Lipo-oligosaccharides of Rhizobium induce infection-related early nodulin gene expression in pea root hairs

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SHORT COMMUNICATION

Lipo-oligosaccharides of *Rhizobium* induce infection-related early nodulin gene expression in pea root hairs

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Summary

This paper shows that lipo-oligosaccharides (Nod factors) synthesized by *Rhizobium* bacteria elicit the induction of infection-related early nodulin genes (*PsENOD5* and *PsENOD12*) in pea root hairs. *R. leguminosarum* bv. *viciae* secretes a mixture of Nod factors containing a C18 fatty acid chain with 4 (C18:4) or 1 double bond (C18:1). Purified Nod factors harbouring either a C18:4 or a C18:1 acyl moiety induce the expression of the pea early nodulin genes, *PsENOD5* and *PsENOD12*, but the kinetics of induction are different. The expression of both early nodulin genes is induced in a transient manner by the purified Nod factors while a mixture of the Nod factors extends the period during which these genes are expressed. In spite of the host-specific nature of the infection process, heterologous Nod factors of *R. meliloti* also induce the expression of *PsENOD5* and *PsENOD12* genes, though with a marked delay compared with the homologous compounds.

Introduction

Infection of roots of leguminous plants by *Rhizobium* bacteria induces the formation of a complete new organ, the so-called root nodule. In these nodules the rhizobia are able to reduce atmospheric nitrogen to ammonia, via a process named symbiotic nitrogen fixation. Nodule organogenesis involves deformation and curling of root hairs, the formation of infection threads and the induction of cell divisions in the normally terminally differentiated root cortex. The latter process, leads to the formation of nodule primordia, which upon penetration by infection threads and subsequent release of the bacteria, develop into root nodules (e.g. Newcomb, 1976, 1981). During the successive steps of root nodule formation, nodule-specific plant genes, called nodulin genes, are induced (Van Kammen, 1984). The nodulin genes whose expression is detectable during early steps of nodule formation are called 'early' nodulin (*ENOD*) genes (Nap and Bisseling, 1989). The expression of two previously identified pea early nodulin genes, *PsENOD5* and *PSENOD12*, is correlated with the infection process. *In situ* hybridization studies on infected roots showed that the *PsENOD5* gene is only expressed in cells containing a growing infection thread. The *PsENOD12* gene is also transcribed in root hairs and cortical cells containing a growing infection thread but in addition, the *PsENOD12* gene is expressed several cell layers in front of the infection thread tip (Scheres *et al.*, 1990a, 1990b). Thus, in this respect *PsENOD12* gene expression differs from that of the *PsENOD5* gene, which suggested that the induction of the *PsENOD5* gene involves intracellular signalling only, while the transcription of the *PsENOD12* gene requires both intra- and intercellular signalling (Nap and Bisseling, 1990). Genetic studies have shown that the nodulation (*nod*) genes of *Rhizobium* play a pivotal role in the induction of initial steps in nodule formation (Long, 1989). The *nod* genes are induced by secreted plant flavonoids as a result of which the bacteria secrete signal molecules named Nod factors (Fisher and Long, 1992; Lerouge *et al.*, 1990; Spaink *et al.*, 1991). The major *R. meliloti* Nod factors are sulphated β-1,4-tetra- and pentasaccharides of D-glucosamine in which the amino group at the non-reducing terminal sugar is acylated with an unsaturated C16 fatty acid chain containing two double bonds, while the other three glucosamines are acetylated (Lerouge *et al.*, 1990; Roche *et al.*, 1991; Schultze *et al.*, 1992). It has been shown that exogenously applied NodRm factors are sufficient to induce root hair deformation (Lerouge *et al.*, 1990), and depolarization of root hair membranes (Ehrhardt *et al.*, 1992) as well as root cortical cell division (Truchet *et al.*, 1991). The sulphate group is a major determinant of host-specific induction of root hair deformation. *R. meliloti* Nod factors containing a sulphate

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group deform alfalfa root hairs, whereas after removal of this group, the factors lose this ability and become efficient inducers of root hair deformation on vetch (e.g. *Vicia sativa*) (Roche *et al.*, 1991). The latter legume, as well as *Pisum sativum* (pea), is a natural host plant for *R. leguminosarum* bv. *viciae*, and is normally unable to establish a symbiosis with wild-type *R. meliloti* strains.

