Nod Factor–Induced Expression of Leghemoglobin to Study the Mechanism of NH_4NO_3 Inhibition on Root Hair Deformation

Renze Heidstra,¹ Gerd Nilsen,² Francisco Martinez-Abarca,³ Ab van Kammen,¹ and Ton Bisseling¹

¹Department of Molecular Biology, Agricultural University, Dreijenlaan 3, 6703 HA, Wageningen, The Netherlands; ²Avdeling for Plantefysiologi og Mikrobiologi, Institutt for Biologi og Geologi, Universitetet i Tromsø, 9037 Tromsø, Norway; and ³Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, Profesor Albareda 1, 18008 Granada, Spain

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

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

al. 1992; Heidstra et al. 1994; Journet et al. 1994; Cook et al. 1995; Vijn et al. 1995).

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

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

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

To test the possible effect of NH_4NO_3 on the formation of a component of the Nod factor perception-transduction system, we studied Nod factor–induced gene expression. Genes that are rapidly activated after the addition of Nod factor are most suitable for that purpose. Previously, it was shown that in vetch, Nod factors induce the expression of *ENOD5* and *ENOD12*, but these genes are activated after root hair deformation has been initiated (Vijn et al. 1995). Therefore, we

Corresponding author: T. Bisseling; fax: +31-317-483584; E-mail: Ton.Bisseling@mac.mb.wau.nl

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searched for additional marker genes, the induction of which occurs shortly after Nod factor application and do not require deformation.

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

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

RESULTS

Identification of Nod factor-induced vetch genes.

We used differential display to identify genes that are induced within the 1- to 3-h time period during which root hair deformation occurs. Total RNA was isolated from the susceptible zone of 300 vetch roots either untreated or treated with 10^{-10} M NodRlv factors for 1 or 3 h. First-strand cDNAs were synthesized with 12 oligo-dT₁₂MN anchor primers. Subsequently, 23 upstream primers (10 mers) were used to ampli-

fy specific subsets of the first-strand cDNAs. By comparing the patterns of cDNA fragments of the different RNA samples obtained with each primer combination, cDNA fragments present in the 1- and/or 3-h samples but absent from the 0-h sample were located. In this way, a total of 34 mRNAs were identified that apparently accumulated after Nod factor addition, 24 of which represented genes that were already induced after 1 h.

The 34 positive cDNAs were excised from the gel, reamplified, and cloned. Nine clones were randomly selected for further characterization. It was established that all nine cDNA clones represented genes that are expressed at very low levels, because we were unable to detect the corresponding mRNAs by Northern (RNA) blot analysis. The cDNA sequences of eight of the nine clones showed no homology to sequences in any of the databases. These sequences contained an oligo-dT₁₂MN anchor primer sequence, and therefore the cDNAs most likely represent the 3' ends of mRNAs. It is known that in general the untranslated 3' ends of mRNAs are not conserved. These clones were not further used.

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

Early expression of leghemoglobin.

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

To study whether de novo protein synthesis is required for the induction of *VsLb1* gene expression, we used cycloheximide (CHX). Preliminary experiments with a concentration series of CHX showed that 1 μM is the minimal concentration capable of completely inhibiting root hair deformation

VfaLbB	CAAGTTTGTG	GAATGGTGGC	TGATCTGCA	ATTCAACTAC	GGACGACGGG	50
VsLb1			CAACTTCGA	GCAAAAGGAA	AAGTAGATGT	19
VfaLbB	AGATATGTGT	TTGAAAGATG	GTACATTGGG	TGCTATCCAC	ATTCAAAAGG	100
VsLb1	AGCTGATGAT	GTTACATTAG	AATATTGGG	TTCTGTCCAT	GTTCAAAAG	69
VfaLbB	GAGTGTGTGA	TCCTCATTTT	GTGGTGGTTA	AAGAAGCTCT	ATTAAAAACA	150
VsLb1	GTGTGATGTA	TCTCCATTTC	ATGGTGTGTTA	AAAAAGCTAT	GTTGAAAAACA	119
VfaLbB	ATAAAGGAAG	CATCGGAGGA	CAAAATGGAGT	GAAGAACTAA	GCATTGCTTG	200
VsLb1	ATAAAATGG	CTGTGGAGGA	TAAATGGAGT	GAGGAATTGG	ATTGTGCTTG	169
VfaLbB	GGAAGTAGCC	TATGATGAGT	TGGCAACGGC	AATTAAAAAG	GCAATGAGTT	250
VsLb1	GGGTAAAGCC	TATGATGAAC	TAGCAGCTGC	GATTAAAGAG	GCAATGGGAT	219
VfaLbB	AAATTCGGGA	ATGAATCATA	ATTATAATA	AAAATTGCAT	CACTAAAACT	300
VsLb1	GGC	CAAAAAA	AAAAA			221

Fig. 1. Alignment of nucleotide sequences of the cDNA clones *VfaLbB* and *VsLb1*. The primers used for the differential display reaction are underlined.

