whether the increased *nod* gene inducing activity found in root exudate of *V. sativa* ssp. *nigra* plants infected with *R. leguminosarum* bv. *viciae* represents a second plant signal in response to *Rhizobium* signals, or that it simply reflects processes that are going on in the root. In the latter case investigations on the role of flavonoids in plant cell differentiation become very interesting.

Note Added in Proof

Recently (Lerouge et al., Nature (London). *344*: 781–784 (1990)), the *R meliloti* host-range singal NodRm1 was identified as a sulfated β-1-4-tetrasaccharide of D-glucosamine in which three amino groups are acetylated and one is acylated with a C16 biunsaturated fatty acid. NodRm1 induces specific root hair deformation on alfalfa plants.

Spaink et al. (Henneke, H. et al., Proceedings of the 5th International Symposium on the Molecular Genetics of Plant-Microbe Interactions, September 1990, Interlaken, Switzerland, APS-press, in press) reported on several symbiotic signals, among which biovar specific signals, of *R leguminosarum* bv. *viciae*. These signals are related to the NodRm1 signal, however the sulfate group at the reducing end of the D-glucosamine chain (which in one case is a pentasaccharide) is lacking and they contain an O-acetyl group, which is *nodL* dependent and required for the induction of increased *nod* gene inducing activity in *Vicia sativa* ssp. *nigra* root exudate. Also the fatty acid chain has a somewhat different composition.

The sulfate group on NodRm1 is involved in host-specificity of *R meliloti*. The determinant(s) of host specificity in the symbiotic signals of *R leguminosarum* bv. *viciae* is(are) not known yet.

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REFERENCES

Banfalvi, Z. and Kondorosi, A. 1989. Production of root hair deforming factors by *Rhizobium meliloti* nodulation genes in *Escherichia coli: HsnD (NodH)* is

involved in the plant host-specific modification of the NodABC factor. *Plant Mol. Biol.* **13:** 1–12.

Bender, G.L., Nayudu, M., Le Strange, K.K., and Rolfe, B.G. 1988. The *nodD1* gene from *Rhizobium* strain NGR234 is a key determinant in the extension of host range to the nonlegume *Parasponia. Mol. Plant-Microbe Int.* **1:** 25–266.

Canter Cremers, H.C.J., Van Brussel, A.A.N., Plazinski, J., and Rolfe, B.G. 1986. Sym plasmid and chromosomal gene products of *Rhizobium trifolii* elicit developmental responses on various legume roots. *J. Plant Physiol.* **122:** 25–40.

Ervin, S.E. and Hubbell, D.H. 1985. Root hair deformations associated with fractionated extracts from *Rhizobium trifolii. Appl. Environ. Microbiol.* **49:** 61–68.

Faucher, C., Maillet, F., Vasse, J., Rosenberg, C., van Brussel, A.A.N., Truchet, G. and Dénarié, J. 1988. *Rhizobium meliloti* host range *nodH* determines production of an alfalfa-specific extracellular signal. *J. Bacteriol.* **170:** 5489–5499.

Faucher, C., Camut, S., Dénarié, J. and Truchet, G. 1989a. The *nodH* and *nodQ* host range genes of *Rhizobium meliloti* behave as Avirulence genes in *R. leguminosarum* bv. *viciae* and determine changes in the production of plant-specific extracellular signals. *Mol. Plant-Microbe interactions.* **2:** 291–300.

Faucher, C., Lerouge, P., Roche, P., Rosenberg, C., Debellé, F., Vasse, J., Cervantès, E., Sharma, S.B., Truchet, G., Promé, J.C. and Dénarié, J. 1989b. The common *nodABC* genes and the *nodH* and *nodQ* host-range genes of *Rhizobium meliloti* determine the production of low molecular weight extracellular signals. In: *Signal Molecules in Plant and Plant-Microbe Interactions.* Nato ASI Series, Series H: Cell Biologie, Vol. 36. B.J.J. Lugtenberg, ed. Springer Verlag, Berlin, Heidelberg. pp 379–386.

Firmin, J.L., Wilson, K.E., Rossen, L., and Johnston, A.W.B. 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. *Nature(London).* **324:** 90–92.

Györgypal, Z., Iyer, N., and Kondorosi, A. 1988. Three regulatory *nodD* alleles of divergent flavonoid-specificity are involved in host-dependent nodulation by *Rhizobium meliloti. Mol. Gen. Genet.* **212:** 85–92.

Hiltner, L. 1900. Ueber die Ursachen, welch die Grösse, Zahl, Stellung und Wirkung der Wurzelknöllchen der Leguminosen bedingen. *Arb. Gsndhtamt., Biol. Abt.* **1:** 177–222.

Horvath, B., Kondorosi, E., John, M., Schmidt, J. Török, I., Györgypal, Z., Barabas, I., Wieneke, U., Schell, J., and Kondorosi, A. 1986. Organization, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. *Cell.* **46:** 335–343.

Horvath, B., Bachem, C.W., Schell, J., and Kondorosi, A. 1987. Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant-signal, interacting with the *nodD* gene product. *EMBO J.* **6:** 841–848.

Innes, R.W., Kuempel, P.L., Plazinski, J., Canter Cremers, H.C.J., Rolfe, B.G., and Djordjevic, M.A. 1985. Plant factors induce expression of nodulation and host-range genes in *R. trifolii*. *Mol. Gen. Genet.* **201:** 426–432.

Kosslak, R.M., Bookland, R., Barkei, J., Paaren, H.E., and Appelbaum, E.R. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. *Proc. Natl. Acad. Sci. USA.* **84:** 7428–7432.