R. leguminosarum bv. *viciae* Nod factors (NodRlv) are structurally related to NodRm factors (Spaink *et al.*, 1991). These compounds are tetra- or pentamers of D-glucosamine, but lack of sulphate group at the reducing end sugar and also the acyl group at the non-reducing terminal sugar is different from the one present in NodRm-IV (C16:2, S) (Spaink *et al.*, 1991). *R. leguminosarum* bv. *viciae* produces Nod factors with two different fatty acid chains. One lipid has a C18 chain containing four double bonds (C18:4) and its synthesis requires the activity of the NodE protein, whereas the other lipid has a C18 fatty acid chain containing only one unsaturated bond (C18:1). Nod factors containing the C18:1 acyl group are still formed by a *nod*E mutant (Spaink *et al.*, 1991). Vetch root hair deformation is induced by Nod factors containing either a C18:4 or a C18:1 lipid moiety, whereas cortical cell division is only triggered by factors containing a C18:4 acyl group (Spaink *et al.*, 1991).

Similarly, only a C18:4 containing compound can elicit the formation of 'pre-infection threads' in the root outer cortex (Van Brussel *et al.*, 1992). These are radial rows of cells in which the nucleus has moved to the centre and the cytoplasm has a radial alignment.

Here we have studied whether *R. leguminosarum* bv. *viciae* Nod factors can induce the expression of pea early nodulin genes. We have selected the *PsENOD5* and *PsENOD12* genes for this purpose, since these genes are induced at an early stage of the *Rhizobium*-plant interaction and are involved in the infection process. We have compared the abilities of different *R. leguminosarum* bv. *viciae* Nod factors to induce expression of these two early nodulin genes and in addition we have tested whether the heterologous *R. meliloti* Nod factors can elicit the activation of these pea genes.

Results

Induction of PsENOD5 *gene expression by bacterial-free medium*

In a former publication (Scheres *et al.*, 1990a) we demonstrated that the expression of the *PsENOD12* gene is induced by bacterial-free culture medium of *R. leguminosarum* bv. *viciae* grown in the presence of the *nod* gene inducer naringenin. In similar experiments we have analysed whether the *PsENOD5* gene expression is also triggered by a bacterial-free medium of an induced culture.

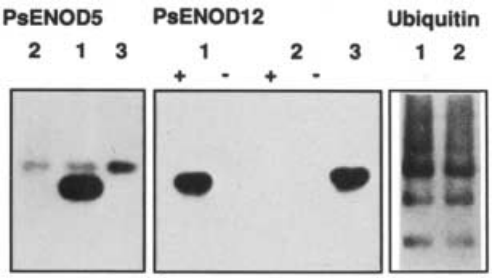

Figure 1. Detection of *PsENOD5*, *PsENOD12* and ubiquitin mRNAs in root hair RNA preparations from pea seedlings.
Pea seedlings (1) were treated with bacterial-free culture medium of *R. leguminosarum* bv. *viciae* grown in the presence of naringenin and the control plants (2) were treated with bacterial growth medium. In panel PsENOD12, lanes 1 and 2 the cDNA synthesis step was carried out in the presence (+) or the absence (–) or reverse transcriptase. In panels PsENOD5 and PsENOD12 lanes 3 represent a PCR amplification on pea genomic DNA. The *PsENOD5* fragments are 418 bp (genomic DNA) and 338 bp (mRNA) long, while the amplified *PsENOD12* DNA is 280 bp long. The length of the ubiquitin fragments are 228, 456, 684, 912 and 1140 bp.

PsENOD5 as well as *PsENOD12* gene expression were studied by a PCR based assay in which ubiquitin (*ubi*) mRNA was used as an internal control (see Experimental procedures). The *PsENOD5* gene contains a small intron of 80 bp (Horvath, unpublished data). Using primers homologous to the regions flanking this intron, amplification of genomic DNA by PCR resulted in a DNA fragment 80 bp longer than the mRNA-derived product (Figure 1, panel PsENOD5, lane 3). Thus, the appearance of a 338 bp long fragment indicated the expression of the *PsENOD5* gene (Figure 1, panel PsENOD5, lane 1), while the 418 bp long band could reflect the presence of remaining genomic DNA (Figure 1, panel PsENOD5).