Table 1. Primers used in the reverse transcriptase polymerase chain reaction assays

Primer	Position
5'-ATCCCATTCGCTTCTTAATC-3'	Complementary to nucleotides 229 to 209 of the <i>VsLb1</i> cDNA
5'-GGAAAAGTAGATGTAGCTGA-3'	Homologous to nucleotides 16 to 35 of the <i>VsLb1</i> cDNA
5'-ACCGCACACACATATCTGT-3'	Complementary to nucleotides 442 to 424 of the <i>VsENOD5</i> cDNA
5'-TGATACTGTTGTCAATGAGG-3'	Homologous to nucleotides 145 to 164 of the <i>VsENOD5</i> cDNA
5'-CTATGTTCTTCTGTAGCATG-3'	Complementary to nucleotides 297 to 278 of the <i>VsENOD12</i> cDNA
5'-TTCTTGCTGCTCTTATCC-3'	Homologous to nucleotides 41 to 59 of the <i>VsENOD12</i> cDNA
5'-ACCACCACGG/AAGACGGAG-3'	Complementary to the 3' end of a repeating ubiquitin unit
5'-ATGCAGATC/TTTTGTGAAGAC-3'	Complementary to the 5' end of a repeating ubiquitin unit

induced by 10^{-10} M NodRlv factor. When 1 μ M CHX and 10^{-10} M NodRlv factor were applied together, no *VsLb1* mRNA was found in the RNA isolated 3 h after application, indicating that de novo protein synthesis is required for the activation of *VsLb1* (Fig. 2).

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

We then compared the expression of *VsLb1* with that of the early nodulin genes *VsENOD5* and *VsENOD12*. Previously, it was shown by RT-PCR that vetch genes *VsENOD5* and *VsENOD12* were induced between 3 and 8 h after application of NodRlv factors (Vijn et al. 1995). However, in that study, primers based on the heterologous sequences from pea were used. We designed new primers complementary to the vetch sequences for quantitative RT-PCR experiments (Table 1). The time course of activation of *VsENOD5* and *VsENOD12* was determined with the same total RNA used for quantifying *VsLb1* mRNA accumulation at 0, 1, 3, and 8 h after NodRlv factor addition. Unlike *VsLb1*, *VsENOD5* and *VsENOD12* appeared to be expressed at a low basal level in the susceptible zone in the absence of NodRlv factor (Fig. 2). This low basal level was not the result of contaminating DNA, since no *VsENOD5* and *VsENOD12* sequences were amplified if the RT reaction was omitted (data not shown). Three hours after NodRlv factor application, the expressions of *VsENOD5* and *VsENOD12* were enhanced 22-fold and fourfold, respectively, and the level of those transcripts was further increased slightly after 8 h. The enhanced expression of both genes required de novo protein synthesis, since the expression remained at a basal level when 1 μ M CHX and 10^{-10} M NodRlv factor were applied together (Fig. 2).

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

Uncoupling of root hair deformation and induction of gene expression.

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

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

We then studied whether Nod factors can induce the expression of *VsENOD5*, *VsENOD12*, and *VsLb1* in the susceptible zone of vetch plants grown in the presence of 20 mM NH_4NO_3 . For this purpose, total RNA was isolated from the susceptible zone of the roots of 60 vetch plants grown in the presence of 20 mM NH_4NO_3 and treated for 3 h with 10^{-10} M NodRlv factor. Upon amplification of *VsLb1* mRNA by RT-PCR, it was found that the amount of *VsLb1* mRNA accumulated after 3 h was similar to that in plants grown in the absence of NH_4NO_3 (Fig. 2). However, only a twofold increase in expression of *VsENOD5* and *VsENOD12* was observed 3 h after NodRlv application when plants were grown in the presence of NH_4NO_3 .

