Accumulation of Transcripts Encoding a Lipid Transfer–Like Protein During Deformation of Nodulation-Competent *Vigna unguiculata* Root Hairs

Andrea Krause,[1] Christian J. A. Sigrist,[1] Irmtraut Dehning,[1] Hans Sommer,[2] and William J. Broughton[1]

[1]L.B.M.P.S., Université de Genève, 1, ch. de l'Impératrice, 1292 Chambésy/Geneva, Switzerland, and [2]Max-Planck-Institute for Plant Breeding, Carl-von-Linné-Weg, 50829 Cologne, Germany
Received 16 July 1993. Revision received 23 February 1994. Accepted 11 March 1994.

A cDNA library was constructed from RNA of *Vigna unguiculata* root hairs harvested 1 day and 4 days after inoculation with *Rhizobium* sp. NGR234. A heterologous probe was used to identify a cDNA clone, the predicted 99-amino-acid sequence of which shares homology with a nonspecific lipid transfer protein (LTP) of *Hordeum vulgare*. Other characteristics, including an estimated molecular weight of 10.4 kD, an isoelectric point of 8.6, and a signal peptide with a hydrophobic region at the amino-terminal end, are shared by most LTPs. A transcript of 630 nt was found in all tissues tested, except nodules. Levels of mRNA increased in root hairs 24 hr after treatment with *Rhizobium* sp. NGR234, with different hormones, or with Nod factors. Amounts of transcripts were dependent on the concentration of Nod factors. Accumulation of transcripts during nodule development correlated with root hair deformation, the first visible step in the *Rhizobium*-legume symbiosis.

Additional keywords: hadulin, root hair cDNA library.

Root nodules are organized structures which develop from newly formed meristems in the cortex of legume roots following interaction with rhizobia. Cytological and ultrastructural analyses have demonstrated that the initial communication between the symbionts occurs at the single-cell level. Rhizobia enter the roots of their hosts through infections initiated in root hairs (Verma and Long 1983). In successful associations, rhizobia bind to the root hairs of the host plant and induce elongation, deformation (Had), branching, and curling (Hac) (Bauer 1981). Electron microscope studies have shown that rhizobia penetrate a degraded region of the plant cell wall, often near the curled part of the root hair. A secondary cell wall is deposited at this site and contributes to the formation of infection threads, which grow toward the base of the infected root hair (Callaham and Torrey 1981). Growing regions of the infection thread are surrounded by plant cytoplasm which contains high concentrations of organelles involved in the synthesis and deposition of membrane and cell wall material (Turgeon and Bauer 1985). Prior to penetration of the root cortex by the infection thread, the cortical cells dedifferentiate and divide to form nodule primordia (Calvert *et al.* 1984). The release of bacteria into plant cells is not well understood, but microscopic studies indicate that the plant plasma membrane envelops bacterial cells as they emerge from discontinuities in the infection thread wall (Robertson *et al.* 1978).

Nodule initiation is a highly specific process, which has been characterized as a two-way molecular conversation. Legume hosts release signal compounds, flavonoids, which stimulate the coordinated expression of bacterial genes required for nodulation (*nod* genes). Nodulation genes encode enzymes involved in the synthesis of Nod factors, which are substituted *N*-acylated oligomers of *N*-acetyl-D-glucosamine. Two different types of *nod* genes have been characterized—common, such as *nodABC,* which are essential for nodulation, and host-specific, which are unique to certain rhizobia and determine host specificity (Fellay *et al.,* in press). Expression of *nod* genes is controlled by regulatory *nodD* genes, whose products are activated by plant flavonoids (Dénarié *et al.* 1992). The ability of the NodD proteins to interact with the various flavonoids of different plants in a specific manner defines one level of host specificity (Horvath *et al.* 1987; Spaink *et al.* 1987).

At extremely low concentrations, purified Nod factors elicit most of the plant responses characteristic of the bacterial infection. Amongst the responses reported are depolarization of root hair membranes, Had, Hac, cortical cell division, and nodule formation on their homologous hosts (Truchet *et al.* 1991; Ehrhardt *et al.* 1992; Mergaert *et al.* 1993; Relić *et al.* 1993). Nodule formation can be mimicked by auxin transport inhibitors and by cytokinins (Allen *et al.* 1953; Hirsch *et al.* 1989; Relić *et al.* 1993). Nod factors also stimulate the expression of plant genes whose products are involved in nodule morphogenesis (Horvath *et al.* 1993). Genes expressed in root hairs after inoculation with *Rhizobium* are called hadulins (Krause and Broughton 1992), while early nodulins (Franssen *et al.* 1992; Govers and Bisseling 1992) are involved in the infection process and the formation of nodules. Late nodulins (Franssen *et al.* 1992; Nap and Bisseling 1990) function during nitrogen fixation. So far, hadulins and early nodulins have been described in a few legumes, but their precise symbiotic function remains unknown. Ultrastructural analyses suggest that plasma membrane as well as cell wall synthesis takes place during the early steps of nodule

Corresponding author: Andrea Krause.

MPMI Vol. 7, No. 3, 1994, pp. 411-418

morphogenesis, indicating that some of the hadulins and early nodulins are involved in their biogenesis. Studies on the relationships between signal exchange and the expression of hadulin and nodulin genes are still in their infancy (Horvath *et al.* 1993). It seems likely, however, that Nod factors exert control over genes whose expression is necessary for nodule development, while the control of those genes required for nodule function resides in the bacteria.

The ability to regulate nodule morphogenesis by exogenous Nod factors, hormones, etc., provides an excellent system for the study of signal transduction and organ development in plants. In our studies, we use the broad-host-range *Rhizobium* sp. NGR234 and the widely compatible legume *Vigna unguiculata.* NGR234 nodulates more than 75 genera of legumes as well as the nonlegume *Parasponia andersonii* (S. G. Pueppke and W. J. Broughton, unpublished), and its broad host range is largely mediated by the excretion of a family of acetylated or sulfated Nod factors (Price *et al.* 1992). Similarly, we used *V. unguiculata* because: 1) relative to other legumes, it has a small genome—540 Mbp (W. J. Broughton, unpublished); 2) it is a true diploid ($2n = 22$); 3) it has a short generation time (60–90 days); 4) linkage maps comprising genomic, random amplified polymorphic DNA, cDNA, and morphological markers are available (Fatokun *et al.* 1993); 5) it possesses one of the broadest capacities to nodulate of all known legumes (Lewin *et al.* 1987); 6) it responds rapidly (Lewin *et al.* 1990) and massively to inoculation with *Rhizobium* (Danso and Owiredu 1988); 7) it grows quickly and well under artificial conditions (Broughton *et al.* 1978; Broughton 1979); 8) it produces a large amount of root hairs (Krause and