Despite using DNase-treated RNA samples in all experiments, the 418 bp long fragment was occasionally still faintly visible after 27 cycles.

None of the *PsENOD12* genes harbours an intron (Govers *et al.*, 1991; Vijn, unpublished data, and Figure 1, PsENOD12 lanes 1 and 3), therefore the contribution of genomic DNA to the amplified *PsENOD12* DNA was tested in a different way. By omitting the cDNA synthesis step we showed that in none of the experiments presented here was *PsENOD12* genomic DNA amplified to a detectable level after 23 cycles (Figure 1, panel PsENOD12, lanes 1 and 2 + and –).

In the experiment presented in Figure 1, root hairs from 100 seedlings were harvested 48 h after application of the medium and total RNA was isolated. After amplification of the relevant cDNA products we have shown that indeed the bacterial-free medium was able to induce *PsENOD12* as well as *PsENOD5* gene expression. Based on these data we have tested whether purified

Nod factors are able to elicit the transcription of these genes.

Induction of PsENOD12 *and* PsENOD5 *gene expression by different NodRlv compounds*

Analysis by Spaink *et al.* (1991) showed that the nature of the acyl moiety of *R. leguminosarum* bv. *viciae* Nod factors is a major determinant in the induction of mitotic activity. Therefore, we compared the inducing activity of two NodRlv factors which only differ in their acyl content. Pea seedlings were treated with 10^{-8} M purified NodRlv-V(Ac, C18:4) and NodRlv-V(Ac, C18:1), and as a control the mixture of NodRlv factors (see Experimental procedures) containing four different Nod metabolites in about equimolar amounts was used. Root hairs were isolated 12, 24 and 48 h after inoculation. As shown in Figure 2 (panel PsENOD12 lane 2) NodRlv-V(Ac, C18:4) induced *PsENOD12* gene expression within 12 h, followed by a marked decrease in the *PsENOD12* mRNA level at 24 h. The *PsENOD5* transcript also accumulated transiently after treatment with NodRlv-V(Ac, C18:4). *PsENOD5* mRNA was also detectable at 12 h, though a higher level of the *PsENOD5* transcript accumulated at 24 h, after which it decreased to a non-detectable level at 48 h (Figure 2, panel PsENOD5). Thus, the *PsENOD5* gene was transcribed during a later time period than the *PsENOD12* gene.

The Nod factor containing the C18:1 acyl moiety also had the ability to induce the expression of both pea early nodulin genes in a transient manner (Figure 2, panels PsENOD12 and PsENOD5, lanes 3). However, in comparison with NodRlv-V(Ac, C18:4)-treated plants there was delay in the timing of induction of both early nodulin genes. The *PsENOD12* gene was expressed at a high level after 24 h, while at this time point the amount of the *PsENOD12* transcript in NodRlv-V(Ac, C18:4)-treated plants had already dropped to a low level (Figure 2, panel PsENOD12, lanes 2 and 3). The *PsENOD5* mRNA was already detectable 12 h after treatment with the C18:4 Nod factor, while only a very low level of *PsENOD5* messenger was detected at this time point in the C18:1 Nod-factor-treated plants. The *PsENOD5* mRNA reached the highest detected level at 24 h in plants treated by either Nod factor (Figure 2, panel PsENOD5, lanes 2 and 3).

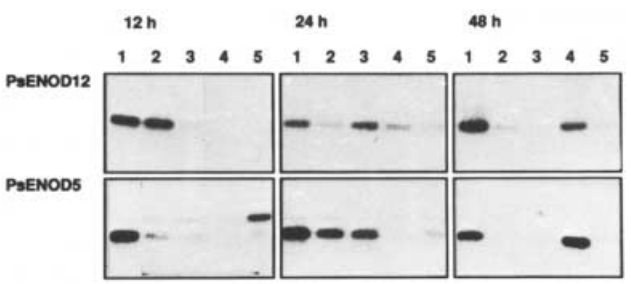

Figure 2. Induction of early nodulin gene expression by Nod factors at different time points.
PsENOD12 and *PsENOD5* gene expression induced by (1) the mixture of the NodRlv factors, (2) NodRlv-V(Ac, C18:4), (3) NodRlv-V(Ac, C18:1), (4) NodRm-IV(C16:2, S)/NodRm-IV(Ac, C16:2, S) and (5) 10 mM Tris-HCl pH 7.5 at 12, 24 and 48 h after application.