These results show that induction of *VsENOD5*, *VsENOD12*, and *VsLb1* gene expression can be uncoupled from root hair deformation. Although in the presence of NH_4NO_3 , *VsENOD5* and *VsENOD12* gene expression is only marginally enhanced by NodRlv factors compared with plants grown in the absence

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

of NH_4NO_3 , *VsLb1* expression is induced to a similar level, showing that Nod factor perception and transduction still occur, even though deformation is not induced.

DISCUSSION

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

Our studies show that the differential display technique is a useful method for identifying genes induced very early during the *Rhizobium* (Nod factor)-plant interaction. A nearly complete screening with the 12 dT₁₂MN anchor and 23 upstream primers resulted in the isolation of 34 putative positive cDNAs representing genes specifically induced by Nod factors within the time that root hair deformation occurs. One of these genes, the expression of which is induced in vetch roots within 1 h after Nod factor application, appeared to be *VsLb1*. However, the induction of this gene is prevented in the presence of CHX and therefore requires de novo protein synthesis, which means that there must be genes induced earlier. In view of the low expression of *VsLb1* after 1 h, differential display may again be used to identify such genes expressed earlier. The characterization of the remaining 33 cloned cDNAs will show whether such genes are already represented in this group.

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

Transgenic studies in which the promoter of nonsymbiotic hemoglobin genes from *Parasponia* and *Casuarina* spp. was used showed that these genes are expressed in the root meristem of tobacco and lotus plants, respectively (Bogusz et al. 1990; Jacobsen-Lyon et al. 1995). It was proposed that the function of hemoglobin in these tissues, where oxygen supply is likely to be limiting, was that of an oxygen carrier for these rapidly respiring cells. Additional support for the oxygen-transport role comes from the study on a barley hemoglobin gene, which was induced under hypoxic conditions (Taylor et al. 1994). Vetch roots are grown in water, which is supposed to cause a poor supply of oxygen (waterlogging). Therefore, it is unlikely that Nod factors cause a rapidly hypoxic condition. Local expression of the *Lb* gene during the initial *Rhizobium*-plant interaction may be involved in facilitating the flux of

oxygen necessary for the mitochondrial respiration in root hairs and cortical cells, as has been reported for the non-symbiotic *Lb* genes. Thus, in addition to carrying oxygen for the bacteroids in nodules, the *Lb* gene may also be employed in the early stages of the interaction to support mitochondrial respiration in cells interacting with the bacteria.

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

VsLb1 now appears to be the earliest and most suitable marker gene of Nod factor-induced processes, since it is induced within 1 h after Nod factor application, whereas *VsENOD5* and *VsENOD12* are induced only after 3 h. In addition, *VsENOD5* and *VsENOD12* are expressed at a low level in control plants, whereas *VsLb1* is not. This previously unobserved low expression in untreated roots probably results from the specific primers complementary to the vetch *VsENOD5* and *VsENOD12* sequences that make the RT-PCR analysis more sensitive. The fact that these basal expression levels were observed in vetch and not in pea and alfalfa may be explained by the fact that we used whole root segments as starting material for RNA isolation and RT-PCR experiments. In contrast, in pea and alfalfa, gene expression only in the epidermis was examined (Pichon et al. 1992; Horvath et al. 1993), thus avoiding the contribution of other tissues to the background level of *VsENOD5* and *VsENOD12* expression.

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

Not only does NH_4NO_3 affect root hair deformation soon after inoculation, but it also inhibits both initial cortical cell divisions and infection thread formation (Malik et al. 1987; Carroll and Mathews 1990). If Nod factor perception and transduction are unaffected, then NH_4NO_3 should cause other

changes affecting these different steps in nodule formation. Since the growth and morphology of plants in general are affected by NH_4NO_3 (Redinbaugh and Campbell 1991), it is likely to have a pleiotropic effect. Thus, although the way in which nodulation is affected remains unclear, Nod factor perception and transduction are unaffected by NH_4NO_3 .

MATERIALS AND METHODS

Plant material and root hair deformation assay.

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

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

RNA isolation.

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

Total RNA was isolated (Pawlowski et al. 1994), and chromosomal DNA contamination was removed from the RNA samples according to Liang et al. (1993). The amount and quality of RNA was determined by spectrophotometry and agarose gel electrophoresis. Only RNA preparations having both A_{260}/A_{280} and $A_{260}/A_{240} \geq 2$ were used for further studies.