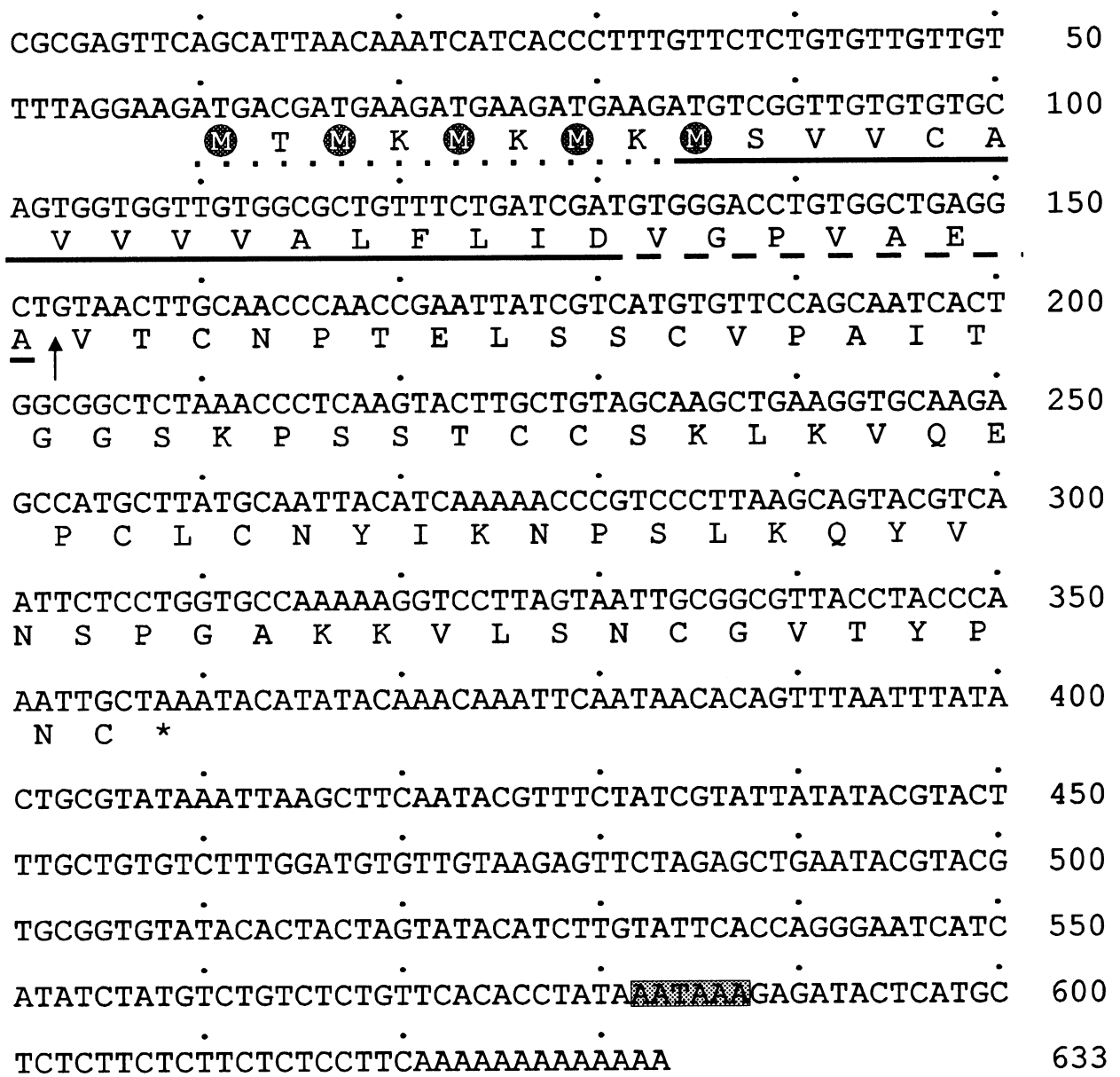

Fig. 1. Nucleotide and predicted amino acid sequence of the 633-bp *Eco*RI fragment of pAKCS9. Translation start points are marked with circles. The signal peptide cleavage site is indicated by an arrow. The different regions of the signal peptide are indicated as follows: region **n** is underlined with dots, region **h** with a straight line, and region **c** with dashes. The polyadenylation site is framed.

Broughton 1992); 9) it can be transformed with *Agrobacterium tumefaciens* (Garcia *et al.* 1987); and 10) it is widely cultivated, especially in Africa, India, and North America.

Here we describe the construction of a cDNA library from mRNA isolated from root hairs of *V. unguiculata* treated with *Rhizobium* sp. NGR234. Using a heterologous probe, we were able to isolate a cDNA clone that corresponds to a hadulin. The deduced protein sequence of pAKCS9 displays strong homology to a nonspecific lipid transfer protein (LTP) of *Hordeum vulgare.* Gene expression of the LTP-like protein was investigated at different stages of nodule development and in different tissues.

RESULTS

Construction of a root hair cDNA library.

To isolate *V. unguiculata* cv. Red Caloona genes involved in the first steps of nodule development, root hairs (the primary target of rhizobia) were harvested 1 day and 4 days after inoculation with *Rhizobium* sp. NGR234 (in the ratio of nine parts on day 1 to one part on day 4). Under our growth conditions, the first morphological symptoms of a compatible interaction, Had and Hac, were visible at that time (Krause and Broughton 1992), and most genes involved in the establishment of a *Rhizobium*-legume interaction should have been activated by then. Root hairs of 10^4 seedlings yielded sufficient poly(A)$^+$ RNA for construction of a cDNA library in λNM1149 using the RNaseH approach (Gubler and Hoffmann 1983; Lapeyre and Amalric 1985).

Isolation and identification of a sequence coding for an LTP-like protein.