Previously, we reported that a *nod*E mutant of *R. leguminosarum* bv. *viciae* does not elicit *PsENOD12* gene expression (Scheres *et al.*, 1990a). As this observation was not consistent with the induction of this gene by NodRlv-V(Ac, C18:1) we repeated the experiments and showed that this mutant was indeed able to trigger *PsENOD12* as well as *PsENOD5* gene expression (data not shown). Most likely, we missed this expression in former experiments since the *PsENOD12* cDNA was only amplified during 16 cycles (Scheres *et al.*, 1990a) instead of the 23 cycles used in these experiments.

The mixture of NodRlv factors also had the ability to induce *PsENOD12* and *PsENOD5* gene expression. However, there was a striking difference in the pattern of induction in pea seedlings treated with one single compound. While NodRlv-V(Ac, C18:4) and NodRlv-V(Ac, C18:1) induced the expression of both early nodulin genes transiently, the mixture of the Nod factors activated these genes during the whole period that the plants were examined (Figure 2, panels PsENOD12 and PsENOD5, lanes 1). Similar *PsENOD5* and *PsENOD12* mRNA accumulation patterns were observed when plants were treated with 10^{-9} M NodRlv factors (data not shown).

Induction of PsENOD12 *and* PsENOD5 *gene expression by heterologous NodRm factors*

NodRm-IV(C16:2, S) and NodRm-IV(Ac, C16:2, S), the major Nod factors produced by *R. meliloti*, induce root hair deformation in a host-specific manner. They deform alfalfa root hairs, but not those of a host plant (vetch) of *R. leguminosarum* bv. *viciae* at 10^{-8}–10^{-12} M (Lerouge *et al.*, 1990; Schultze *et al.*, 1992). To test whether the pea early nodulin genes are induced in a similar host-specific manner, pea seedlings were treated with 10^{-8} M of a mixture of NodRm-IV(C16:2, S) and NodRm-IV(Ac, C16:2, S) (see Experimental procedures) and the levels of *PsENOD12* and *PsENOD5* mRNA in root hairs were determined at different time points after inoculation (Figure 2, lanes 4). Surprisingly, both early nodulin genes were induced by these heterologous compounds, but the highest level of expression was observed at 48 h (Figure 2, panels PsENOD12 and PsENOD5, lanes 4). So, there was a significant delay in the induction of these two plant genes when compared with NodRlv-treated pea seedlings.

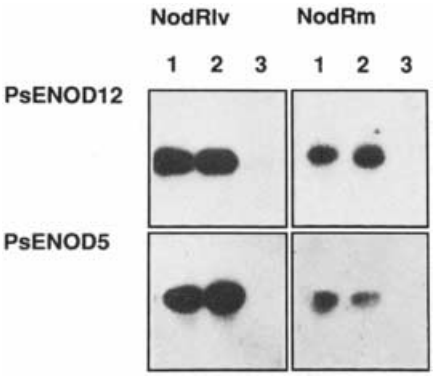

Figure 3. Induction of early nodulin gene expression by homologous and heterologous Nod factors at different concentrations.
PsENOD12 and *PsENOD5* gene expression induced by NodRlv (the mixture of the NodRlv factors) and NodRm (NodRm-IV(C16:2, S)/NodRm-IV(Ac, C16:2, S)) at different concentrations: (1) 10^{-9}, (2) 10^{-10} M; (3) no Nod factors, at 48 h after application.

Observations of Schultze *et al.* (1992) showed that NodRm factors when applied at relatively high concentrations (higher than 10^{-8} M) can induce root hair deformation on a non-host plant. Therefore, we studied whether NodRm factors can induce the expression of pea early nodulin genes at concentrations lower than 10^{-8} M. Pea seedlings were treated with 10^{-9} and 10^{-10} M of the mixture of NodRm-IV(C16:2, S) and NodRm-IV(Ac, C16:2, S), and as a control the mixture of NodRlv factors was applied at the same concentrations. Root hairs were harvested 48 h after treatment. As shown in Figure 3 (panels PsENOD12 and PsENOD5) application of 10^{-9} and 10^{-10} M (lanes 1 and 2, respectively) of either NodRlv or NodRm factors induced the expression of both early nodulin genes. Therefore, the heterologous compounds elicit the expression of pea early nodulin genes at concentrations, which trigger root hair deformation in a host-specific way (Schultze *et al.*, 1992).