Long, S.R. 1989. *Rhizobium*-legume nodulation: Life togehter in the underground. *Cell.* **56**: 203–214.

Maxwell, C.A., Hartwig, U.A., Joseph, C.M., and Philips, D.A. 1989. A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti*. *Plant Physiol.* **91:** 842–847.

Mulligan, J.T. and Long, S.R. 1985. Induction of *Rhizobium meliloti nodC* expression by plant root exudate requires *nodD*. *Proc. Natl. Acad. Sci. USA.* **82:** 6609–6613.

Peters, N.K., Frost, J.W., and Long, S.R. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* genes. Science. **233:** 977–980.

Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L., and Rolfe, B.G. 1986. Flavones induce expression of nodulation genes in *Rhizobium*. *Nature* (*London*). **323:** 632–635.

Rossen, L., Shearman, C.A., Johnston, A.W.B., and Downie, J.A. 1985. The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. *EMBO J.* **4:** 3369–3373.

Rostas, K., Kondorosi, E., Horvath, B., Simoncsits, A., and Kondorosi, A. 1986. Conservation of extended promoter regions of nodulation genes in *Rhizobium*. *Proc. Natl. Acad. Sci. USA.* **83:** 1757–1761.

Scheres, B., Van de Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Wolters, A.M., Gloudemans, T., Van Kammen, A., and Bisseling, T. 1990. The ENOD12 early nodulin is involved in the infection process during the Pea-*Rhizobium leguminosarum* interaction. *Cell.* **60:** 281–294.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. 1988. *Rhizobium meliloti nodA* and *nodB* genes are involved in generating compounds that stimulate mitosis of plant cells. *Proc. Natl. Acad. Sci. USA.* **85:** 8578–8582.

Schofield, P.R. and Wayson, J.M. 1986. DNA sequence of *Rhizobium trifolii* nodulation genes reveals a reiterated and potentially regulatory sequence preceding *nodABC* and *nodFE*. *Nucleic Acids Res.* **14:** 2891–2903.

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E., and Lugtenberg, B. 1986. Promoters and operon structure of the nodulation region of the *Rhizobium leguminosarum* symbiosis plasmid pRL1JI. In: *Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions*, ASI Series, Vol H 4. B. Lugtenberg, ed. Springer Verlag, Berlin, Heidelberg, pp. 55–68.

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Pees, E., and Lugtenberg, B.J.J. 1987a.

Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. *Plant Mol. Biol.* **9:** 27–39.

Spaink, H.P., Wijffelman, C.A., Pees, E., Okker, R.J.H., and Lugtenberg, B.J.J. 1987b. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. *Nature (London).* **328:** 337–340.

Spaink, H.P., Okker, R.J.H., Wijffelman, C.A., Tak, T., Goosen-deRoo, L., Pees, E., Van Brussel, A.A.N., and Lugtenberg, B.J.J. 1989. Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. *J. Bacteriol.* **171:** 4045–4053.

Van Brussel, A.A.N., Zaat, S.A.J., Canter Cremers, H.C.J., Wijffelman, C.A., Pees, E., Tak, T., and Lugtenberg, B.J.J. 1986. Role of plant root exudate and sym plasmid-localized nodulation genes in the synthesis by *Rhizobium leguminosarum* of Tsr factor which causes thick and short roots on common vetch. *J. Bacteriol.* **165:** 517–522.

Vincent, J.M. 1980. Factors controlling the Legume-*Rhizobium* symbiosis. In: *Nitrogen Fixation.* W.E. Newton, and Orme-Johnson, eds. Univ. Park Press, Baltimore, pp. 103–129.

Wijffelman, C.A., Zaat, B., Spaink, H., Mulders, I., Van Brussel, A.A.N., Okker, R., De Maagd, R., and Lugtenberg, B.J.J. 1986. Induction of *Rhizobium nod* genes by flavonoids: differential adaptation of promoter, *nodD* gene and inducers for various cross-inoculation groups. In: *Recognition in Microbe-plant Symbiotic and Pathogenic interactions,* NATO ASI Series, Vol H 4. B. Lugtenberg, ed. Springer Verlag, Berlin, Heidelberg, pp. 123–135.

Zaat, S.A.J., Van Brussel, A.A.N., Tak, T., Pees, E., and Lugtenberg, B.J.J. 1987a. Flavonoids induce *Rhizobium leguminosarum* to produce *nodDABC* gene-related factors that cause thick, short roots and root hair responses on common vetch. *J. Bacteriol.* **169:** 3388–3391.

Zaat, S.A.J., Wijffelman, C.A., Spaink, H.P., Van Brussel, A.A.N., Okker, R.J.H., and Lugtenberg, B.J.J. 1987b. Induction of the *nodA* promoter of *Rhizobium leguminosarum* Sym plasmid pRL1JI by plant flavanones and flavones. *J. Bacteriol.* **169:** 198–204.

Zaat, S.A.J., Wijffelman, C.A., Mulders, I.H.M., Van Brussel, A.A.N., and Lugtenberg, B.J.M. 1988a. Root exudates of various host plants of *Rhizobium leguminosarum* contain different sets of inducers of *Rhizobium* nodulation genes. *Plant Physiol.* **86:** 1298–1303.

Zaat, S.A.J. 1988b. Molecular signals in the early *Rhizobium*-legume interaction. PhD thesis, Leiden University, The Netherlands.

Zaat, S.A.J., Schripsema, J., Wijffelman, C.A., Van Brussel, A.A.N., and Lugtenberg, B.J.J. 1989. Analysis of the major inducers of the *Rhizobium nodA* promoter from *Vicia sativa* root exudate and their activity with different *nodD* genes. *Plant Mol. Biol.* **13:** 175–188.