By differentially screening a cDNA library prepared from root segments of *V. unguiculata* cv. Pink Eye Purple Hull, Trese and Pueppke (1991) isolated *Vu*C, which is homologous to transcripts preferentially expressed in nonnodulated roots and is strongly repressed after inoculation with *R. fredii.* To the best of our knowledge, neither the sequence nor the possible symbiotic role of *Vu*C is known. Northern analysis has shown, however, that the 600-bp insert of p*Vu*C hybridizes to a root transcript of 800 nt. This suggests that p*Vu*C does not contain a full-length insert, and for that reason we decided to use it to isolate the complete cDNA from *V. unguiculata* cv. Red Caloona. Using randomly labeled *Vu*C as a probe, we isolated 11 positive plaques (out of 1×10^5 recombinant phages screened) from the root hair cDNA library. After plaque purification and isolation of the phage DNA, the size of each insert was determined, and the clone carrying the longest insert was chosen for further analysis. This insert was subcloned into pBluescript IISK(+), and the recombinant plasmid was designated pAKCS9.

pAKCS9 contains an insert of 633 bp with a poly(A) tail of 13 nt (Fig. 1). A polyadenylation signal is located 40 nt upstream of the poly(A) tail. Only one open reading frame is present, with an untranslated region of 59 nt at the 5′ end and another of 277 nt at the 3′ end. The open reading frame has five potential translation start codons between nucleotides 60 and 86. None of them match the consensus sequence for plant translation initiation sequences, however (Lütcke *et al.* 1987). On the assumption that the first methionine is used, the predicted amino acid sequence consists of 99 residues, with an estimated molecular weight of 10.4 kD and an isoelectric point of 8.6. By the method of von Heijne (1986), a signal peptide of 31 amino acids which carries all necessary elements, including a potential cleavage site, was identified in the amino-terminal region (Fig. 1). Von Heijne's (1985) techniques distinguished three different regions in the signal peptide: region **n** consists of eight positive amino acids, region **h** has 17 hydrophobic amino acids, and region **c** possesses six polar amino acids.

A search of nucleic acid and protein data bases revealed significant sequence homology of the insert of pAKCS9 to a nonspecific LTP (NLT2) of *H. vulgare* (Jakobsen *et al.* 1989) and to a 253-bp clone (TAT3D7) from *Arabidopsis thaliana* (Bardet *et al.* 1992). At the DNA level, the overall homology was 44% with NLT2 and 56% with TAT3D7. The deduced amino acid sequence of the insert displayed 41 and 48% identity with NLT2 and TAT3D7 and 63 and 68% similarity to them, respectively (Fig. 2). These homologies start from the first methionine, indicating that it is most probably used as the translation start. Furthermore, there are eight conserved cysteine residues which are characteristic of LTPs (Fig. 2).

Genomic organization.

To determine the number of genes coding for the LTP-like protein in *V. unguiculata,* genomic DNA was digested with

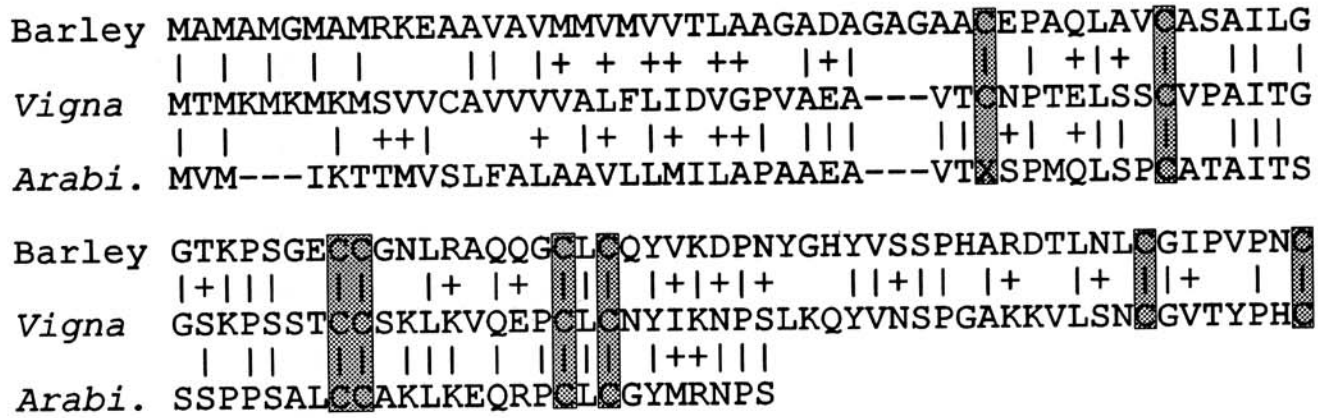

Fig. 2. Deduced amino acid sequence of clone pAKCS9 compared with sequences of a nonspecific lipid transfer protein (NLT2) of barley (*Hordeum vulgare*) and clone TAT3D7 from *Arabidopsis thaliana.* The eight conserved cysteines are shadowed.

various restriction enzymes, and Southern transfer analysis performed with the 633-bp insert of pAKCS9 as the probe (Fig. 3). This insert hybridized strongly to a single band of DNA formed after digestion with the restriction enzymes *Bam*HI, *Eco*RI, and *Hin*cII, indicating that only one copy of the LTP-like gene is present in the genome. Internal restriction sites are responsible for the two hybridizing bands seen when DNA was digested with *Acc*I or *Cla*I.

Expression of the LTP *in vivo*.

Northern blot analysis using total RNA isolated from six different tissues showed that the insert of pAKCS9 hybridized only to a transcript of 630 nt, suggesting that pAKCS9 carries a full-length cDNA insert. With the exception of 5-wk-old nodules, transcripts of the LTP-like gene were visible in all tissues tested. LTP-like mRNAs were most abundant in young leaves. Densitometric analysis showed that the amount of LTP mRNA in flower buds and young pods was approximately 90 and 70%, respectively, of that found in leaves. Transcript levels were reduced by a factor of 11 in 4-day-old roots and by a factor of five in uninoculated root hairs (Fig. 4).