Discussion

This is the first report on the induction of early nodulin gene expression elicited by purified *Rhizobium* Nod factors. We have compared the inducing activity of the Nod factors, NodRlv-V(Ac, C18:4) and NodRlv-V(Ac, C18:1), which differ only in their fatty acid moiety. We have shown that both factors have the potential to induce the expression of *PsENOD5* as well as *PsENOD12* genes. *In situ* hybridization showed that the spatial distribution of *PsENOD5* and *PsENOD12* mRNAs in infected roots is different (Scheres *et al.*, 1990b). These data suggested that these two genes are induced by different mechanisms (Nap and Bisseling, 1990). Here we have shown that the same Nod factors can induce the expression of both plant genes, indicating that the same receptor mediates the induction of both early nodulin genes. Therefore, we postulate that the difference in mechanism of gene induction is caused by secondary signals generated after perception of the Nod factors.

The patterns of *PsENOD5* and *PsENOD12* mRNA accumulation after treatment with a mixture of NodRlv factors markedly differed from those induced by individual factors. The major difference concerned the period during which the *PsENOD5* and *PsENOD12* mRNAs were present in root hairs. This period was significantly longer in seedlings exposed to a mixture of Nod factors than in those that were treated with single Nod factors. The latter induced a transient mRNA accumulation. This might be caused by transient expression of the *PsENOD5* and *PsENOD12* genes or by a marked drop of the Nod factor concentration during the experiment. We think that the latter explanation is rather unlikely since identical concentrations of the mixture of Nod factors induce expression during a longer period. At this moment it is unknown why the mixture of Nod factors induces early nodulin gene expression during an extended period. One explanation might be that the different factors complement one another. However, we can not exclude the possibility that co-purified compounds present in the intermediate fractions have a synergistic effect. Several studies have shown that only root cells present in a relatively small zone, just above the root tip, are susceptible to interaction with rhizobia (Bauer, 1981). Thus, the extended duration of early nodulin gene expression might increase the chance that *Rhizobium* induces early nodulin gene expression in the susceptible zone of the root, which might be the biological significance of the secretion of a mixture of Nod factors.

Studies of Pichon *et al.* (1992) showed that the *Medicago truncutula MtENOD12* gene is induced by *Rhizobium* in root hairs as well as in epidermal cells at a stage preceding root hair formation. Since we only could analyse the accumulation of mRNAs in root hairs, our observations are restricted to the events occurring in these cells.

Nod factors containing either a C18:4 or C18:1 acyl moiety are able to induce *PsENOD5* and *PsENOD12* gene expression, but the timing of induction is different. The C18:4-containing factor induced expression at an earlier time point than the factor with the C18:1 fatty acid. This difference in timing was observed in all experiments, though the exact time point of induction varied. To obtain more information on the timing of early nodulin gene expression, we are planning to use cytological methods on serial sections of roots.

A major characteristic of the *Rhizobium*–legume interaction is its host-specific nature. Therefore, we studied whether Nod factors of *R. meliloti*, which are unable to induce root hair deformation and cell division in the host

plants of *R. leguminosarum* bv. *viciae* (Roche *et al.*, 1991), can elicit pea early nodulin gene expression at a concentration that causes host-specific responses. Surprisingly, both pea early nodulin genes are activated by the heterologous *R. meliloti* Nod factors. However, this induction is first detectable after a marked delay. We propose that this delay may be caused by a modification (e.g. by removal of the sulphate group) of NodRm factors by the plant before an interaction of the NodRm factors with the putative pea Nod factor-receptor can take place.

Our experiments show that Nod factors trigger the expression of infection-related early nodulin genes, and thus induce processes correlated to the infection process. The observation of Van Brussel *et al.* (1992) on the formation of 'pre-infection threads' also demonstrates the role of Nod factors in the induction of infection-related processes. However, in their experiments only a C18:4 Nod factor provoked the formation of these structures while the expression of early nodulin genes is induced by Nod factors containing a C18:1 or C18:4 acyl moiety. This suggests that eliciting infection-related early nodulin gene expression has less stringent requirements to the Nod factor structure than the induction of a rather complex process, like 'pre-infection thread' formation.