Differential display of NodRlv factor-induced genes in vetch.

Differential display, in which total RNA isolated from susceptible zones of vetch roots 0, 1, and 3 h after Nod factor treatment was used, was performed essentially as described by Liang and Pardee (1992) with slight modifications. Reverse transcription was performed with 0.5 μg of total RNA in a 20- μl reaction volume with 300 U of M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Duplicate cDNA samples, starting with the same amount of RNA, were prepared

for the amplification by PCR in order to minimize errors in the PCR procedure that lead to spurious bands in one lane but not in the other. A ramp of 3 s/ $^{\circ}\text{C}$ was included in the PCR during the heating from the annealing until the extension temperature was reached, resulting in better amplification when oligo-dT primers containing two pyrimidines as anchor were used. The PCR conditions were as follows: 94 $^{\circ}\text{C}$ for 30 s, 40 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 30 s for 40 cycles followed by 72 $^{\circ}\text{C}$ for 5 min. After the PCR procedure, the reaction mixture was evaporated in a Speed Vac and redissolved in 5 μl of a dye solution of formamide and water (1:1), and the amplified cDNAs were separated on a 6% DNA sequencing gel. The PCR products were labeled with ^{32}P -dATP or preferably with ^{33}P -dATP, because the latter results in a much better resolution of the labeled fragments after separation on a DNA sequencing gel. The recovery and reamplification of the cDNA probes were done according to Liang et al. (1993). The reamplified cDNA probes were cloned into the pGEM-T vector (Promega, Madison, WI). For at least 12 positives of each ligation, it was determined whether only one or several different cDNAs were cloned originating from the same band on the sequencing gel.

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

RT-PCR.

The amounts of *VsLb1*, *VsENOD5*, *VsENOD12*, and *VsPRP1* mRNA were quantified by using the RT-PCR method. Hybridization to the corresponding ^{32}P -labeled cloned inserts allowed the measurement of amplified products during the exponential phase of the PCR amplification. The amplification efficiency for each primer pair was determined by sampling a PCR reaction every two cycles between eight and 28 cycles. This procedure was repeated for PCR reactions at different annealing temperatures and MgCl_2 concentrations to determine the optimal values. The PCR samples were analyzed by agarose gel electrophoresis followed by Southern blotting (see below). At approximately the cycle number that was chosen to quantify gene expression, the amount of PCR product increased exponentially. Ubiquitin (*Ubi*) mRNA was used as an internal control (Horvath et al. 1993). The *Ubi* gene consists of five multiple units (Watts and Moore 1989), and the *Ubi* primers hybridize with the ends of each single unit.

Total RNA, 2.5 μg , was reverse transcribed in a total volume of 20 μl in 10mM Tris-HCl, pH 8.8, 50 mM KCl, 5 mM MgCl_2 containing 1 mM dNTPs, 20 U of RNAGuard, 1 μg of oligo-dT₁₂₋₁₈ (Pharmacia, Uppsala, Sweden), and 25 U of AMV reverse transcriptase (Stratagene, La Jolla, CA). The reaction mixture was incubated for 10 min at room temperature and then for 1 h at 42 $^{\circ}\text{C}$. After they were heated for 5 min at 95 $^{\circ}\text{C}$, the samples were diluted with water to 200 μl .

If aliquots of the cDNA sample are amplified in separate PCR reactions, the same batch of cDNA can be used in the analysis of multiple mRNAs. The cDNAs were amplified separately to avoid differences in amplification efficiencies between primer pairs. For the PCR reaction, 5 μl of the cDNA sample was amplified in a total volume of 50 μl in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl_2 containing 100 μM dNTPs, 100 ng of 3' and 5' primer (Table 1), and 1 U of *Taq* DNA polymerase. The *VsLb1* cDNA was amplified

during 25 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s), while amplification of *VsENOD12* and *VsENOD5* was carried out during 23 cycles (94°C, 30 s; 58°C, 30 s; 72°C, 30 s). *Ubi* cDNA was amplified during 12 cycles (94°C, 30 s; 60°C, 30 s; 72°C, 30 s). Amplified DNA samples (10 µl) were run on a 1.5% agarose gel, and after alkaline blotting to Nytran-N⁺ membrane (Schleicher and Schuell, Dassel, Germany), hybridized to ³²P-labeled inserts of the corresponding cDNA clones (Sambrook et al. 1989). Each experiment was performed twice.

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

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