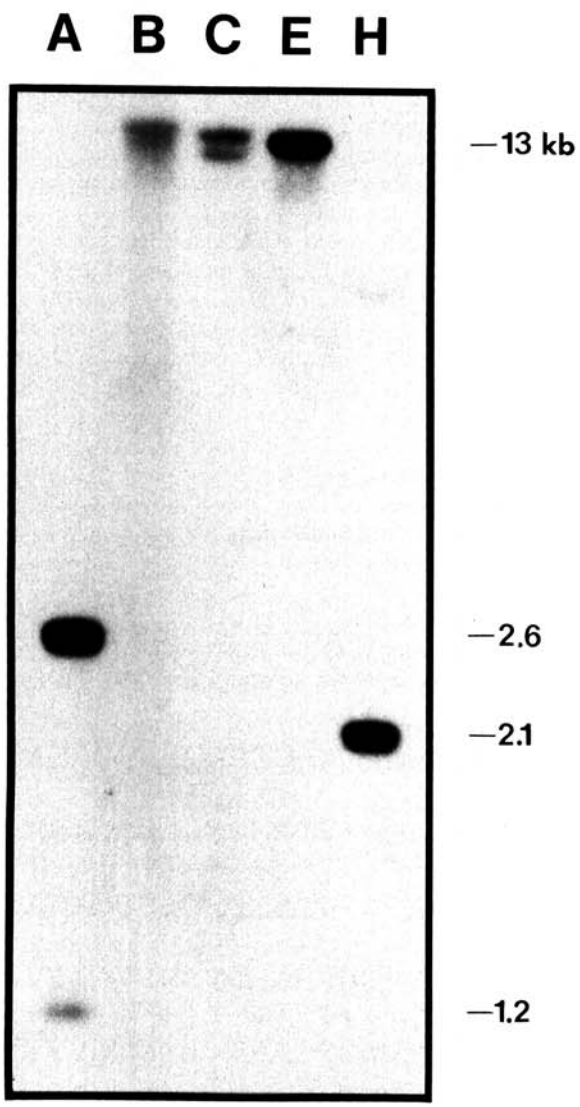

Fig. 3. Southern blot hybridization of 5 µg of total DNA from *Vigna unguiculata* digested with *Acc*I (lane A), *Bam*HI (lane B), *Cla*I (lane C), *Eco*RI (lane E), and *Hin*cII (lane H) and probed with the 633-bp insert of pAKCS9.

To examine the role of the LTP-like protein in nodule development, mRNA levels were determined in root hairs 1 day after inoculation with either *Rhizobium* sp. NGR234 or the NodABC$^-$ mutant NGRΔnodABC, as well as in mature nodules and pseudonodules. Significantly, five times more LTP-like transcripts were found in root hairs treated with the wild-type *Rhizobium* than in those sprayed with either the mutant or water (Fig. 4). The amounts of transcripts visible after inoculation with *Rhizobium* sp. NGR234 attained the levels found in young leaves. LTP-like transcripts were found neither in nodules nor in pseudonodules.

An obvious question is whether the *Rhizobium*-stimulated increases of LTP-like transcripts could be mimicked by Nod factors, which also induce Had, Hac, and the formation of pseudonodules on *V. unguiculata* (B. Relić and W. J. Broughton, unpublished). It was of further interest to compare their effect with that of auxins, gibberellins, and cytokinins on the accumulation of LTP-like transcripts, since the latter also provoke pseudonodulation on *Vigna* (B. Relić and W. J. Broughton, unpublished). To do this, mRNA levels were determined in root hairs harvested 1 day after treatment with different concentrations of hormones or acetylated Nod factors (NodNGR[Ac]). Interestingly, Nod factors induced the accumulation of LTP-like mRNAs in 24 hr or less (Fig. 5). Increases in transcript levels were dependent on the concentration of Nod factors, with an optimum at about 1×10^{-6} M. NodNGR[Ac] at 5×10^{-6} M lowered transcript accumulation by a factor of about four, while treatment with 1×10^{-7} M reduced transcript levels by a factor of about three. All hormones induced a sixfold accumulation of LTP-like transcripts (data not shown). Figure 6 shows a comparison of the effects of Nod factors and kinetin. No correlation exists between the accumulation of the mRNAs coding for the LTP-like gene and the formation of pseudonodules.

DISCUSSION

To identify marker genes for the development of *V. unguiculata* nodules, we constructed a cDNA library of mRNA isolated from root hairs harvested 1 day and 4 days after inoculation with *Rhizobium*. Root hair deformation occurs 1 day after inoculation, and by day 4 the bacteria have penetrated the root (Krause and Broughton 1992). The library was screened with *Vu*C, a clone isolated by differential screening of a root segment cDNA library of *V. unguiculata* cv. Pink Eye Purple Hull (Trese and Pueppke 1991). *Vu*C is homologous to a gene that is strongly repressed after inoculation with *R. fredii* strain USDA257. Expression of *Vu*C in nonnodulated roots is greatest 2.5 days after inoculation and declines thereafter. In this system, *Vu*C hybridizes to a transcript of 800 nt. Although the nucleotide sequence of *Vu*C is not available, we believe that the insert of pAKSC9 differs greatly from *Vu*C for the following reasons. First, at 630 nt, the transcript is considerably smaller. Second, in contrast to *Vu*C, the transcript accumulates in root hair tissue after inoculation with *Rhizobium*. Third, the expression of the two clones during nodule development is different. Nevertheless,

they must share limited sequence homology, since the cDNA insert of pAKCS9 cross-hybridizes with *Vu*C.

Interestingly, the cDNA insert of pAKCS9 shares 44% DNA homology and 63% protein similarity with an LTP of *H. vulgare* (Jakobsen *et al.* 1989). In addition to 30–70% DNA sequence homologies (Tsuboi *et al.* 1991), LTPs share the following characteristics: 1) the presence of eight cysteine residues at conserved locations (Sossountzov *et al.* 1991); 2) a molecular weight of 9–14 kD (Chasan 1991); 3) an isoelectric point of approximately 9 (Chasan 1991); and 4) a signal peptide (Bernhard *et al.* 1991). As these criteria are also applicable to pAKCS9, it seems likely that it carries an insert which codes for an LTP-like protein.

It is thought that as LTPs facilitate the transfer of lipids between membranes *in vitro* (Chasan 1991; Kader 1990), they play a role in membrane biogenesis. Although the physiological function of LTPs has yet to be demonstrated *in vivo,* Sossountzov *et al.* (1991) believe that LTPs participate in membrane biogenesis by transporting lipids from their place of synthesis, the endoplasmic reticulum, to other membranes. In contrast, Sterk *et al.* (1991) and Thoma *et al.* (1993) suggested that LTPs might be involved in cutin formation, while Molina and Garcia-Olmedo (1993) believe that LTPs play a role in the defense against bacterial and fungal pathogens. Nevertheless, since the expression of genes encoding LTPs is modulated in certain tissues during plant development, they are an early marker of plant differentiation (Chasan 1991; Sterk *et al.* 1991)

High levels of LTP-like transcripts in young tissues of the aerial parts of the plant and low amounts in the root systems are in agreement with the observations of Molina and Garcia-Olmedo (1993) and Thoma *et al.* (1993). After inoculation with *Rhizobium,* the amount of LTP-like transcripts increases dramatically, but they are absent from nodules. Inoculation with a Nod$^-$ mutant had no effect on transcription of the LTP-like gene, showing that *nod* genes are necessary for its induction. Since Nod factors are the products of the *nod* genes, their ability to induce transcription of the LTP-like gene is good evidence that they are the symbiotic signals which regulate the activity of the LTP-like protein. Under our conditions, this rapid rise in LTP-like transcripts corresponds to Had (Krause and Broughton 1992). As no LTP-like transcript was visible in nodules, but transcripts were relatively abundant in treated root hairs, it appears that the LTP-like gene is transiently expressed during nodule development. It thus conforms to the definition of a hadulin (Krause and Broughton 1992) and provides a marker for the first visible step in the *Rhizobium*-legume interaction: root hair deformation.