Studies presented in this paper together with previously reported observations show that Nod factors are involved in all early steps of nodule formation. Nod factors are sufficient to induce root hair deformation and cortical cell divisions (Lerouge *et al.*, 1990; Spaink *et al.*, 1991; Truchet *et al.*, 1991) and the involvement in infection is demonstrated by the induction of the early nodulin genes *PsENOD5* and *PsENOD12* (this paper and the function of infection threads (Van Brussel *et al.*, 1992)). The major challenge now is to understand the mechanisms by which Nod factors can elicit such a variety of responses. Here we have shown that assays based on the induction of early nodulin gene expression could be useful tools to obtain clues on such mechanisms.

Experimental procedures

Inoculation of plant material

Pea (*Pisum sativum* L. cv. Rondo) plants were cultured as described previously by Bisseling *et al.* (1978). In order to harvest equal amounts of root hairs 3-day-old pea seedlings were treated with Nod factors for the 48 h time point; while 4-day-old seedlings were used for the 24 and 12 h treatments. Thus, root hairs were always isolated (Gloudemans *et al.*, 1989) from 5-day-old seedlings. Each experiment involved 100 pea seedlings and 1 ml bacterial-free culture medium (Scheres *et al.*, 1990a) or a solution of Nod factors (in 10 mM Tris-HCl pH 7.5) was pipetted on to the tap root of each seedling.

Total RNA isolation

Total RNA was isolated using the hot phenol method with subsequent LiCl precipitation as described by De Vries *et al.* (1982). RNA samples were dissolved in water (1 μg μl^{-1}) and carbohydrates were removed by adding KAc to 0.3 M final concentration, followed by chloroform extraction and ethanol precipitation (2.5 vol.). The RNA (1 μg μl^{-1}) was dissolved in 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ and contaminating DNA was digested with 10 U DNase (Boehringer) for 30 min at 37°C. After ethanol precipitation the RNA preparations were dissolved in water (1 μg μl^{-1}). The total RNA preparations were quantified using spectrophotometry and agarose gel electrophoresis. Only RNA preparations having both A_{260}/A_{280} and A_{260}/A_{240} ≥2 were used for further studies. The integrity of the RNA samples was examined by 2% agarose gel electrophoresis (Sambrook *et al.*, 1989).

PCR assays to detect PsENOD12 *and* PsENOD5 *mRNA*

The *PsENOD12* and *PsENOD5* mRNA levels in root hair RNA preparations were determined by the following PCR assay: 1 μg of total RNA and 200 ng of synthetic oligonucleotide 1 or 3 (Table 1) were incubated in 10 μl annealing buffer (250 mM KCl, 10 mM Tris-HCl pH 8.3, 1 mM EDTA) first for 3 min at 85°C and subsequently for 30 min at 52°C (*PsENOD12*) or 48°C (*PsENOD5*). After gradual cooling to 42°C, 15 μl of reverse transcription buffer (24 mM Tris-HCl pH 8.3, 16 mM $MgCl_2$, 8 mM DTT, 0.4 mM dNTPs) including 2.5 U AMV reverse transcriptase (Life Sciences, Inc.) was added and cDNA was synthesized at 42°C for 45 min. Then 75 μl amplification buffer (33.3 mM KCl, 7.2 mM Tris-HCl pH 8.3, gelatin 0.01 w/v) supplemented with 1 μl of 25 mM of dNTPs, 1.5 U *AmpliTaq* DNA Polymerase (Perkin Elmer, Cetus) and 200 ng of oligonucleotide 2 (*PsENOD12*) or 4 (*PsENOD5*; Table 1) was added to the cDNA samples. The *PsENOD12A* cDNA was amplified during 23 cycles (92°C, 1.5 min; 52°C, 1.5 min; 72°C, 3 min); while the amplification of the *PsENOD5* cDNA was carried out during 27 cycles (92°C, 1.5 min; 48°C, 1.5 min; 72°C, 3 min). The amplified DNA samples were separated on 2% agarose gel, and after blotting to *Hybond-N* + (Amersham) it was hybridized to ^{32}P-labelled inserts of pPsENOD12A or pPsENOD5.