One possible role of the LTP-like protein in nodule development could be due to its ability, *in vitro,* to transport lipids between membranes. Cytological and ultrastructural analyses suggest that membrane synthesis occurs during root hair curling and in front of the growing infection thread (Turgeon and Bauer 1985; Ridge and Rolfe 1986). Perhaps the LTP-like protein is involved in the biogenesis of these new membranes. Another possibility is based on the observation that

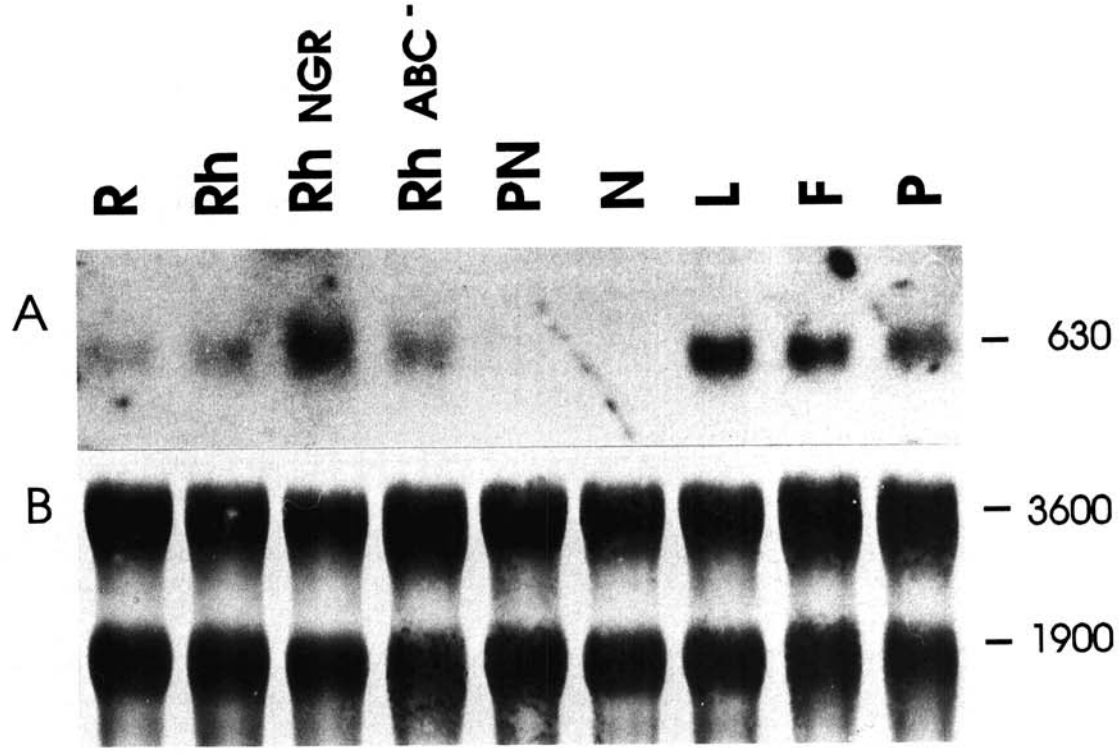

Fig. 4. Differential expression of the gene for a lipid transfer–like protein of *Vigna unguiculata* in different tissues following inoculation with *Rhizobium.* Total RNA (10 µg) was extracted from roots (R), root hairs (RH), root hairs inoculated with *Rhizobium* sp. NGR234 (Rh NGR), root hairs inoculated with *Rhizobium* sp. NGRΔ*nodABC* (Rh ABC$^-$), pseudonodules (PN), nodules (N), leaves (L), flowers (F), and seed pods (P). **A,** Hybridization against the 633-bp fragment of pAKCS9 under stringent conditions. **B,** Amounts and integrities of ribosomal RNA were confirmed by staining the filter with methylene blue (Sambrook *et al.* 1989).

LTPs preferentially accumulate in the outer cell layers of exposed surfaces (Molina and Garcia-Olmedo 1993), particularly in the cell walls of epidermal cells (Thoma *et al.* 1993). This leads us to speculate that the transport of Nod factors from the plasma membrane to their target may be coupled to binding with the LTP-like protein via the acyl chain. This would explain why structural variation in the fatty acid has such an influence on the specificity of Nod factors (Spaink *et al.* 1991; Dénarié and Cullimore 1993).

MATERIALS AND METHODS

Bacteria.

Bacterial strains, plasmids, and bacteriophages used in this study are listed in Table 1. *Rhizobium* strains were grown as described by Krause and Broughton (1992). *Escherichia coli* XL1-Blue was grown in Luria-Bertani medium, while POP13 was cultivated in NZYM medium (Sambrook *et al.* 1989).

Plant growth and inoculation.

V. unguiculata (L.) Walp. cv. Red Caloona was obtained from Wright Stephenson & Co. (Seven Hills, N.S.W., Australia). Seeds were surface-sterilized, germinated, treated with bacteria, and cultivated, and the root hairs were harvested as described by Krause and Broughton (1992). In other experiments, root hairs were isolated from seedlings 1 day after treatment with 6-benzylaminopurine (5×10^{-6} and 10^{-5} M), gibberellic acid (10^{-7} and 5×10^{-6} M), kinetin (5×10^{-6} and 10^{-5} M), α-naphthaleneacetic acid (10^{-8} and 5×10^{-6} M), and NodNGR[Ac] of *Rhizobium* sp. NGR234 (10^{-7}, 5×10^{-6}, and 10^{-6} M). As a control, sterile water was sprayed on seedlings. Root hairs were stripped from the roots of uninoculated seedlings; RNA was then prepared from both fractions. Young leaves, young seed pods, flower buds, and 5-week-old nodules were harvested from plants inoculated with *Rhizobium*

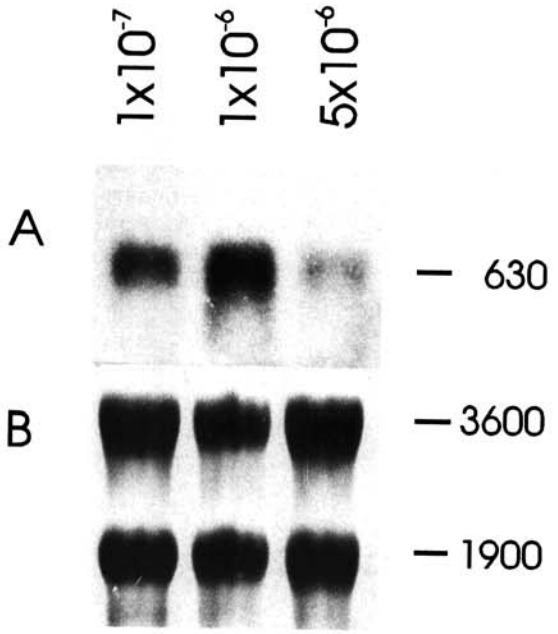

Fig. 5. Induction of lipid transfer–like gene expression in *Vigna unguiculata* root hairs by Nod factors at different molar concentrations. **A,** Hybridization with the cDNA coding for a lipid transfer–like protein. **B,** Amounts of rRNA obtained by staining the filter with methylene blue.

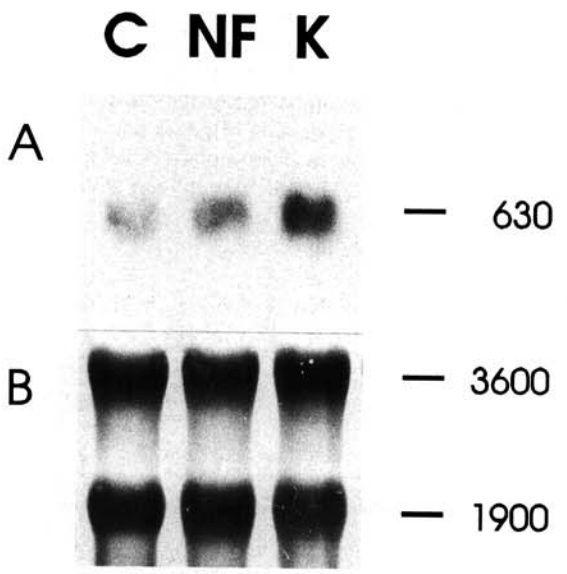

Fig. 6. Northern blot analysis of 10 μg of total RNA extracted from *Vigna* root hairs following treatment with acetylated Nod factors (NF) (10^{-7} M) or kinetin (K) (10^{-5} M). Water was used as a control (C). **A,** Hybridization with pAKCS9. **B,** Filter stained with methylene blue.

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

	Characteristics	Source or reference
Rhizobium sp. NGR234		
NGR234	Rif^r derivative of NGR234	Lewin *et al.* 1990
NGRΔ*nodABC*	*nodABC*$^-$ mutant of NGR234, Nod$^-$ on *Vigna*, Sp^r, Rif^r	B. Relić, unpublished
NGRΔ*fixDE*	NGR234 mutant, Fix$^-$ on *Vigna*, Sp^r, Rif^r	B. Relić, unpublished
Escherichia coli		
POP13	Hif derivative of POP101	Murray 1983
XL1-Blue	recA$^-$ (*rec*A1, *lac*$^-$, *end*A1, *gyr*A96, *thi*, *hsd*R17, *lac*I^q, supE44, F′proAB, *lacZ*DM15, Tn 10)	Bullock *et al.* 1987
Phage		
λNM1149	λ*b*538, srIλ3°, *imm*434, *sr*Iλ4°, *sr*Iλ5°, *shn*dIIIλ6°	Murray 1983
Plasmids		
pBluescript IISK(+)	pMB1, phage f1, *lacZα*$^+$, Amp^r	Stratagene, La Jolla, CA
p*VuC*	cDNA clone isolated by differential screening from root segments of *Vigna* inoculated with *R. fredii* USDA257, subcloned in pGEM7Zf+, Amp^r	Trese and Pueppke 1991
pAKCS9	*Eco*RI subclone of a cDNA clone in pBluescript IISK (+), Amp^r	This study

sp. NGR234 and grown in Magenta jars (Lewin *et al.* 1990). Pseudonodules were collected 5 wk after inoculation of seedlings with a Fix⁻ mutant of NGR234 (B. Relić, unpublished).

DNA and RNA isolation and analysis.

Total DNA of *V. unguiculata* was isolated as described by Schwarz-Sommer *et al.* (1984). All other recombinant DNA methods were according to Sambrook *et al.* (1989).

To construct a cDNA library, total cellular RNA was isolated from root hairs harvested 1 day and 4 days after treatment with *Rhizobum* sp. NGR234. Isolation of the RNA and purification on Qiagen-tip 100 was performed according to the manufacturer's protocol (Qiagen, Düsseldorf, Germany). mRNA was separated from total RNA by passage through an oligo(dT)-cellulose column (Sambrook *et al.* 1989). For Northern blot analysis, total plant RNA was extracted as described by Chandler *et al.* (1983). After separation of 10 μg of total RNA on denaturing agarose gels and transfer to nylon membranes, the amount and the integrity of ribosomal RNA was confirmed by staining the filter with methylene blue (Sambrook *et al.* 1989). Standard techniques were used for Southern and Northern hybridizations (Sambrook *et al.* 1989). Verification that equal amounts of RNA were loaded was obtained by densitometric analysis of the stained filters (QuantiScan, Biosoft, Cambridge, UK). Densitometry was also used to calculate the relative amounts of transcripts in the Northern blots.

Construction and screening of the root hair cDNA library.

Double-stranded (ds) cDNA was synthesized from 1 μg of poly(A)⁺ RNA isolated from root hairs treated with *Rhizobium* sp. NGR234, using the RNaseH method (Gubler and Hoffmann 1983; Lapeyre and Amalric 1985). The ends of the ds-cDNA were repaired with T4 DNA polymerase (Sommer *et al.* 1990), and the ds-cDNA was methylated (Sambrook *et al.* 1989). *Eco*RI linkers were added as described by Sambrook *et al.* (1989). After digestion with *Eco*RI, the excess linkers were removed on a Qiagen-tip 5, and the ds-cDNA was ligated into the *Eco*RI restriction site of the λNM1149 vector (Murray 1983). The ligation mixture was packaged *in vitro* and plated with the Hfl strain POP13 (Murray 1983). The number of recombinants obtained was 2×10^5.