Table 1. Oligonucleotides used in the PCR assays

	Oligonucleotides	Positions
1	5'-CATAAGATGGTTTTGTCACG-3'	Compl. to nt 301–282 of the *PsENOD12A* cDNA (Govers *et al.*, 1991; Scheres *et al.*, 1990a)
2	5'-CTTGTCCTCACTAGTGTTGT-3'	Identical to nt 21–40 of *PsENOD12A* cDNA (Scheres *et al.*, 1990a)
3	5'-CATCAACATAGGTAACGAAG-3'	Compl. to nt 384–365 of the *PsENOD5* cDNA (Scheres *et al.*, 1990b)
4	5'-CTTCTATTTTCCTACTCAGA-3'	Identical to nt 46–65 of *PsENOD5* cDNA (Scheres *et al.*, 1990b)
5	5'-ACCACCACG$^{G}/_{A}$AGACGGAG-3'	Compl. to the 3' end of a repeating ubiquitin unit
6	5'-ATGCAGAT$^{C}/_{T}$TTTGTGAAGAC-3'	Homologous to the 5' end of a repeating ubiquitin unit

The *PsENOD12* gene family consists of two members, *PsENOD12A* and *B*. According to Govers *et al.* (1991) their expression shows the same pattern; these two genes are expressed in the same tissues at similar levels, and both are inducible by Nod factors (Horvath, unpublished data). Here we used a *PsENOD12A*-specific oligonucleotide (Table 1, oligonucleotide 1). Southern blot analyses indicate that the pea genome only contains one *PsENOD5* gene (Scheres *et al.*, 1990b; Matvienko unpublished data).

We used ubiquitin (*ubi*) mRNA as an internal control. In control experiments we demonstrated that the level of *ubi* gene expression was not affected by application of Nod factors (Figure 1, panel ubiquitin) and that simultaneous reverse transcription and amplification of *ubi* mRNA together with the *PsENOD* transcript had no adverse effects on the results. The *ubi* gene of pea is built up of five multiple units (Watts and Moore, 1989) and the oligonucleotides 5 and 6 match to the ends of each single unit, thus after amplification five bands are visible on agarose gels.

We tested the semi-quantitative nature of the PCR assays as described by Scheres *et al.* (1990a). We showed that the amount of DNA formed after 23/27 cycles is proportional to the initial concentration of the RNA.

In each experiment the presence of *PsENOD5*, *PsENOD12* and the control *ubi* mRNAs was studied on the same RNA preparation, the reverse transcription and amplification were carried out simultaneously for the *ubi* mRNA and *PsENOD* transcript of interest. For each RNA preparation early nodulin mRNA levels were determined by at least two independent experiments. The kinetics of early nodulin gene induction was studied on seedlings treated at the same time with different Nod factors and the kinetics demonstrated in the Results was based on two or more independent experiments.

Purification of the Nod factors

Rhizobium strains LPR5045.pIJ1089 ('wild type') and LPR5045.pIJ1089 (*nod*E::Tn5) both overproducing the Nod factors (Spaink *et al.*, 1991) were used to purify these compounds. The purification of Nod factors produced by the 'wild-type' and the '*nod*E mutant' strains was carried out according to Spaink *et al.* (1991). The fractions containing the four major Nod factors and the intermediate fractions synthesized by the 'wild-type' strain (Spaink *et al.*, 1991) were pooled to generate the mixture of Nod factors. NodRlv-V(Ac, C18:4) was purified from the mixture of Nod factors produced by the 'wild type' strain, while NodRlv-V(Ac, C18:1) was isolated from a flavonoid induced culture of the '*nod*E mutant' strain according to Spaink *et al.* (1991). The NodRm factors were purified according to Lerouge *et al.* (1990).

The concentrations of the NodRlv factors were determined by comparing the HPLC pattern (C18 reversed phase, 206 nm) with an internal standard made by dissolving 1 mg NodRlv factor in 100 ml 60/40 acetonitrile/water.

The concentration of the mixture of the Nod factors represents the total amount of Nod factor molecules present. The mixture of the Nod factors contains the four major compounds in approximately equimolar concentrations.

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