Recombinant phages were replicated on nitrocellulose filters (BA85, Schleicher & Schuell, Dassel, Germany) as described by Sambrook *et al.* (1989). Hybridizations with randomly labeled *Vu*C (Trese and Pueppke 1991) were performed overnight at 62° C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, and 0.1% (w/v) sodium dodecyl sulfate (SDS), containing herring sperm DNA 100 (μg ml^{-1}). Filters were washed twice in 2× SSC and 0.1% (w/v) SDS for 10 min and once in 0.1× SSC and 0.1% (w/v) SDS.

DNA sequencing.

Inserts of recombinant phages were re-cloned into the *Eco*RI site of the pBluescript IISK(+) vector (Stratagene, La Jolla, CA). Deletions were made using exonuclease III (Henikoff 1987). A double-stranded dideoxy chain termination method was used to determine the sequence (Sequenase II, USB, Cleveland, OH). DNA sequence data were analyzed with PC/Gene (IntelliGenetics Inc., Mountain View, CA) and the Blast network service (National Center for Biotechnology Information, Bethesda, MA; Altschul *et al.* 1990; Gish and States 1993).

ACKNOWLEDGMENTS

We are very grateful to A. Trese and S. G. Pueppke for providing *Vu*C. B. Relić donated Fix⁻ nodules as well as *Rhizobium* strains NGRΔ*nodABC* and NGRΔ*fixDE*, and S. Jabbouri provided Nod factors of *Rhizobium* sp. NGR234. We thank T. Estrada-Garcia, R. Fellay, and M. Löbler for critical reading of the manuscript and M. Shayya for help in sequencing the second strand of pAKCS9. We are grateful to D. Gerber for her constant support. Financial assistance was provided by the University of Geneva, the Roche Research Foundation and the Fonds National Suisse de la Récherche Scientifique (Project 31-30950.91).

LITERATURE CITED

Allen, E. K., Allen, O. N., and Newman, A. S. 1953. Pseudonodulation of leguminous plants induced by 2-bromo-3,5-dichlorobenzoic acid. Am. J. Bot. 40:429-435.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Liptman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Bardet, C., Axelos, M., Tremousaygue, D., Lebas, M., Lagravere, T., and Lescure, B. 1992. The *Arabidopsis thaliana* transcribed genome: The GDR cDNA program. EMBL Data Library Release 34.0, March 1993. Accession no. Z17513.

Bauer, W. D. 1981. Infection of legumes by rhizobia. Annu. Rev. Plant Physiol. 32:407-449.

Bernhard, W. R., Thoma, S., Botella, J., and Somerville, C. R. 1991. Isolation of a cDNA clone for spinach lipid transfer protein and evidence that the protein is synthesized by the secretory pathway. Plant Physiol. 95:164-170.

Broughton, W. J. 1979. Effect of light intensity on net assimilation rates of nitrate-supplied or nitrogen-fixing legumes. Pages 285-299 in: Photosynthesis and Plant Development. R. Marcelle, H. Clysters, and M. van Poucke, eds. Dr. Junk, The Hague.

Broughton, W. J., Hoh, C.-H., Behm, C. A., and Tung, H.-F. 1978. Development of the nitrogen fixing apparatus in the legumes *Centrosema pubescens* Benth., and *Vigna unguiculata* L. Walp. Planta 139:183-192.

Bullock, W. O., Fernandez, J. M., and Short, J. M. 1987. XL1-Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. Biotechniques 5:376-379.

Callaham, D. A., and Torrey, J. G. 1981. The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium.* Can. J. Bot. 59: 1647-1664

Calvert, H. E., Pence, M. K., Pierce, M., Malik, N. S. A., and Bauer, W. D. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can. J. Bot. 62:2375-2384.

Chandler, P. M., Higgins, T. J. V., Randall, P. J., and Spencer, D. 1983. Regulation of legumin levels in developing pea seeds under conditions of sulfur deficiency. Plant Physiol. 71:47-54.

Chasan, R. 1991. Lipid transfer proteins: Moving molecules? Plant Cell 3:842-843.

Danso, S. K. A., and Owiredu, J. D. 1988. Competitiveness of introduced and indigenous *Bradyrhizobium* strains for nodule formation in three soils. Soil Biol. Biochem. 20:305-310.

Dénarié, J., and Cullimore, J. 1993. Lipo-oligosaccharide nodulation factors: A new class of signaling molecules mediating recognition and morphogenesis. Cell 74:951-954.

Dénarié, J., Debellé, F., and Rosenberg, C. 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46:497-531.

Ehrhardt, D. W., Atkinson, E. M., and Long, S. R. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod-factors. Science 256:998-1000.

Fatokun, C. A., Danesh, D., Menancio-Hautea, D. I., and Young, N. D. 1993. A linkage map for cowpea [*Vigna unguiculata* (L.) Walp.] based on DNA markers (2N = 22). Pages 6256-6258 in: Genetic Maps: Lo-

cus Maps of Complex Genomes. S. J. O'Brien, ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fellay, R., Rochepeau, P., Relić, B., and Broughton, W. J. Signals to and emanating from *Rhizobium* largely control symbiotic specificity. In: Pathogenesis and Host-Parasite Specificity in Plant Diseases: Histopathological, Biochemical, Genetic and Molecular Basis. K. Kohomoto, R. P. Singh, and U. S. Singh, eds. Pergamon Press, Oxford. (In press.)

Franssen, H. J., Nap, J.-P., and Bisseling, T. 1992. Nodulins in root nodule development. Pages 598-624 in: Biological Nitrogen Fixation. G. Stacey, R. H. Burris, and H. J. Evans, eds. Chapman and Hall, New York.

Garcia, J. A., Hille, J., Vos, P., and Goldbach, R. 1987. Transformation of cowpea *Vigna unguiculata* with a full-length DNA copy of cowpea mosaic virus m-RNA. Plant Sci. 48:89-98.

Gish, W., and States, D. J. 1993. Identification of protein coding regions by database similarity search. Nat. Genet. 3:266-272.

Govers, F., and Bisseling, T. 1992. Nodulins in root nodule development: Function and gene regulation. Pages 31-37 in: Nitrogen Metabolism of Plants. Proc. Phytochem. Soc. Eur., vol. 33. K. Mengel and D. J. Pilbeam, eds. Oxford Science Publications, Oxford.

Gubler, U., and Hoffmann, B. J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25:263-269.

Henikoff, S. 1987. Unidirectional digestion with exonuclease III in DNA sequence analysis. Methods Enzymol. 155:156-167.

Hirsch, A. M., Bhuvaneswari, T. V., Torrey, J. G., and Bisseling, T. 1989. Early nodulin genes are induced in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA 86:1244-1248.

Horvath, B., Bachem, C. W. B., 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.

Horvath, B., Heidstra, R., Lados, M., Moerman, M., Spaink, H. P., Promé, J.-C., van Kammen, A., and Bisseling, T. 1993. Lipo-oligosaccharides of *Rhizobium* induce infection-related early nodulin gene expression in pea root hairs. Plant J. 4:727-733.

Jakobsen, K., Klemsdal, S. S., Aalen, R. B., Bosnes, M., Alexander, D., and Olsen, O.-A. 1989. Barley aleurone cell development: Molecular cloning of aleurone-specific cDNAs from immature grains. Plant Mol. Biol. 12:285-293.

Kader, J.-C. 1990. Intracellular transfer of phospholipids, galactolipids and fatty acids in plants cell. Subcell. Biochem. 16:69-111.

Krause, A., and Broughton, W. J. 1992. Proteins associated with root-hair deformation and nodule initiation in *Vigna unguiculata.* Mol. Plant-Microbe Interact. 5:96-103.

Lapeyre, B., and Amalric, F. 1985. A powerful method for the preparation of cDNA libraries: Isolation of cDNA encoding a 100-kDa nucleolar protein. Gene 37:215-220.

Lewin, A., Cervantes, E., Wong, C.-H., and Broughton, W. J. 1990. *nodSU,* two new *nod* genes of the broad host range *Rhizobium* strain NGR234 encode host-specific nodulation of the tropical tree *Leucaena leucocephala.* Mol. Plant-Microbe Interact. 3:317-326.

Lewin, A., Rosenberg, C., Meyer z. A., H., Wong, C.-H., Nelson, L., Manen, J.-F., Stanley, J., Dowling, D. N., Dénarié, J., and Broughton, W. J. 1987. Multiple host-specificity loci of the broad host-range *Rhizobium* sp. NGR234 selected using the widely compatible legume *Vigna unguiculata.* Plant Mol. Biol. 8:447-459.

Lütcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. 1987. Selection of AUG initiation codons differs in plant and animals. EMBO J. 6:43-48.

Mergaert, P., van Montagu, M., Promé, J.-C., and Holsters, M. 1993. Three unusual modifications, a D-arabinosyl, an *N*-methyl, and a carbamoyl group, are present on the Nod factors of *Azorhizobium caulinodans* strain ORS571. Proc. Natl. Acad. Sci. USA 90:1551-1555.

Molina, A., and Garcia-Olmedo, F. 1993. Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. Plant J. 4:983-991.

Murray, N. E. 1983. Phage lambda and molecular cloning. Pages 395-432 in: Lambda II. R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Nap., J.-P., and Bisseling, T. 1990. Nodulin function and nodulin gene regulation in root nodule development. Pages 181-229 in: Molecular Biology of Symbiotic Nitrogen Fixation. P. M. Gresshoff, ed. CRC Press, Boca Raton, FL.

Price, N. P. J., Relić, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S. G., Maillet, F., Dénarié, J., Promé, J.-C., and Broughton, W. J. 1992. Broad-host-range *Rhizobium* species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are *O*-acetylated or sulphated. Mol. Microbiol. 6:3575-3584.

Relić, B., Talmont, F., Kopcinska, J., Golinowski, W., Promé, J.-C., and Broughton, W. J. 1993. Biological activity of *Rhizobium* sp. NGR234 Nod-factors on *Macroptilium atropurpureum.* Mol. Plant-Microbe Interact. 6:764-774.

Ridge, R. W., and Rolfe, B. G. 1986. Sequence of events during the infection of the tropical legume *Macroptilium atropurpureum* Urb. by the broad-host-range, fast growing *Rhizobium* ANU240. J. Plant Physiol. 122:121-137.

Robertson, J. G., Lyttleton, P., Bullivant, S., and Grayston, G. F. 1978. Membranes in lupin root nodules. I. The role of Golgi bodies in the biogenesis of infection threads and peribacteroid membranes. J. Cell Sci. 30:129-149.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schwarz-Sommer, Z., Gierl, A., Klösgen, R. B., Wienand, U., Peterson, P. A., and Saedler, H. 1984. The Spm (En) transposable element controls the excision of a 2-kb insert at the wx-m8 allele of *Zea mays.* EMBO J. 3:1021-1028.

Sommer, H., Beltran, J.-P., Huijser, P., Pape, H., Lönnig, W.-E., Saedler, H., and Schwarz-Sommer, Z. 1990. *Deficiens,* a homeotic gene involved in the control of flower morphogenesis in *Antirrhinum majus:* The protein shows homology to transcription factors. EMBO J. 9:605-613.

Sossountzov, L., Ruiz-Avila, L., Vignols, F., Jolliot, A., Arondel, V., Tchang, F., Grosbois, M., Guerbette, F., Miginiac, E., Delseny, M., Puigdomenèch, P., and Kader, J.-C. 1991. Spatial and temporal expression of a maize lipid transfer protein gene. Plant Cell 3:923-933.

Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium.* Nature 354:125-130.

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

Sterk, P., Booij, H., Schellekens, G. A., van Kammen, A., and de Vries, S. C. 1991. Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3:907-921.

Thoma, S., Kaneko, Y., and Somerville, C. 1993. A non-specific lipid transfer protein from *Arabidopsis* is a cell wall protein. Plant J. 3:427-436.

Trese, A. T., and Pueppke, S. G. 1991. Cloning of cowpea (*Vigna unguiculata*) genes that are regulated during initiation of nodulation. Mol. Plant-Microbe Interact. 4:46-51.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J.-C., and Dénarié, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351:670-673.

Tsuboi, S., Suga, T., Takishima, K., Mamiya, G., Matsui, K., Ozeki, Y., and Yamada, M. 1991. Organ-specific occurrence and expression of the isoforms of nonspecific lipid transfer protein in castor bean seedlings, and molecular cloning of a full-length cDNA for a cotyledon-specific isoform. J. Biochem. 110:823-831.

Turgeon, B. G., and Bauer, W. D. 1985. Ultrastructure of infection-thread development during the infection of soybean by *Rhizobium japonicum.* Planta 163:328-349.

Verma, D. P. S., and Long, S. 1983. The molecular biology of the *Rhizobium*-legume symbiosis. Int. Rev. Cytol. 14:211-245.

von Heijne, G. 1985. Signal sequences. The limits of variation. J. Mol. Biol. 184:99-105.

von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. 14:4683-4690.